Molecular Architecture of a Complex between an Adhesion Protein from the Malaria Parasite and Intracellular Adhesion Molecule 1*

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Background: PIEMP1 proteins cause Plasmodium falciparum-infected erythrocytes to bind human tissues during malaria.

Results: The IT4VAR13 ectodomain is rigid, elongated, and monomeric, presenting a binding site for its ligand, ICAM-1.

Conclusion: The IT4VAR13 ectodomain is unlike that of VAR2CSA, a PIEMP1 that adopts a compact structure with multiple domains contributing to ligand binding.

Significance: PIEMP1 proteins have evolved diverse architectures to facilitate ligand recognition.

The adhesion of Plasmodium falciparum-infected erythrocytes to human tissues or endothelium is central to the pathology caused by the parasite during malaria. It contributes to the avoidance of parasite clearance by the spleen and to the specific pathological consequences of cerebral and placental malaria. The PIEMP1 family of adhesive proteins is responsible for this sequestration by mediating interactions with diverse human ligands. In addition, as the primary targets of acquired, protective immunity, the PIEMP1s are potential vaccine candidates. PIEMP1s contain large extracellular ectodomains made from CIDR (cysteine-rich interdomain regions) and DBL (Duffy-binding-like) domains and show extensive variation in sequence, size, and domain organization. Here we use biophysical methods to characterize the entire ~300-kDa ectodomain from IT4VAR13, a protein that interacts with the host receptor, intercellular adhesion molecule-1 (ICAM-1). We show through small angle x-ray scattering that IT4VAR13 is rigid, elongated, and monomeric. We also show that it interacts with ICAM-1 through the DBLβ domain alone, forming a 1:1 complex. These studies provide a first low resolution structural view of a PIEMP1 ectodomain in complex with its ligand. They show that it combines a modular domain arrangement consisting of individual ligand binding domains, with a defined higher order architecture that exposes the ICAM-1 binding surface to allow adhesion.

Malaria, caused by the parasite Plasmodium falciparum, remains one of the deadliest diseases affecting humanity. In 2010, there were an estimated 216 million episodes and 655,000 fatalities (1). The most severe symptoms occur during the erythrocytic phase of the parasite life-cycle and are associated with sequestration of parasitized erythrocytes within the microvasculature (2). This protects the parasite from detection and destruction by the spleen and causes pathology due to accumulation of infected erythrocytes in tissues, resulting in inflammation and occlusion of blood flow. Cerebral malaria is a major complication in the development of severe disease and is linked to erythrocyte accumulation within cerebral vessels and characterized by neurological symptoms such as impaired consciousness and seizures (2, 3).

Cytoadhesion is mediated by parasite-encoded PIEMP1 proteins that are exposed on the surfaces of infected erythrocytes (4). These multidomain proteins are encoded by ~60 highly divergent var genes (5). In most cases the parasite only expresses one PIEMP1 at a time (6). As well as allowing the parasite to evade immune responses, switching of var gene expression can alter its adhesion phenotype (4). The PIEMP1 proteins are major targets for acquired, protective immunity that prevents severe disease and are, therefore, targets for vaccine development (7).

Numerous human receptors for P. falciparum have been identified (8) with CD36 and intercellular adhesion molecule-1 (ICAM-1),2 most commonly found to interact with infected erythrocytes (9, 10). ICAM-1 is a transmembrane glycoprotein with five extracellular immunoglobulin-like domains (D1–D5) and a short cytoplasmic tail. It is expressed at basal levels on endothelial cells, is greatly up-regulated during malaria-induced inflammation (2), and is important for efficient adhesion in vitro (11). Studies differ in their conclusions on the importance of ICAM-1 binding for the development of severe or cerebral disease. One recent work shows a correlation between

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The abbreviations used are: ICAM-1, intercellular adhesion molecule-1; DBL, Duffy-binding-like; CIDR, cysteine-rich interdomain region; SPR, surface plasmon resonance; SEC, size-exclusion chromatography; SAXS, small-angle x-ray scattering; AUC, analytical ultracentrifugation; RU, response units.

2 The abbreviations used are: ICAM-1, intercellular adhesion molecule-1; DBL, Duffy-binding-like; CIDR, cysteine-rich interdomain region; SPR, surface plasmon resonance; SEC, size-exclusion chromatography; SAXS, small-angle x-ray scattering; AUC, analytical ultracentrifugation; RU, response units.
ICAM-1 binding and cerebral malaria (12), and another showed increased, although not statistically significant, ICAM-1 binding in isolates from patients with clinical malaria compared with asymptomatic malaria (13). Infected erythrocytes also co-localize with ICAM-1 in patients who died of cerebral malaria (2), and vessels with higher ICAM-1 levels have higher levels of sequestration (14). However, although ICAM-1 may contribute to cerebral accumulation, it is not required for binding to endothelial cells derived from human brain tissue (15).

PIEMP1s have large modular ectodomains containing different numbers and combinations of Duffy binding-like (DBL) domains and cysteine-rich interdomain regions (CIDR). DBL and CIDR domains have been classified into different types (α-γ) based on sequence identity (16). The DBLβ domains have been shown to contribute to ICAM-1 binding (17, 18). However, it is uncertain whether single domains from PIEMP1 proteins fully mimic the ligand binding phenotypes of intact ectodomains. Indeed VAR2CSA, a PIEMP1 involved in pregnancy-associated malaria, binds its ligand, chondroitin sulfate proteoglycan with 100,000-fold greater affinity than any of its individual DBL domains (19, 20).

