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Structural and Functional Insights into the Malaria Parasite Moving Junction Complex

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Abstract

Members of the phylum Apicomplexa, which include the malaria parasite Plasmodium, share many features in their invasion mechanism in spite of their diverse host cell specificities and life cycle characteristics. The formation of a moving junction (MJ) between the membranes of the invading apicomplexan parasite and the host cell is common to these intracellular pathogens. The MJ contains two key parasite components: the surface protein Apical Membrane Antigen 1 (AMA1) and its receptor, the Rhopty Neck Protein (RON) complex, which is targeted to the host cell membrane during invasion. In particular, RON2, a transmembrane component of the RON complex, interacts directly with AMA1. Here, we report the crystal structure of AMA1 from Plasmodium falciparum in complex with a peptide derived from the extracellular region of PRON2, highlighting clear specificities of the P. falciparum RON2-AMA1 interaction. The receptor-binding site of PIAMA1 comprises the hydrophobic groove and a region that becomes exposed by displacement of the flexible Domain II loop. Mutations of key contact residues of PRON2 and PIAMA1 abrogate binding between the recombinant proteins. Although PRON2 contacts some polymorphic residues, binding studies with PIAMA1 from different strains show that these have little effect on affinity. Moreover, we demonstrate that the PRON2 peptide inhibits erythrocyte invasion by P. falciparum merozoites and that this strong inhibitory potency is not affected by AMA1 polymorphisms. In parallel, we have determined the crystal structure of PIAMA1 in complex with the invasion-inhibitory peptide R1 derived by phage display, revealing an unexpected structural mimicry of the PRON2 peptide. These results identify the key residues governing the interactions between AMA1 and RON2 in P. falciparum and suggest novel approaches to antimalarial therapeutics.

Introduction

Plasmodium spp., and P. falciparum in particular, are devastating global pathogens that place nearly half the human population at risk to malaria, leading to more than 250 million cases yearly and over one million deaths [1]. The success of the malaria parasite can be attributed to its intracellular lifestyle, invading host cells both in liver and blood stages. Invasion of red blood cells is an active process involving a moving junction (MJ), which is formed by intimate contact between erythrocyte and parasite membranes and is thought to be coupled to the parasite’s actin-myosin motor [2,3]. A number of merozoite antigens, either exposed on the surface or stored in secretory organelles, play a role in the invasion process [4]. One of these is Apical Membrane Antigen 1 (AMA1), a type-one transmembrane protein secreted from the micronemes to the merozoite surface and present at the MJ [5,6]. AMA1 is highly conserved in the Plasmodium genus [6] and, moreover, in the Apicomplexa phylum to which Plasmodium belongs [7,8], suggesting a common functional role in diverse host cell invasion scenarios. In the apicomplexan organism Toxoplasma gondii, the receptor for AMA1 was shown to be Rhopty Neck Protein 2 (RON2), a component of the parasite-derived RON protein complex that is secreted into the host cell during invasion and integrated into the host cell membrane [9,10]. This interaction was subsequently confirmed in P. falciparum as well [11,12]. Apicomplexans thus provide both receptor and ligand to drive active invasion.

In many malaria-endemic regions, P. falciparum has become resistant to classic drugs, such as chloroquine, and is rapidly developing resistance to recently introduced drugs. Since both
Author Summary

Malaria arises from infection of erythrocytes by single-cell parasites belonging to the genus Plasmodium, the species P. falciparum causing the most severe forms of the disease. The formation of a moving junction (MJ) between the membranes of the parasite and its host cell is essential for invasion. Two important components of the MJ are Apical Membrane Antigen 1 (AMA1) on the parasite surface and the Plasmodium rhoptry neck (RON) protein complex that is translocated to the erythrocyte membrane during invasion. The extra-cellular region of RON2, a component of this complex, interacts with AMA1, providing a bridge between the parasite and its host cell that is crucial for successful invasion. The parasite thus provides its own receptor for AMA1 and accordingly this critical interaction is not subject to evasive adaptations by the host. We present atomic details of the interaction of AMA1 with the carboxy-terminal region of RON2 and shed light on structural adaptations by each apicomplexan parasite to maintain an interaction so crucial for invasion. The structure of the RON2 ligand bound to AMA1 thus provides an ideal basis for drug design as such molecules may be refractory to the development of drug resistance in P. falciparum.

AMA1 and RON2 are specific to Apicomplexa and essential for invasion, interruption of the AMA1-RON2 interaction presents an ideal new target for the design and development of inhibitors. This is supported by the recent observation that the invasion-inhibitory peptide R1 [13,14] blocks interaction between AMA1 and the RON complex in P. falciparum [15], but due to the polymorphism of AMA1, the effectiveness of this peptide inhibitor is limited to a subset of parasite isolates. Interestingly, R1 does not prevent apical contact but no formation of a functional MJ ensues from this event [15].

Crystal structures of AMA1 in complex with invasion-inhibitory antibodies [16,17] have implicated a hydrophobic groove on Domain I (DI) of AMA1 as being critical for function. The topological nature of the AMA1 groove [18] is conserved in T. gondii AMA1 [19], and contains a number of residues that are conserved or semi-conserved across Plasmodium species, as well as other members of Apicomplexa [21], suggesting that it contributes to the receptor-binding site of AMA1. This was recently confirmed by the crystal structure of TgAMA1 in complex with a synthetic peptide, TgRON2sp, which inserts in the groove of TgAMA1 [22].

Here, we report the crystal structure of the complex formed between AMA1 and peptide segments of RON2, which, together with our previous structural results on the TgAMA1-TgRON2 co-structure [22], highlights a conserved, crucial interaction in apicomplexan host cell invasion. Functional characterization of hot-spot residues driving AMA1-RON2 complex formation leads to a deeper understanding of key interactions occurring at the MJ of P. falciparum and reveals the molecular basis of cross-strain reactivity while preserving specificity for the species. We also describe the crystal structure of AMA1 in complex with the invasion-inhibitory peptide R1 [14], and show that this peptide presents an intriguing structural mimicry of RON2. Collectively, our results provide an important structural basis for designing cross-strain reactive molecules that inhibit invasion by P. falciparum.

Results

PfRON2sp specifically binds to PfAMA1

From the 67-residue construct, PfRON2-5, that we previously showed to have affinity for PfAMA1 [11], and guided by the TgAMA1-TgRON2sp structure [22], we synthesized two analogous PfRON2 peptides: PfRON2sp1 (residues 2021–2059; numbering from the initiation methionine in PF14_0495), and PfRON2sp2 (residues 2027–2055). Significantly, there is no polymorphism in this sequence among P. falciparum isolates. Both constructs incorporate a disulphide-bound β-hairpin loop proposed to be critical in complex formation [22] while PfRON2sp2 is truncated at both the N- and C-termini (Fig. 1A). Since the extracellular region of PfRON2 is non-polymorphic, we determined the affinity of both peptides for PfAMA1 by Surface Plasmon Resonance (SPR) measurements using the 3D7, CAMP, FVO and HB3 proteins to explore the possible effects of AMA1 polymorphisms. The affinity of PfAMA1 from 3D7 for PfRON2sp1 is 25-fold higher than for PfRON2sp2 (Fig. 1B to E, Table 1), highlighting a moderate, yet influential, role for the N- and C-terminal tails. Interestingly, Kd values for the PfRON2sp peptides showed no significant variation in binding to PfAMA1 from the four strains.

PfRON2sp1 and PfRON2sp2 were co-crystallized with the first two ectoplasmic domains (DI, DII) of recombinant PfAMA1 3D7 or CAMP strains, respectively. The co-structure of PfAMA1 3D7 PfRON2sp1 (PDB entry code 3ZWZ) was refined to 2.2 Å resolution, while PfAMA1 CAMP PfRON2sp2 (PDB entry code 3SRI) was refined to 1.6 Å resolution (Tables 2, 3). The two co-structures overlay with a root mean square deviation (rmsd) of 0.81 Å in 304 Cα positions, and the two peptides alone overlay with a rmsd of 0.34 Å over the complete length of the modeled PfRON2sp2 (25 Cα) (Fig. 2A). These data confirm that the reduced affinity of PfRON2sp2 is due to the truncated N- and C-termini. Since PfRON2sp1 is more biologically relevant than its truncated counterpart, it is used for the following analyses unless otherwise noted.

PfRON2sp1, traced from Thr2023 to Leu2058, includes a disulphide bridge between Cys2037 and Cys2049 and makes several direct contacts with PfAMA1 (Fig. S1), resulting in a total buried surface area of 3154 Å² (1441 Å² for PfAMA1 and 1713 Å² for PfRON2sp1). Overall, the binding paradigm established by TgAMA1-TgRON2sp [22] is maintained, with an N-terminal helix seated at one end of the AMA1 receptor-binding groove and extended through an ordered coil to a disulphide-closed β-hairpin loop, generating a U-shaped conformation (Fig. 2A). Similarly, exposing a functional receptor-binding groove on AMA1 requires displacement of the extended non-polymorphic DII loop, which adopts a disordered state (not modeled between Lys351 to Ala387); this region is stabilized by DI in apo PfAMA1 (Fig. 2B).

Intriguingly, the backbone of the N-terminal helix and additional coil of PfRON2sp1 (2024-QQAKDIGAG-2032) overlays remarkably well with a section of the apo PfAMA1 DII loop (360-YEKKIEGFK-368) (rmsd<0.4 Å), which also includes a helical region (Fig. 2B - box 1). Three water molecules buried by the DII loop in the apo form are retained in the receptor-binding state and facilitate a network of hydrogen bonds that bridge PfAMA1 DI to either the DII loop or PfRON2sp in apo PfAMA1 or the receptor complex, respectively (Fig. 2C). The majority of intermolecular contacts are formed by the segment Lys351 to Arg2041, a residue specific to the P. falciparum species, located at the tip of the β-hairpin with its guanidyl group fitting snugly into a preformed pocket of PfAMA1 (Fig. 2D).
R1 occupies the PfRON2sp-binding site on PfAMA1

The invasion-inhibitory peptide R1, comprising 20 residues (VFAEFLPLSKFGSMHLK) [14], has been shown by nuclear magnetic resonance (NMR) to bind to the PfAMA1 hydrophobic groove, but this study gave little structural detail of the interaction [15]. We therefore crystallized PfAMA1 3D7 (DI and II) with R1 to compare with the PfRON2 complex. Surprisingly, two molecules of R1 are bound to PfAMA1, which we denote respectively as the major peptide (R1-major), lying deeply in the binding groove, and the minor peptide (R1-minor), lying above R1-major and making fewer contacts with PfAMA1 (Fig. 3 and Table S1). Several solvent molecules bridge directly between
PfAMA1 and R1-major. As in the PfAMA1-PfRON2sp complex, the N-terminus of R1-major binds to a region of PfAMA1 that becomes exposed after displacement of the DII loop.