The multimeric state of PIEMP1s and the stoichiometry of engagement with their receptors are also unclear. Two DBL-containing proteins involved in invasion, Plasmodium vivax Duffy-binding protein (21) and P. falciparum erythrocyte binding antigen 175 (EBA-175) (22), exist as dimers in their crystal structures, and the putative interfaces used to interact with binding partners include contributions from both monomers. It has also been suggested that dimerization of DBL domains is necessary for ligand binding in PIEMP1 proteins (21).

There are currently no structures available for any PIEMP1 or constituent domain bound to its ligand, yet the molecular mechanisms of PIEMP1 recognition of host receptors are of paramount importance to understand the role of cytoadherence in severe malaria and the mechanisms of antigenic variation. They may also guide the development of vaccines through identification of potential vaccine targets. Two DBLβ domains from IT4VAR13 (residues 811–1201), IT4VAR16 (835–1228), IT4VAR27 (919–1323), IT4VAR31 (810–1212), and IT4VAR41 (836–1228) were cloned into a modified pET15b vector, and the hexahistidine-tagged proteins were expressed in Escherichia coli Origami B cells (Novagen) at 25 °C. Cells were pelleted and lysed, and proteins were purified using nickel-nitrilotriacetic acid-Sepharose (Qiagen). The hexahistidine tags were removed by incubation overnight at 4 °C with 1 mg tobacco etch virus (TEV) protease for every 10 mg of protein before passing through a nickel-nitrilotriacetic acid column to remove TEV, tag, and uncleaved material. The domains were further purified on a Superdex 200 16/60 size-exclusion chromatography column (GE Healthcare) in 20 mM Tris, pH 8.0, 150 mM NaCl.

ICAM-1D1D2 (UNIPROT ID P05362, 1–485) and ICAM-1D1D5 (1–212) fused to human IgG1 Fc were transiently expressed in COS-7 cells and purified by Protein A-affinity chromatography. The Fc tag was cleaved from ICAM-1D1D5-Fc using endoproteinase GluC. ICAM-1D1D2 (28–212) was transiently expressed in HEK293T cells and purified using Ni2+-affinity chromatography.

Circular Dichroism—IT4VAR13 and IT4VAR13DBL at 0.4 mg ml−1 were dialyzed into 50 mM phosphate buffer, pH 7.2. Spectra were recorded using an Aviv Model 410 spectrometer (Aviv Biomedical) at 25 °C. Measurements were taken in a 0.1-cm path length cell at 0.5-nm intervals between 180 and 290 nm with a 1-s averaging time for each data point. Three consecutive recordings were made, averaged, and corrected for absorption by buffer alone. Secondary structure estimation was performed using the CDSSTR method from DichroWeb (23).

Thermal Shift—IT4VAR13DBL was mixed at 0.5 mg ml−1 with SYPRO orange dye (Invitrogen) (1:250) in 20 mM Tris, pH 8.0, 150 mM NaCl. Buffer-alone controls were run in the same plate. The samples were heated in an iCycler IQ real-time PCR detection system (Bio-Rad) from 35 to 50 °C in 0.5 °C increments. The fluorescence intensity was measured with a charge-coupled device using an excitation and emission wavelength of 490 and 525 nm, respectively.

Surface Plasmon Resonance (SPR)—SPR experiments were carried out using a BIACore T100 instrument (GE Healthcare). All experiments were performed in 10 mM HEPES, pH 7.2, 150 mM NaCl, 50 mM EDTA, and 0.05% Tween 20 at 20 °C. Protein A was immobilized on a CM5 chip (GE Healthcare) by amine coupling. ICAM-1-Fc was captured onto the Protein A surface. Concentration series of full-length IT4VAR13 or DBLβ domains were flowed over the ICAM-1-Fc-bound surface at 30 ml/min for 240 s followed by buffer for 300 s. After each run, the biosensor chip was regenerated using 20 mM glycine, pH 1.5, which breaks the Protein A-Fc interaction. The specific binding response to ICAM-1 was obtained by subtracting the response given by analytes to an uncoupled Protein A surface. No binding was observed to Fc alone. The kinetic sensorgrams were fitted to a global 1:1 interaction model to allow calculation of $k_\text{on}$, $k_\text{off}$, and $K_D$, using BLAevaluation software 2.0.3 (GE Healthcare).

Analytical Size-exclusion Chromatography (SEC)—SEC was performed using a Superdex 200 10/300 column (GE Health-
Analytical Ultracentrifugation—Sedimentation velocity experiments were conducted using a Beckman Optima XL-1 analytical ultracentrifuge (Beckman Coulter) at 20 °C. All samples were prepared in 50 mM Tris, pH 8.0, 150 mM NaCl. Reference and sample were loaded into a double-sector centriplate and mounted in a Beckman An-60 Ti rotor. Sample concentrations were selected to give an absorbance of 1.0 at a wavelength of 280 nm. This required 3.2 μM for IT4VAR13, 5.5 μM for IT4VAR13DBLβ, and 10 μM for ICAM-1DIS. Components were mixed in 1:1 molar ratios to form complexes, again giving total absorbance of 1.0 at 280 nm wavelength. Centrifugation rates were chosen based on the predicted size of the species, and absorbance at 280 nm was measured across the sample cell every 2 min for 70–200 scans during centrifugation. Multiple scans were fitted to a continuous size distribution using SEDFIT (24). The solvent density and viscosity and the partial specific volumes for the different samples were calculated using SEDNTERP (25). The solvent viscosity was 0.010312 poise, and solvent density was 1.00585 g ml⁻¹. The partial specific volumes based on the amino acid sequence for the proteins were 0.7231 cm³ g⁻¹ (IT4VAR13), 0.7222 cm³ g⁻¹ (IT4VAR13DBLβ), and 0.7358 cm³ g⁻¹ (ICAM-1DIS).

Small-angle X-ray Scattering (SAXS)—SAXS data were collected at the ID14-3 beamline at the European Synchrotron Radiation Facility using a wavelength (λ) of 0.931 Å. The sample-to-detector distance was 2.43 m, resulting in scattering vectors, q, ranging from 0.04 to 0.61 Å⁻¹. The scattering vector is defined as 4π sin(q) / λ. The scattering angle. Scattering was detected using a Pilatus image reader.

Samples were prepared at 5.0, 2.5, 1.25, 0.63, and 0.31 mg ml⁻¹ in 20 mM Tris, pH 8.0, 150 mM NaCl. Complexes were mixed in a 1:1 molar ratio for 30 min to give a total combined protein concentration of 5 mg ml⁻¹ before producing the dilution series. All experiments were performed at 20 °C. Ten consecutive frames each with an exposure time of 10 s were recorded for proteins and buffer. Each frame was carefully inspected for protein radiation damage before averaging unaffected images.

SAXS data were normalized to the intensity of the incident beam and averaged, and background was subtracted using PRIMUS (26, 27). They were investigated for aggregation using Guinier plots (28). The scattering curves were extrapolated to zero concentration, and composite curves were generated by scaling and merging low concentration data with high concentration data to counter effects due to protein-protein concentration.

The distance distribution function (P(r)) was derived using indirect Fourier transform (29), from which the radius of gyration (Rg) and the maximum particle dimension (Dmax) were estimated. The Porod volume of the hydrated particle was calculated as described (30). Ab initio shape reconstructions were calculated using DAMMIF (31) and averaged with DAMAVER (32). DAMMIF was run in slow mode using 50 spherical harmonics for the processing of IT4VAR13.
FIGURE 1. The DBLβ domain of IT4VAR13 binds ICAM-1D1D2 with nanomolar affinity. a, shown is a schematic of IT4VAR13 and ICAM-1. IT4VAR13 contains seven domains: five DBL domains (DBLα (gray), β (red), δ (magenta), γ (teal), and ζ (light blue)) and two CIDR domains (CIDRα and γ (both blue)). ICAM-1 has five immunoglobulin-like domains (D1–D5 (yellow)). Both proteins have a single transmembrane (TM) domain. IT4VAR13 domain boundaries were set according to Rask et al. (16), and ICAM-1 domain boundaries were according to Pfam. b, shown is a size-exclusion chromatogram of IT4VAR13 and IT4VAR13DBLβ, with purity shown by SDS-PAGE. IT4VAR13 is predominantly monomeric but with a shoulder indicating the presence of higher order aggregates that elute near the void volume. IT4VAR13DBLβ is similarly predominantly monomeric, with a low percentage of dimer. mAu, milliabsorbance units. c, shown is a representative fluorescence melting curve for IT4VAR13DBLβ in 20 mM Tris, pH 8.0, 150 mM NaCl. The temperature was altered in 0.5 °C increments. The y axis shows fluorescence (arbitrary units). IT4VAR13DBLβ has a melting temperature (Tm) of 47.5 °C. d, secondary structure analysis by CD is shown. Spectra were recorded between 190 and 240 nm for IT4VAR13 (●) and IT4VAR13DBLβ (ƒ). For each protein, three measurements were averaged, normalized for buffer absorption, and deconvoluted using an experimental model. Fitting residuals for IT4VAR13 (blue) and IT4VAR13DBLβ (red) are shown. IT4VAR13 is composed of 46% α-helix and 18% β-strand, and IT4VAR13DBLβ is composed of 57% α-helix and 6% β-strand. Shown are SPR sensograms (upper panels) with fitting residuals (lower panels) for the binding of IT4VAR13 to ICAM-1D1D5-Fc at concentrations of 5, 10, 20, 30, 40, and 50 nM (e) and IT4VAR13DBLβ to ICAM-1D1D5-Fc (10, 20, 30, 40, and 50 nM) (f). Binding was conducted with a 4-min association phase and 6-min dissociation phase at a constant flow rate of 30 ml min⁻¹. In each case the lower panel shows residuals from binding.
VAR2CSA for placental chondroitin sulfate proteoglycan (19, 20).