R1-major makes several direct contacts with PfAMA1 (113 interatomic distances <3.8Å), including 19 hydrogen bonds and a salt bridge between the amino group of Lys-P11 (R1 peptide residues numbers are prefixed by P) and the Asp227 carboxylate group of PfAMA1 (Table S1A). Contacts made by R1-minor to PfAMA1 are fewer (26 contacts <3.8Å) and include only five hydrogen bonds (Table S1B). Interactions between R1-major and R1-minor are maintained by a total of 24 interatomic contacts, including three hydrogen bonds (Table S1C). In total, 3025Å² of molecular surface is buried between PfAMA1 and the two peptides, with R1-major contributing about 75% to this area. The buried surface between R1-major and R1-minor is 563Å², reflecting the smaller number of close interatomic contacts between these two components.

Since the structure of the PfAMA1 3D7-R1 complex revealed two bound peptide molecules, binding measurements of R1 to PfAMA1 3D7 were made by isothermal titration calorimetry (ITC) to examine the stoichiometry (Fig. S2). The measured $K_D$ of 145nM is comparable with previous measurements by SPR [13] (recapitulated in Table S2). In contrast, PfRON2sp binding to all the PfAMA1 proteins tested (Table 1) with a higher affinity than for R1 peptide. Consistent with these values, PfRON2sp displayed a higher capacity to inhibit red cell invasion by Pfalciparum 3D7 than the R1 peptide (Fig. 5). Moreover, PfRON2sp shows cross-strain inhibition of invasion as expected from its biological function (Table 1), contrasting with the more restricted strain specificity of R1 (Fig. 3, Table S2) [14].

The PfAMA1 3D7-R1 crystal structure shows that three polymorphic residues (175, 224 and 225) contact R1-major (Table S2). The 224 polymorphism, Met/Leu, is conservative and since contacts are formed by the main chain only, this should not affect R1 specificity. The 3D7 and D10 antigens both carry Tyr175 and Ile225; for the W2mef and HB3 antigens, residue 175 is Tyr and Asp, respectively, and residue 225 is Asn in both. Thus, polymorphisms at positions 225 and possibly 175 appear to be determinant for the 3D7 specificity of R1 at the major peptide-binding site (Table S2A). R1-minor contacts polymorphic residue 230, which is Lys in all strains studied (Table S2B). As our data suggest a weak affinity for this binding site, however, it is unlikely that this polymorphism has a significant effect on the specificity for R1. We examined these polymorphisms further using the mutant PfAMA1 Dico3 [23], which differs only at residue 175 for the 3D7-contacting residues (Table S2A), and a 3D7 mutant with the substitution Ile225Asp, which we call 3D7mut. The equilibrium $K_D$, determined from the SPR steady-state responses to R1 binding, was 15.2±1.9 μM for 3D7mut and 22.3±3.3 μM for Dico3, showing a reduction in affinity of over 200-fold with respect to the native 3D7 antigen (Fig. 6, Table S2C). This affinity is comparable to that observed for HB3 and W2mef [13] (recapitulated in Table S2), and confirms that both Tyr175 and Ile225 are important for the strain-specific recognition of R1. Tyr175, located at the tip of a flexible DI loop that is solvent-exposed in the apo antigen [10], becomes buried by R1-major and forms a hydrogen bond to this ligand via the phenol group. Ile225 is also buried by R1-major, forming a pair of hydrogen bond via its main chain to the R1-major main chain.

Table 1. Apparent equilibrium dissociation constants $K_D$ (nM) for the binding of peptides PfRON2sp1 and PfRON2sp2 to AMA1 from different strains of P. falciparum.

| Strain   | PfRON2sp1 | PfRON2sp2 |
|----------|-----------|-----------|
| 3D7      | 20.3±6.3  | 520±74    |
| CAM                  | 14.6±3.8  | 165±42    |
| PVV                  | 9.2±3.0   | 80±15     |
| HB3                  | 18.3±4.6  | 680±180   |

Independent experiments were performed at least three times and the values represent the mean ± SD.

PfAMA1 Polymorphisms at positions 175 and 225 are determinant for the 3D7 specificity of R1

R1 is strain specific, binding to PfAMA1 from the 3D7 (cognate antigen) and D10 strains, but with much reduced affinity to the HB3 or W2mef proteins, as determined by ELISA [14] or SPR [13] measurements (recapitulated in Table S2). In contrast, PfRON2sp1 bound to all the PfAMA1 proteins tested (Table 1) with a higher affinity than for R1 peptide. Consistent with these values, PfRON2sp1 displayed a higher capacity to inhibit red cell invasion by Pfalciparum 3D7 than the R1 peptide (Fig. 5). Moreover, PfRON2sp1 shows cross-strain inhibition of invasion as expected from its biological function (Table 1), contrasting with the more restricted strain specificity of R1 (Fig. 3, Table S2) [14].

The PfAMA1 3D7-R1 crystal structure shows that three polymorphic residues (175, 224 and 225) contact R1-major (Table S2). The 224 polymorphism, Met/Leu, is conservative and since contacts are formed by the main chain only, this should not affect R1 specificity. The 3D7 and D10 antigens both carry Tyr175 and Ile225; for the W2mef and HB3 antigens, residue 175 is Tyr and Asp, respectively, and residue 225 is Asn in both. Thus, polymorphisms at positions 225 and possibly 175 appear to be determinant for the 3D7 specificity of R1 at the major peptide-binding site (Table S2A). R1-minor contacts polymorphic residue 230, which is Lys in all strains studied (Table S2B). As our data suggest a weak affinity for this binding site, however, it is unlikely that this polymorphism has a significant effect on the specificity for R1. We examined these polymorphisms further using the mutant PfAMA1 Dico3 [23], which differs only at residue 175 for the 3D7-contacting residues (Table S2A), and a 3D7 mutant with the substitution Ile225Asp, which we call 3D7mut. The equilibrium $K_D$, determined from the SPR steady-state responses to R1 binding, was 15.2±1.9 μM for 3D7mut and 22.3±3.3 μM for Dico3, showing a reduction in affinity of over 200-fold with respect to the native 3D7 antigen (Fig. 6, Table S2C). This affinity is comparable to that observed for HB3 and W2mef [13] (recapitulated in Table S2), and confirms that both Tyr175 and Ile225 are important for the strain-specific recognition of R1. Tyr175, located at the tip of a flexible DI loop that is solvent-exposed in the apo antigen [10], becomes buried by R1-major and forms a hydrogen bond to this ligand via the phenol group. Ile225 is also buried by R1-major, forming a pair of hydrogen bond via its main chain to the R1-major main chain.

R1 mimicry of PfRON2

While R1-major follows the general contour of the receptor-binding groove, it does so in a linear rather than the U-shaped conformation adopted by PfRON2sp1 (Fig. 4A). R1-minor occupies a similar region in space as the second strand of the PfRON2sp β-hairpin, contacting the same DI loop of PfAMA1 but running in the opposite direction to form a parallel two-stranded β-sheet with the major peptide (Fig. 4A). Portions of R1-major exhibit structural similarity to PfRON2, displaying a 1.2 Å rmsd in the twelve Cα positions (PfRON2sp1, Ala2031 to Met2042; R1-major, Phe-P5 to Met-P16) (Fig. 4A). Moreover, sequence alignment based on the structural superposition reveals a remarkable similarity between the central regions of the two ligands; the segments Ala2031-Met2042 of PfRON2 and Phe-P5-Met-P16 of R1 have five identical amino acids and two conservative differences (Fig. 4B). R1-major residue Arg-P15 contributes the most contacts to PfAMA1 and is positioned within the same pocket of PfAMA1 as PfRON2 Arg2041 (Fig. 4A - box 3) where it maintains six of the seven hydrogen bonds observed for PfAMA1-PfRON2sp. Interestingly, while PfRON2 mimicry is observed in the cystine loop-binding region (Phe2038/Phe-P12 to Arg2041/Arg-P15), R1-major establishes clear anchor points in the hydrophobic groove different from PfRON2; Phe-P2 and Phe-P5 brace the peptide N-terminus in the region exposed by displacement of the DII loop, with Phe-P3 occupying the pocket left vacant by Phe367 of PfAMA1 (Fig. 4A - box 1).
is cis and is thus important for the β-hairpin conformation). Consistent with the structure, mutation of Arg2041 to Ala abrogated binding to PfAMA1 (Fig. 7B). Similar effects were observed with Pro2044, Phe2038 and Pro2033 mutations, the latter also shown to be a key residue in the TgAMA1-TgRON2 interaction [22].

Similarly, a subset of key PfAMA1 residues was also chosen for mutation: Phe183 (an invariant residue that contributes to the hydrophobic groove and that interacts with Phe2038 of PfRON2 via aromatic interactions), Asn223 (which makes important polar interactions with PfRON2), residue 225 (a polymorphic residue that contributes many contacts to PfRON2 in the structure both the CAMP (Asn225) and 3D7 (Ile225) complexes), Tyr234 (which makes polar contacts to Arg2041 of PfRON2) and Tyr251 (which has been suggested by previous studies to be important [12,25]). A clear role for Phe183 in the PfAMA1-PfRON2 complex formation was evident when expressed on the surface of BHK-21 cells and tested for their ability to bind GST-PfRON2-5 fusion protein (Fig. 7C). A less pronounced role of Tyr234 was observed and none for the remaining residues, including Tyr251. Although these conclusions differ from those of others [12,25], these results are consistent with the limited contacts shown by this residue in the structures and with our earlier findings on the TgAMA1-TgRON2 interaction, where the equivalent TgAMA1 residue, Tyr230, had a minimal effect on the binding.

### Table 2. Crystallographic parameters, data collection statistics and refinement summary.

|                  | PfAMA1 3D7-PfRON2sp1 | PfAMA1 CAMP-PfRON2sp2 | PfAMA1 3D7-R1 |
|------------------|----------------------|-----------------------|--------------|
| Spacegroup       | P2₁                  | P2₁                   | P2₁,2₁,2₁    |
| a, b, c (Å)      | 70.15, 38.26, 70.75  | 70.72, 38.14, 72.08  | 38.32, 144.32, 145.64 |
| α, β, γ (deg.)   | 90, 99.73, 90        | 90, 97.72, 90        | 90, 90, 90  |
| Wavelength (Å)   | 0.9795               | 0.9537                | 0.9791       |
| Resolution range (Å) | 45.41-2.10         | 46.97-1.60          | 40.28-2.15 |
|                  | (2.21-2.10)          | (1.69-1.60)          | (2.25-2.15) |
| Measured reflections | 109520              | 153050               | 156625      |
| Unique reflections | 22041               | 48207                | 42798       |
| Redundancy       | 5.0 (5.0)            | 3.2 (3.2)            | 3.7 (2.5)   |
| Completeness (%) | 100.0 (100.0)        | 94.9 (92.8)          | 95.3 (75.7) |
| Rmerge           | 0.140 (0.470)        | 0.056 (0.618)        | 0.075 (0.485) |

Values in parenthesis are for the last resolution shell. doi:10.1371/journal.ppat.1002755.t002