The binding of IT4VAR13DBL to ICAM-11D1D5-Fc was investigated using the same chip surface. The binding curves showed a partially biphasic interaction with a major component that fitted with an affinity ($K_D = 2.6 \text{ nM}$) and kinetic parameters ($k_a = 3.5 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$, $k_d = 1.0 \times 10^{-4} \text{ s}^{-1}$), almost identical to the parameters observed for the entire IT4VAR13 ectodomain (Fig. 1f). We attribute the minor binding component, with extremely fast on and off rates, to nonspecific adhesion of IT4VAR13DBL to the ICAM-11D1D5-Fc-coated surface. Indeed, although IT4VAR13 is monomeric in solution, IT4VAR13DBL shows some propensity to dimerize and aggregate with removal of the DBL domain from its ectodomain context presumably exposing normally buried surfaces and causing some “stickiness” (see Fig. 4). Nevertheless, the similarity in binding parameters of the principal interactions of IT4VAR13DBL and IT4VAR13 for ICAM-1 is consistent with a modular architecture for IT4VAR13 in which a single DBL domain contributes the ICAM-1 binding site.

**Comparison of ICAM-1 Binding by DBL Domains from the IT4 Strain—**As well as IT4VAR13, six other P. falciparum proteins from the *P. falciparum* IT4 isolate have been shown to bind ICAM-1 (IT4VAR1, -14, -16, -27, -31, -41) (17). The seven DBL domains from these PfEMP1 proteins share 46% sequence identity (Fig. 2a). The DBL domains of four of these were expressed in *E. coli* and purified to homogeneity (Fig. 2b–e). The data were fitted to a one-site binding model, and the kinetic parameters determined are given in Table 2. In all cases comparison of the maximum binding levels with the amount of ICAM-11D1D5 coupled to the chip surface suggests formation of 1:1 complexes. ITVAR31DBL bound ICAM-11D1D5-Fc with the lowest affinity (144 nM). This is a more than 50-fold lower affinity than the strongest ICAM-1 binder (IT4VAR131DBL, 2.8 nM) and is consistent with the observation that parasite lines expressing ITVAR31 bind ICAM-1 weakly (42, 43).

The ability of different DBL domains to bind simultaneously to ICAM-11D1D5 was also studied using SPR. IT4VAR27DBL was initially bound to ICAM-11D1D5 followed by an injection of IT4VAR13DBL (Fig. 2f). Had the two domains been capable of binding both simultaneously and independently, the expected response would have been considerably higher than that observed. Instead, the increase in material bound to the surface upon injection of IT4VAR13DBL was comparable to the decrease caused by the dissociation of IT4VAR27DBL, making it likely that IT4VAR13DBL binds to an overlapping binding site on ICAM-1.

![Image](image_url)

**TABLE 1**

Kinetic parameters derived from SPR

| MW          | ICAM-1 capture levels | $k_a$ | $k_d$ | $K_D$ |
|-------------|-----------------------|-------|-------|-------|
| IT4VAR13-ICAM-11D1D5-Fc | 313.9 | 119.9 ± 2.8 | 2.7 ± 0.004 | 7.5 ± 0.01 | 2.8 |
| IT4VAR13DBL,ICAM-11D1D5-Fc | 45.3 | 102.8 ± 0.5 | 3.5 ± 0.013 | 9.0 ± 0.03 | 2.6 |

**FIGURE 2.** Characterization by SPR of the interactions of ICAM-1 with multiple DBL domains from the *P. falciparum* IT4 isolate. a: Shown is a phylogenetic tree of seven PfEMP1 DBL domains known to bind ICAM-1 from the *P. falciparum* IT4 isolate. Shown are sensorgrams (upper panels) with resulting residuals when fit to a one-site kinetic model (lower panel). Shown are SPR sensorgrams (upper panels) with fitting residuals (lower panels) for the binding of IT4VAR16DBL(1, 5, 10, 20, 30, 40, 50, 100, 250, 500, and 1000 nM) (c), IT4VAR31DBL(0.05, 0.1, 0.25, 1.25, and 10 μM) (d), and IT4VAR41DBL (1, 5, 10, 20, 30, 40, 50, 100, 250, 500, and 1000 nM) (e) to ICAM-11D1D5-Fc with an association phase of 4 min and a dissociation phase of 6 min at a flow rate of 30 μl min⁻¹. f: DBL domains from IT4VAR13 and IT4VAR27 recognize ICAM-1 with overlapping binding sites. Expected binding levels assuming IT4VAR13DBL bound to ICAM-1 in a mode independent and unaffected by the binding of IT4VAR27DBL are shown with a dashed line. Actual binding levels shown by a solid line.

**IT4VAR13 Is Monomeric and Binds to ICAM-1 with a 1:1 Stoichiometry—**The oligomeric states of PfEMP1s are uncertain. However, crystal structures suggest that DBL domain-containing proteins involved in erythrocyte invasion function as dimers (21, 22), and PfEMP1s have been predicted to do the same (21). Here we have used both analytical SEC and sedimentation velocity analytical ultracentrifugation (AUC) to assess
the oligomeric states of IT4VAR13 and IT4VAR13<sub>DBL</sub> both alone and in complex with ICAM-1.

IT4VAR13 is predominantly monomeric in solution with SEC revealing a single major peak (Fig. 3a). This was confirmed by AUC, which showed 92% of the protein to be monomeric, with a sedimentation coefficient of 9.2 S, corresponding to a mass of around 285 kDa (Fig. 3b), whereas 8% was found in a peak consistent with a dimer. A frictional coefficient, <i>f</i><sub>0</sub>, of 1.7 suggests that the ectodomain is elongated but unlikely to exist in a fully extended conformation. Isolated IT4VAR13<sub>DBL</sub> shows a greater propensity to form dimers both by SEC and AUC (Fig. 4), but despite the removal of neighboring domains, some 75–80% of the protein in still in the form of a monomer, with a sedimentation coefficient of 3.2 S consistent with a mass of around 50 kDa. IT4VAR13<sub>DBL</sub> is, therefore, no different from the PfEMP1 DBL domains structurally characterized to date, which all exist as monomers within their crystals (44–47).