### Table 3. Refinement statistics.

|                  | PfAMA1 3D7-PfRON2sp1 | PfAMA1 CAMP-PfRON2sp2 | PfAMA1 3D7-R1 |
|------------------|----------------------|-----------------------|--------------|
| Resolution (Å)   | 34.87–2.10 (2.15–2.10) | 35.04-1.60 (1.64-1.60) | 37.06-2.15 (2.15-2.21) |
| R<sub>cryst</sub>/R<sub>free</sub> | 0.164/0.201 (0.202/0.241) | 0.176/0.195 (0.230/0.247) | 0.171/0.214 (0.215/0.249) |
| No. of atoms     | Protein A/B/C/D/E/F 2377/259 | 2309/190 | 2375/2385/157/60/135/77 |
|                  | Solvent 226           | 265       | 450         |
|                  | Glycerol 30            | N/A       | N/A         |
| B-values (Å<sup>2</sup>) | Protein A/B/C/D/E/F 17.3/29.9 | 27.5/48.3 | 36.4/40.5/50.6/77.8/61.5/92.6 |
|                  | Solvent 28.7           | 37.6      | 46.1        |
|                  | Glycerol 39.1          | N/A       | N/A         |
| r.m.s. deviation from ideality | Bond lengths (Å) 0.015 | 0.010 | 0.010 |
|                  | Bond angles (deg.) 1.52 | 1.05      | 1.10        |
| Ramachandran statistics | Most favoured 97.6% | 96.7% | 96.3% |
|                  | Allowed 2.4%           | 3.3%      | 3.7%        |
|                  | Disallowed 0.0%        | 0.0%      | 0.0%        |

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Discussion

The structure of PfAMA1 in complex with the extracellular region of its receptor PfRON2 and the accompanying functional analysis reveal atomic details of the interaction between two key partners at the MJ. The binding site on PfAMA1 includes the hydrophobic groove and a region that becomes exposed by displacement of the flexible DII loop from its apo conformation.

Comparison of residues from both components at the PfAMA1-PfRON2 interface with those of other apicomplexan homologs underscores the separate co-evolution of the receptor-ligand pair in members of the phylum.

The DII loop displays a strong propensity for mobility in P. falciparum and P. vivax AMA1 structures [19], particularly at its N- and C-terminal extremities (weak or absent electron density); the central region of the DII loop is more structured and...
stabilized by contacts with DI, and is better defined in some of these AMA1 structures. Here, we show that the DII loop is displaced by \(\text{PfRON2sp}\), as well as by the R1 peptide. In \(T. gondii\), the DII loop is 14 residues shorter than in the Plasmodium orthologs and appears less mobile [20] but nonetheless is readily displaced by \(TgRON2sp\) [22]. Flexibility may therefore have an important functional role: it protects a significant portion of the binding site in apo AMA1 against the host’s immune response but can be readily displaced to extend the hydrophobic groove for effective binding to RON2. The anti-\(\text{PfAMA1}\) invasion-inhibitory monoclonal antibody 4G2, which binds to the N- and C-termini of the DII loop [19], probably prevents its displacement for effective binding to \(\text{PfRON2}\). The absence of polymorphisms in the DII loop in spite of immune targeting of this region underlines its important functional role [21].

We have previously demonstrated an evolutionary constraint on the AMA1–RON2 interaction within apicomplexan parasites [11]. Our functional analysis of the \(TgAMA1-TgRON2sp\) co-structure suggested that the cystine loop initially anchors the receptor to the hydrophobic groove, causing expulsion of the DII loop to promote interaction throughout the entire binding site [22]. Comparison of the \(TgAMA1-TgRON2sp\) and \(\text{PfAMA1-PfRON2sp}\) co-structures reveals that the cystine loop, while conserved across the two genera, is the most divergent region within the RON2 (Fig. 8). The separate co-evolution of the AMA1-RON2 pair in Apicomplexa is clearly illustrated by the difference between the cystine loop conformations of \(\text{PfRON2sp}\) and \(TgRON2sp\). In particular, this allows Arg2041 to access the specific \(\text{PfAMA1}\) pocket (Fig. 8), where it participates in an intricate network of polar interactions. From mutagenesis, we have demonstrated a crucial role of

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**Figure 3. Structure of \(\text{PfAMA1}\) complexed with R1 peptide.** (A) The co-crystal structure of \(\text{PfAMA1}\) (blue surface) with R1 reveals two bound peptides, R1 major (yellow) and R1 minor (purple). (B) Detailed analysis of interactions at the \(\text{PfAMA1–R1-major, PfAMA1–R1-minor, and R1-major–R1-minor}\) interfaces. Surface representation of \(\text{PfAMA1}\) (blue), with R1-major (yellow) and R1-minor (purple) shown as cartoons. Box 1 – R1-major anchors its N-terminus to \(\text{PfAMA1}\) through 3 backbone hydrogen bonds. Box 2 – the central region of the \(\text{PfAMA1}\) apical groove is occupied by R1-major through both hydrophobic and polar interactions. Box 3 – R1-minor forms most of its anchor points to \(\text{PfAMA1}\) through the apical loops and does not contact the base of the groove, which is occupied by R1-major. Panel 4 – Backbone hydrogen bonds between R1-minor and R1-major generate a β-sheet, while R1-major is further pinned to the \(\text{PfAMA1}\) groove through 3 hydrogen bonds. Panel 5 – R1-major integrates into \(\text{PfAMA1}\) with the use of an arginine knob-in-hole interaction stabilized by 6 hydrogen bonds, which is also exploited by \(\text{PfRON2sp}\).
doi:10.1371/journal.ppat.1002755.g003
Arg2041 in complex formation (Fig. 7B). Moreover, this region of the cystine loop also appears to play an influential role in species selectivity as superposition of \( \text{PfAMA1}_1 \)-\( \text{PfRON2sp} \) onto \( \text{PfAMA1}_2 \)-\( \text{PfRON2sp} \) shows that Arg2041 would be sterically hindered at the interface but Thr, the equivalent residue in \( \text{PvRON2} \) from \( P. \text{vivax} \), can be accommodated (Fig. 9A). This accounts for our prior observation that the original 67-residue segment of \( \text{PfRON2} \) does not bind to \( \text{PvAMA1} \) [11].