ICAM-1<sup>1D1D5</sup> prepared by removal of the Fc tag was also predominately monomeric, as expected (48). SEC generated a single peak (Fig. 3a). This was confirmed by AUC (Fig. 3c, Table 3), which revealed more than 80% of the sample to form a primary species with a sedimentation coefficient of 3.2 S, corresponding with a mass of around 60 kDa, consistent with a glycosylated ICAM-1<sup>1D1D5</sup> monomer (Fig. 3c). Smaller peaks corresponding to masses of around 120 (10%) and 200 kDa (<5%) are most likely due to dimers. ICAM-1<sup>1D1D5</sup> has an <i>f</i><sub>0</sub> of 1.7 consistent with electron micrographs that show that ICAM-1 assumes a bent rod-like shape 18.7 nm in length (49).

To investigate whether binding to ICAM-1 induces multimerization, IT4VAR13 was mixed with a small excess of ICAM-1<sup>1D1D5</sup> (black, continuous line) from a calibrated Superdex S200 column. Shown are continuous sedimentation coefficient distributions that best describe the AUC data, with fitting residuals inset for IT4VAR13 (gray, wide dashes), ICAM-1<sup>1D1D5</sup> (in a ratio of 1:1.1) and analyzed by SEC. The ICAM-1<sup>1D1D5</sup> domain alone multimerizes more readily than IT4VAR13, which all exist as monomers within their crystals (44–47).

We confirmed this 1:1 stoichiometry by analyzing the IT4VAR13<sub>DBL</sub>-ICAM-1<sup>1D1D5</sup> complex. In this case, a greater variety of species was observed, consistent with the observation that the DBLβ domain alone multimerizes more readily than intact ectodomain. By both SEC and AUC, the predominant species observed in a mixture of IT4VAR13<sub>DBL</sub> and ICAM-1<sup>1D1D5</sup> is a 1:1 complex. In SEC, ICAM-1<sup>1D1D5</sup> was mixed with a
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There is a small excess (1:1.1) of IT4VAR13\textsuperscript{DBL\&beta}, leading to the formation of a predominant 1:1 complex, with a smaller amount of 2:2 complex. In AUC, two main peaks were observed, with sedimentation coefficients of 3.4 S (\(~\sim 50\) kDa and consistent with free IT4VAR13\textsuperscript{DBL\&beta} and ICAM-1\textsuperscript{1D1D5}) and 5.9 S (\(~\sim 120\) kDa, consistent with a complex of one IT4VAR13\textsuperscript{DBL\&beta} and one ICAM-1\textsuperscript{1D1D5}).

Therefore, IT4VAR13 and ICAM-1\textsuperscript{1D1D5} are both predominantly monomeric in solution and combine to form a 1:1 complex. When removed from its ectodomain context, IT4VAR13 shows an increased and presumably artifactual propensity to multimerize but still forms a predominately 1:1 complex. In AUC, two main peaks were observed, with sedimentation coefficients of 3.4 S (\(~\sim 50\) kDa and consistent with free IT4VAR13\textsuperscript{DBL\&beta} and ICAM-1\textsuperscript{1D1D5}) and 5.9 S (\(~\sim 120\) kDa, consistent with a complex of one IT4VAR13\textsuperscript{DBL\&beta} and one ICAM-1\textsuperscript{1D1D5}).

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**Low Resolution Structures of IT4VAR13\textsuperscript{DBL\&beta} Bound to ICAM-1 Fragments**—There are currently no structures of PfEMP1 proteins or domains bound to protein ligands. To understand better the architecture of the complex between PfEMP1 and ICAM-1, we performed SAXS with IT4VAR13\textsuperscript{DBL\&beta} either alone or in complex with ICAM-1\textsuperscript{1D1D2} or ICAM-1\textsuperscript{1D1D5} (Fig. 5). The radius of gyration, determined from Guinier plots, is 3.4 nm for the IT4VAR13\textsuperscript{DBL\&beta}-ICAM-1\textsuperscript{1D1D2} complex and 4.2 nm for the IT4VAR13\textsuperscript{DBL\&beta}-ICAM-1\textsuperscript{1D1D5} complex compared with 3.0 nm for IT4VAR13\textsuperscript{DBL\&beta} alone (Table 4). Concomitant increases in the Porod volume and apparent molecular weight were consistent with SEC and AUC data in suggesting that the interaction is predominantly 1:1. When compared with the distance distribution function for IT4VAR13\textsuperscript{DBL\&beta}, both complexes have a slightly skewed profile with a tail extending toward a \(D_{\text{max}}\) of 12 nm for the shorter complex and 18.5 nm for the longer complex, indicating that ICAM-1 protrudes from the DBL\&beta domain, resulting in an elongated particle (Fig. 5b).