An additional feature of the \( \text{PfRON2sp} \) cystine loop region is the presence of a \textit{cis} peptide bond between Ser2043 and Pro2044; the Ser-Pro-Pro segment contributes negligible buried surface area but is important for maintaining the \( \beta \)-hairpin conformation for efficient complex formation. Sequence alignment reveals that the Pro duo (Pro2044–Pro2045) is preserved in all analyzed \textit{Plasmodium} species (Fig. 8A) and is thus likely important for specific recognition of AMA1. We propose that it provides necessary internal structure at the tip of the cystine loop and places the disulfide bond in the proper orientation to brace the AMA1–RON2 interaction. The influential role of Pro2044 is confirmed by mutagenesis where substitution with Ala, which would disfavor the \textit{cis} peptide bond, abrogates \( \text{PfAMA1}_1 \)-\( \text{PfRON2sp1} \) binding (Fig. 7B).

While \( T. \text{gondii} \) does not share the conserved proline pair, its cystine loop is two residues shorter (Fig. 8A), which mirrors the narrower groove of \( TgAMA1 \). Altogether, the overall U-shape

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**Figure 4. Structural mimicry of \( \text{PfRON2} \) by peptide R1 in binding to \( \text{PfAMA1} \).** (A) Top (left) and end-on (right) views of \( \text{PfAMA1}_1 \)-\( \text{PfRON2sp1} \) (orange cartoon) overlayed on \( \text{PfAMA1}_2 \)-\( \text{R1-major} \) (yellow)/\( \text{R1-minor} \) (purple), show that the \( \text{PfAMA1} \) groove is capable of accepting only \( \text{PfRON2sp1} \) or the two R1 peptides at one time. Box 1 shows that Phe-P5 of R1 mimics Phe367 of the DII loop, while boxes 2 and 3 highlight spatial conservation of a phenylalanine anchor at the center of the groove, and a knob-in-hole interaction incorporating the peptide Arg-P15. R1-major is shown in yellow, \( \text{PfRON2sp1} \) in orange and apo \( \text{PfAMA1} \) in green. (B). Comparison of the R1 and \( \text{PfRON2sp1} \) sequences reveals five identical (red) and two similar (blue) residues. doi:10.1371/journal.ppat.1002755.g004

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**A**

**B**

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R1        VFAEFLPLFSKFGSRMHILK
PfRON2sp1 DITQQAKDIGAGPVASCFTTRMSPQQIICLNSVNTALS
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Arg2041 in complex formation (Fig. 7B). Moreover, this region of the cystine loop also appears to play an influential role in species selectivity as superposition of \( \text{PfAMA1} \) structure [19] onto \( \text{PfAMA1}_1 \)-\( \text{PfRON2sp} \) shows that Arg2041 would be sterically hindered at the interface but Thr, the equivalent residue in \( \text{PvRON2} \) from \( P. \text{vivax} \), can be accommodated (Fig. 9A). This accounts for our prior observation that the original 67-residue segment of \( \text{PfRON2} \) does not bind to \( \text{PvAMA1} \) [11].

An additional feature of the \( \text{PfRON2sp} \) cystine loop region is the presence of a \textit{cis} peptide bond between Ser2043 and Pro2044; the Ser-Pro-Pro segment contributes negligible buried surface area but is important for maintaining the \( \beta \)-hairpin conformation for efficient complex formation. Sequence alignment reveals that the Pro duo (Pro2044–Pro2045) is preserved in all analyzed \textit{Plasmodium} species (Fig. 8A) and is thus likely important for specific recognition of AMA1. We propose that it provides necessary internal structure at the tip of the cystine loop and places the disulfide bond in the proper orientation to brace the AMA1–RON2 interaction. The influential role of Pro2044 is confirmed by mutagenesis where substitution with Ala, which would disfavor the \textit{cis} peptide bond, abrogates \( \text{PfAMA1}_1 \)-\( \text{PfRON2sp} \) binding (Fig. 7B).

While \( T. \text{gondii} \) does not share the conserved proline pair, its cystine loop is two residues shorter (Fig. 8A), which mirrors the narrower groove of \( TgAMA1 \). Altogether, the overall U-shape
architecture of RON2 in complex with AMA1 appears to be remarkably well maintained within apicomplexan parasites but specific features are clearly visible in the cystine loop of PfRON2 and TgRON2, highlighting how a receptor-ligand complex has evolved to maintain a common and crucial event in the biology of these parasites.

Although the PfAMA1-PfRON2 interface is highly conserved, five polymorphic residues of PfAMA1 contact the non-polymorphic PfRON2sp [26]. Of these, however, only residue 225 (Asn/Ile) varies significantly. The remaining polymorphisms should not affect binding as they involve main chain contacts only (residues 172, 174, 187 and 224). Our study allows a detailed structural assessment of polymorphism at residue 225 since complexes with PfAMA1 from the 3D7 (Ile225) and CAMP (Asn225) strains were determined. The 3D7 and CAMP orthologs both maintain two hydrogen bonds between the main chain of residue 225 and PfRON2 Thr2039. However, Ile225 presents a deep pocket to Arg2041 with apolar contacts formed between the aliphatic regions of these two side chains, while Asn225 presents a shallower pocket to Arg2041 with the Asn225 amide group stacking against the guanidyl group. Nonetheless, our binding studies by SPR show no significant difference in the affinity of these two PfAMA1 homologs for PfRON2sp2. Sequence variations at PfRON2-interacting positions, 172 (Glu/Gly), 187 (Glu/Asn) and 225 (Ile/Asn) are represented by the strains 3D7, CAMP, FVO and HB3 that we have analyzed by SPR; the very similar KD constants, ranging from approximately 10 to 20 nM, confirm that these exert little effect in the strength of the interaction.