For each complex, 20 low resolution shape reconstructions were derived from the experimental data using \textit{ab initio} modeling. As only one of the binding partners (ICAM-1) has a known high resolution structure, rigid body modeling was not employed, and the resultant envelopes were derived solely from scattering data. The models were averaged, and structures of IT4VAR13\textsuperscript{DBL\&beta} (from a homology model based on EBA-175 (PDB ID 1ZRL) and DBL3x (PDB ID 3BQK) and ICAM-1\textsuperscript{1D1D2} (PDB ID 1IAM)) were simultaneously docked into the shorter envelope with a 1:1 stoichiometry (Fig. 6a).

The envelope obtained for IT4VAR13\textsuperscript{DBL\&beta}-ICAM-1\textsuperscript{1D1D5} was longer in comparison (160 Å compared with 127 Å), consistent with additional domains extending away from DBL\&beta. The IT4VAR13\textsuperscript{DBL\&beta}-ICAM-1\textsuperscript{1D1D2} complex was modeled into the globular domain with the ICAM-1 D3 domain positioned below the D2 domain (Fig. 6b). However, domains D4 and D5 could not be positioned into the envelope, most likely due to flexibility of ICAM-1. Although there is substantial contact between the D1 and D2 domains, there is a flexible hinge between D2 and D3, and the ICAM-1\textsuperscript{1D1D5} crystal structure shows a further kink of \(~\sim 160°\) between domains D3 and D4 (49, 50). Although D1 and D2 are rigidly held in place relative to the DBL\&beta domain by extensive D1-DBL\&beta contacts, there is enough conformational flexibility in the D2-D3 linker to allow multiple

![FIGURE 4. Stoichiometry of the interaction between IT4VAR13\textsuperscript{DBL\&beta} and ICAM-1. Stoichiometry was determined by analytical SEC (a) and ultracentrifugation (b). mA\textsubscript{u}, milliabsorbance units. a, shown is a size-exclusion chromatogram of IT4VAR13\textsuperscript{DBL\&beta} (gray, wide dashes), ICAM-1\textsuperscript{1D1D2} (black, short dashes) and IT4VAR13\textsuperscript{DBL\&beta}-ICAM-1\textsuperscript{1D1D5} (black, continuous line). b, shown is continuous sedimentation coefficient distribution that best describes the AUC data for IT4VAR13\textsuperscript{DBL\&beta} incubated with ICAM-1\textsuperscript{1D1D5} for 30 min before centrifugation with the continuous sedimentation coefficient distribution for IT4VAR13\textsuperscript{DBL\&beta} inset for comparison. The distribution for ICAM-1\textsuperscript{1D1D5} is in Fig. 3c.]

| TABLE 3 |
|---|
| Summary of AUC sedimentation velocity results |
| Theoretical molecular weights of monomeric and 1:1 species are given in parentheses. |

|                  | Frictional coefficient \((f/f_0)\) | Sedimentation coefficient \((S)\) | MW\textsubscript{app} | Predicted species |
|------------------|-----------------------------------|---------------------------------|-----------------|------------------|
| IT4VAR13 (314 kDa) | 1.66                               | 4.2                             | 89.1            | Truncation       |
| 1D1D5 (50 kDa)    | 1.67                               | 3.2                             | 118.0           | Dimer           |
| IT4VAR13:ICAM-1D1D5 (364 kDa) | 1.74 | 3.3                             | 71.0            | ICAM-1D1D5 monomer |
| IT4VAR13:ICAM-1D1D5 (45 kDa) | 1.37 | 3.1                             | 51.9            | Monomer         |
| IT4VAR13:ICAM-1D1D5 (95 kDa) | 1.53 | 3.4                             | 51.0            | Unbound monomeric IT4VAR13\textsuperscript{DBL\&beta}/ICAM-1D1D5 |

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conformations of D3-D5 relative to the DBLβ-D1D2 complex, disordering these domains relative to D2. As the SAXS profile is a weighted average over all accessible structures (51), such flexibility broadens the density around D3 and causes D4 and D5 to be absent from the averaged envelope. A similar degree of disorder was seen in cryo-EM images of ICAM-1-D1D5 bound to human rhinovirus (52) and in higher resolution reconstructions of the coxsackievirus A21-ICAM-1-D1D5 complex (53) with D4 and D5 not observed due to disorder in either case.

In both cases docking positioned the convex surface of DBLβ in contact with the D1 domain of ICAM-1. DBL domains have previously been described as containing three subdomains (54). Both subdomain 2 and the proximal end of subdomain 3 are positioned close to ICAM-1 here, suggesting a large protein-protein interface and consistent with previous analysis from chimeric proteins and truncations (55–57). Residues equivalent to the mutations that affect the binding of IT4VAR16 to ICAM-1 (17) are located within this interface. Mutational studies that suggested that the interface lies on the “BED” side (that containing β-strands B, E, and D) of the ICAM-1 D1 domain (40, 41) are also consistent with this solution. The D2 domain is in close proximity to DBLβ and may also be capable of forming some interactions with subdomain 2. The observed interface is similar to that proposed by Bertonati and Tramontano (58) from in silico docking, albeit with the orientation that ICAM-1 approaches the DBL domain rotated ~75°.

The Low Resolution Structure of IT4VAR13 and Its Complex with ICAM-1—To see how domains are organized within the PfEMP1 ectodomain and how they accommodate binding to ICAM-1, we collected SAXS data for both IT4VAR13 and the IT4VAR13-ICAM-1-D1D5 complex (Fig. 5c). The $R_g$ for the complex (8.6 nm) is larger than that observed for IT4VAR13 (8.1 nm), but both species have a similar $D_{max}$, indicating that ICAM-1 does not bind in a head-to-head arrangement that increases the maximum particle diameter (Fig. 5d). A Kratky plot (data not shown) indicates that the ectodomain forms a rigid structure.

Low resolution shape reconstructions were derived from the experimental data using $ab initio$ modeling. The IT4VAR13 envelope has dimensions of $260 \times 146 \times 50$ Å (Fig. 6c) and adopts a zig-zag conformation that deviates considerably from a rod-like structure, as expected from an $f/f_0$ of 1.7. This is very different from the compact architecture of VAR2CSA, the only other ectodomain characterized to date. However, neither IT4VAR13 nor VAR2CSA consists of a series of independent domains joined by flexible linkers. To provide more detailed structural insight, homology models of the individual domains were built using known crystal structures, allowing 75% of the IT4VAR13 ectodomain to be modeled. Models for these seven domains were docked into the envelope, having been restrained such that each domain follows in sequential order as dictated by the primary structure.

The SAXS-derived structure of the IT4VAR13-ICAM-1-D1D5 complex, determined independently from that of IT4VAR13 and solely from scattering data, reveals an ectodomain architecture very similar to that in the absence of ligand, suggesting few if any conformational changes take place on ligand binding (Fig. 6d). The main difference is the additional mass immediately adjacent to DBLβ, which is consistent in size and position with the mass attributed to ICAM D1-D3 in reconstructions of data from the IT4VAR13DBLβ-ICAM-1D1D5 complex. Therefore, we ob-

![Image](https://via.placeholder.com/150)

**FIGURE 5.** SAXS analysis of IT4VAR13-ICAM-1 complexes. a, shown is theoretical scattering calculated from $ab initio$ reconstructions (continuous lines with IT4VAR13DBLβ in red, IT4VAR13DBLβ-ICAM-1D1D2 in blue, and IT4VAR13DBLβ-ICAM-1D1D5 in green) superimposed onto experimental scattering intensity curves (squares). Guinier plots are inset. b, shown are distance distribution functions, P(r), plots for IT4VAR13DBLβ (squares, red; IT4VAR13DBLβ-ICAM-1D1D2 (circles, blue), and IT4VAR13DBLβ-ICAM-1D1D5 (triangles, green)). c, theoretical scattering is calculated from $ab initio$ reconstructions for full-length IT4VAR13 ectodomain (continuous lines with IT4VAR13 in red and IT4VAR13-ICAM-1D1D5 in blue) superimposed onto the experimental scattering intensity curves (squares). d, shown are P(r) plots for IT4VAR13DBLβ (squares, red) and IT4VAR13DBLβ-ICAM-1D1D5 (circles, blue). The P(r) functions were calculated from the scattering intensity (I(0)) and normalized to unity at their maxima.

**TABLE 4**

| Domain | $R_g$ (theoretical) | $R_g$ (NN) | $D_{max}$ | Volume | MW | Recons. | SD | χ² |
|--------|---------------------|-----------|-----------|--------|-----|---------|----|----|
| IT4VAR13DBLβ (45 kDa) | 2.6 | 2.97 ± 0.02 | 12.5 | 106.0 | 62.3 | 0.46 ± 0.03 | 2.7 |
| IT4VAR13DBLβ-ICAM-1D1D2 (70 kDa) | 3.1 | 3.41 ± 0.01 | 12.0 | 141.4 | 85.2 | 0.55 ± 0.02 | 4.4 |
| IT4VAR13DBLβ-ICAM-1D1D5 (95 kDa) | 4.0 | 4.21 ± 0.00 | 18.5 | 154.9 | 91.1 | 0.68 ± 0.04 | 4.6 |
| IT4VAR13 (314 kDa) | 8.1 | 8.1 ± 0.00 | 27.5 | 556.3 | 227.2 | 0.78 ± 0.06 | 1.3 |
| IT4VAR13-ICAM-1D1D5 (364 kDa) | 8.3 | 8.6 ± 0.00 | 26.5 | 649.4 | 382.0 | 1.06 ± 0.03 | 3.7 |

The theoretical radius of gyration ($R_g$) was calculated using HydroPro (36) using atomic coordinates from docking into $ab initio$ SAXS shape reconstructions. Experimental $R_g$ values were derived from the Guinier plot using AutoRg (27). The maximum particle diameter ($D_{max}$) was calculated using Gnom, and the Porod volume of the hydrated particle (Volume) was calculated as described (30). The apparent molecular weight was estimated from Porod volume/1.7. Theoretical molecular weights of monomeric and 1:1 species are given in parentheses. Twenty low resolution shape reconstructions were derived from the experimental data using $ab initio$ modeling and the mean normalized spatial discrepancy (NSD), used to quantify the agreement between individual reconstructions (35). The χ² for the fit of the best model to the experimental data is shown.
served contact only between ICAM-1 and DBL and see no significant architectural rearrangements of the rigid ectodomain and no multimerization upon ligand binding.

**DISCUSSION**

Sequestration of parasitized erythrocytes to the brain microvasculature through the interaction of PfEMP1s with host receptors including ICAM-1 is associated with cerebral malaria. Cytoadherence is a potential target of novel therapeutics to combat malaria, especially as it persists after conventional drugs have killed the parasite (59). To develop a greater understanding of the molecular underpinnings of pathogen-host receptor interactions, we used biophysical techniques to characterize the interaction between a PfEMP1 and human ICAM-1.