Peptide R1 shows a more restricted specificity as it binds strongly to the cognate 3D7 and closely related D10 antigens but only weakly to orthologs that do not carry the same polymorphic amino acids at position 175 or 225 (Table S2). Tyr175 in PfAMA1 3D7 makes a hydrogen bond to the main chain of R1-major but, as this residue is located in a flexible loop with some freedom to adapt to the PfAMA1-R1 interface, it is unclear why the Asp175 polymorphism leads to reduced affinity. In the case of Ile225 of PfAMA1 3D7, the main chain forms two hydrogen bonds to the main chain of R1-major but the preference of R1 for the Ile225 polymorphism remains unexplained as it contrasts with PfRON2sp where main chain hydrogen bonds are also formed by both Ile225 (3D7) and Asn225 (CAMP) to the main chain of PfRON2. This emphasizes that specificity differences may present subtleties that are difficult to decipher. Here, the crystal structure of R1 in complex with the 3D7mut (Ile225Asn) and Dico3 (Tyr175Asp) mutants of PfAMA1 would provide invaluable insights into this question. Taken together, these results highlight that unlike the natural ligand PfRON2, R1, which was selected by phage display, is highly susceptible to polymorphisms.

R1 exhibits a close structural similarity to PfRON2, with the major/minor peptide pair displaying a similar boomerang form as PfRON2, binding to the same region of PfAMA1 and following the same general contour of the binding-site groove. Our structural data show that binding of R1-minor is dependent upon prior binding of R1-major as it lies above the latter in the binding groove and makes fewer contacts to PfAMA1. This, indeed, is consistent with the ITIC
measurements that show a stoichiometry of 1:1, indicating a weaker affinity for the minor peptide-binding site. R1-major is thus favored as the principle inhibitor of the interaction with PfRON2, but this does not preclude a contribution by the minor peptide-binding site at high peptide concentrations.

Therapeutic strategies aimed at inhibiting the interaction between PfAMA1 and PfRON2 should be very effective in treating malaria as they address a critical phase in the life cycle of the parasite and, importantly, should not be compromised by polymorphism since the PfAMA1-PfRON2 interface is highly conserved. Our results provide a structural basis for designing inhibitors against the most virulent malaria parasite. The PfRON2sp1 peptide used in this study has a very high affinity to PfAMA1 and is very efficient at inhibiting invasion. Moreover, in contrast to the less strongly binding peptide R1, PfRON2sp1 is not strain specific. Structural details of the PfAMA1-PfRON2 interaction offer the possibility to design molecules with the desired specific inhibitory properties by in silico screening and structural validation. The binding of PfRON2 Arg2041 to a specific pocket on PfAMA1 could be a critical target region. Indeed, the
important role played by Arg-P15 at the PmA1-A1-R1 interface closely mirrors the equivalent interaction in the PmA1-PRon2 complexes and, interestingly, the same pocket is occupied by Arg and Lys in Pf AMA1 [23]; it includes the PfAMA1 FVO prodomain (amino acids 25–96) and one additional mutation to minimize proteolytic cleavage of PfAMA1 (Ile225—>Asn) [23].

**Peptide synthesis**

A 39-residue peptide corresponding to residues 2021 to 2059 of Pf Ron2 (PfRon2sp1) was synthesized by Kinexus (Vancouver, Canada) and disulfide cyclized. Lyophilized PfRon2sp1 was solubilized in 100% DMSO and subsequently diluted in HBS (20 mM HEPES pH 7.5, 150 mM NaCl) for use in co-crystallization and functional studies. Peptides PfRon2sp2 (residues 2027 to 2054) and R1 were synthesized by PolyPeptide (Strasbourg, France) and solubilized in 3.5% DMSO for subsequent use.

**Crytalization and data collection**

Crystals of PfAMA1 3D7 PfRon2sp1 were grown in 30% PEG400, 100 mM Tris-HCl pH 8.5, 200 mM tri-sodium citrate dihydrate and the protein (5 mg/mL final concentration) incubated with PfRon2sp1 (1:2 molar excess). A crystal in cryoprotectant buffer was flash cooled at 100 K and diffraction data collected on beamline 9-2 at SShift (Stanford Synchrotron Radiation Laboratory, Stanford, US). Crystals of PfAMA1 CAMP PfRon2sp2 were obtained in 20% PEG 4000, 0.1 M Tris/HCl pH 8.6, 0.1 M sodium acetate and 20% isopropanol and the protein (6.4 mg/mL final concentration) incubated with PfRon2sp2 (1:5 molar excess). Diffraction data were collected from a crystal in cryoprotectant buffer at 100 K on beamline ID9 at European Synchrotron Radiation Facility (Grenoble, France). Crystals of PfAMA1 3D7 R1 were obtained in 15% PEG 4000, 0.1 M Tris/HCl pH 8.5, 0.1 M sodium acetate and 10% isopropanol and the protein (5.4 mg/mL final concentration) incubated with R1 (1:6 molar excess). Diffraction data were collected at 100 K on beamline PROXIMA 1 at SOLEIL (St. Aubin, France).