Here we show using SEC, AUC, and SAXS that IT4VAR13 is monomeric, and we present its low resolution structure. The protein has a rigid elongated shape that shows considerable deviation from a canonical rod with the DBL2 and CIDR2 domains protruding from the longest axis. The SASS-derived structures of VAR2CSA (20, 60) show a compact organization that dispelled the notion that PfEMP1 domains are ordered as “beads on a string.” Here, we show that PfEMP1s can form alternative global shapes, dependent on the combination of domains present. VAR2CSA is unusual among PfEMP1 proteins, as it comprises six DBL domains and a single CIDR domain. IT4VAR13 has a more typical domain architecture, and the rigid, elongated arrangement may be more frequently observed.

If PfEMP1 proteins are generally modular arrays of ligand binding domains, why have a rigid architecture, with all of the resulting structural constraints on protein evolution? A possible reason is to reduce the surface area that is exposed to immune detection. A rigid architecture, formed by the close packing of domains, will hide surfaces that would otherwise be exposed in a flexible molecule. PfEMP1 proteins are under diversifying selection to evade immune detection and yet must maintain binding properties needed for cytoadhesion. Perhaps rigid ectodomains help reduce immune detection without imposing costly constraints on the evolu-

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**FIGURE 6. SAXS-derived architectures of IT4VAR13-ICAM-1 complexes.** Models of the IT4VAR13DBLICAM-1D1D2 (a) and IT4VAR13DBLICAM-1D1D5 (b) complexes are based on ab initio SAXS envelopes. IT4VAR13DBL (red) was homology-modeled using EBA-175 (PDB ID 1ZRL) and DBL3x (PDB ID 3BQK) as templates. ICAM-1 domains 1 (D1) and 2 (D2) were extracted from PDB ID 1IAM, and domain 3 was from PDB ID 1P53 (all yellow). ICAM-1 residues identified as reducing infected erythrocyte adhesion under flow conditions are shown as blue spheres. c, a low resolution structure of IT4VAR13 was determined from SAXS data showing front and side views. All domains were modeled using homology to known structures. d, a low resolution structure of the IT4VAR13-ICAM-1D1D5 complex was determined from SAXS data. ICAM-1D1D5 exclusively contacts IT4VAR13DBL.
tions, the maintenance of immune diversity or the maintenance of ligand binding.

Previous studies identified DBLβ as an ICAM-1 binding domain. Here we use SPR to confirm this observation and show that isolated DBLβ domains bind to ICAM-1 with the same affinity as intact ectodomain, suggesting that it alone mediates ICAM-1 binding. Indeed, comparison of SAXS reconstructions of IT4VAR13 in the presence and absence of ICAM-1 show a single addition of mass due to ICAM-1 that lies immediately adjacent to the DBLβ domain. Although these SAXS-derived envelopes are low resolution, docking in the ICAM-1 structure, and a model of DBLβ gives an interface consistent with previous mutagenesis studies that predict that the convex surface of DBLβ interacts with the BED face of ICAM-1 (17, 39).

The elongated ectodomain structure positions the DBLβ domain ~150 Å above the erythrocyte membrane surface. This, together with the clustering of PfEMP1s on knobs that protrude 110–160 nm from the red blood cell will position the ICAM-1 binding site for ready access to its ligand (61). The interaction of a domain close to the tip of the ectodomain with domains at the tip of ICAM-1, therefore, allows efficient recognition. The elongated nature of the ectodomain also makes it possible for other domains in the PfEMP1 to mediate interactions with other receptors, allowing binding synergy.

The tight nanomolar affinity (~3 nM) and slow dissociation rate ($K_d = 7.5 \times 10^{-4}$ s$^{-1}$) is consistent with the need for the interaction to be strong enough to pull the infected erythrocyte out of circulation to permit accumulation despite blood flow or to cause rolling adhesion under shear stress. This interaction is stronger and has a slower dissociation constant than interactions involved in selectin-mediated rolling adhesion of leukocytes from the bloodstream (61). Affinities in the nanomolar range (3–144 nM), characterized by slow dissociation kinetics, were also observed for other DBLβ domains from the $P$. falciparum IT4 isolate, suggesting a consistent mode of interaction with ICAM-1 within the family.

The stoichiometry of the engagement of DBL domains with their receptors has been under debate. Studies of DBL domains from proteins that mediate interactions involved in invasion have led to the suggestion that dimerization is necessary for ligand binding (21). For the interaction between PfEMP1 and ICAM-1, SPR, AUC, SEC, and SAXS all reveal a 1:1 stoichiometry, and multimerization is not required for, or driven by, binding.

These studies reveal many differences between IT4VAR13 and VAR2CSA, the only other intact PfEMP1 ectodomain with a low resolution structure determined to date. VAR2CSA adopts a compact structure with more than a single DBL domain required to form a high affinity, specific binding site for its carbohydrate ligand (19, 20). In contrast, IT4VAR13 is rigid but elongated, positioning a single ICAM-1 binding DBLβ domain close to its tip for efficient recognition of this protein ligand. This highlights some of the degree of diversity available to this adaptable adhesion protein, with changes in domain organization and use modulating the ability of these antigens to recognize their ligands as they perform their dual role of adhesion and immune evasion.

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