**Data processing, structure solution and refinement**

Diffraction data were processed using Imosfilm [30] or XDS [31] and Scala [32] in the CCP4 suite of programs [33]. Crystallographic parameters and data collection statistics are given in Table 2. Initial phases were obtained by molecular replacement using PHASER [34] or AMoRe [35] with the unliganded PfAMA1 structure (PDB 1Z40). Tracing of the PfRon2 and R1 peptides, and addition of solvent molecules, was performed manually in COOT [36] and refinement was performed with Refmac5 [37] or autobUSTER (Global Phasing Ltd, Cambridge, UK). A summary of refinement statistics is given.

**Materials and Methods**

**Recombinant protein production**

(i) Baculovirus insect cell expression: A synthetic codon-optimized gene encoding DI and DII of PfAMA1 3D7 [27] (residues 104–438; numbering based on the initiation methionine, PF11_0344) (GenScript) was subcloned into a modified pAcGP67B vector (Pharmingen) for expression in insect cells using established protocols [20]. Final yield of recombinant protein was approximately 3 mg per L of culture.

(ii) P. pastoris expression: Synthetic genes were optimized for PfAMA1 coding of residues 97–442, from strains 3D7 (Genbank accession number U33277), CAMP (accession number M34552) and HB3 (accession number U33277). Potential N-glycosylation sites were mutated and genes were cloned EcoRI-KpnI in the pPicZalphA vector (Invitrogen), resulting in an 11-residues sequence extension followed by myc-epitope and hexa-His tags at the C-terminus, expressed in P. pastoris, and purified as described before [28]. Yield after purification was approximately 20 mg per L of culture. PfAMA1 FVO (residues 25–545, no tags, accession number AJ277646) was produced as described before [29]. The Dico3 protein was modified compared to the published protein [23]; it includes the PfAMA1 FVO prodomain (amino acids 25–96) and one additional mutation to minimize proteolytic cleavage of Lys376—>Arg (B. Faber, unpublished results). The PfAMA1 3D7 mutant (Ile225—>Asn, residues 25–545, no tags) mutant was generated by site-directed mutagenesis (Genscript) and produced in P. pastoris in a similar fashion to the native protein [29].
in Table 3. All molecular representation figures were generated in the PyMOL Molecular Graphics System, version 1.2r3pre, Schrödinger, LLC. Coordinates and structure factors have been deposited in the Protein Data Bank with the following entry codes: PfAMA1-PfRON2sp1, 3ZWZ; PfAMA1-PfRON2sp2, 3SRJ; PfAMA1-R1, 3SRJ.

Binding studies by SPR

SPR measurements were made with a Biacore 2000 instrument (Biacore AB). AMA1 proteins diluted in 10 mM sodium acetate pH 4.5 for 3D7, CAMP, HB3 and FVO strains, or pH 4.0 for 3D7mut and Dico3, were covalently immobilized by an amine-coupling procedure on CM5 sensor chips (GE Healthcare). The reference flow cell was prepared by the same procedure in absence of protein. Binding assays were performed at 25°C in PBS and 0.005% Tween 20 by injecting a series of peptide (PfRON2sp1 and PfRON2sp2 on 3D7, CAMP, HB3 and FVO, and R1 on 3D7mut and Dico3) concentrations at a constant flow rate of 5 μL/min. A heterologous peptide was used to verify the absence of non-specific binding. Peptide dissociation was realized by injecting the running buffer, and the surface was regenerated by injecting glycine/HCl pH 1.5 followed by SDS 0.05%. Control flow cell sensorgrams were subtracted from the ligand flow cell sensorgrams and averaged buffer injections were subtracted from analyte sensorgrams. For peptide R1, steady-state signals (Req) were obtained directly from the plateau region of the sensorgrams, while for PfRON2sp peptides, estimated values of Req were obtained by extrapolation from the experimental curves since the association phase did not reach a final equilibrium state. All calculations were made using the BIAevaluation 4.2 software (Biacore AB). The saturation curves obtained by plotting Req versus the peptide concentration were fitted with a steady-state
model to obtain the $R_{\text{max}}$ and the apparent equilibrium dissociation constants, $K_D$. To normalize the response for the different ligands, these curves were reported as the percentage of bound sites (ratio $R_b/R_{\text{max}}$) versus the analyte concentration.

**Isothermal calorimetry.** ITC measurements were made using a ITC200 calorimeter (MicroCal). PfAMA1 3D7 (P. falciparum) and peptide R1 were diluted in PBS to final concentrations of 0.6 μM and 55 μM, respectively. PfAMA1 3D7 (initial volume 200 μL) was titrated at 25 °C by consecutive injections of the peptide R1 (2 μL aliquots at 3 min intervals). Raw data were normalized and corrected for the heat of dilution of R1 in PBS. Binding stoichiometry was determined by fitting the final data to a 1:1 interaction model using the OriginLab software (OriginLab).

**P. falciparum cultures and invasion assays**

The P. falciparum cell cultures and the invasion assays were performed as described previously [11]. Briefly, highly synchronized P. falciparum 3D7 and HB3 schizonts (1.5% hematocrit, 1.5% parasitemia) were incubated with R1 or PfRON2sp1 peptides. Blood smears were collected 16 hours post-invasion and used for ring-stage parasites counting. The results presented are representative of three independent experiments, each performed in triplicate.

**Transient transfection experiments and cell binding assays**

Cell binding assays using PfAMA1-expressing BHK-21 cells and recombinant GST-PfRON2-5 fusion proteins were performed as previously described [11]. Although not quantitative, this cell-binding assay truly reflects the interaction between AMA1 and RON2 as we carefully checked all the experimental steps as well as the image recording as described below. Transfections were carried out using Lipofectamine Reagent (Invitrogen) as instructed by the manufacturer with $3 \times 10^5$ BHK-21 cells grown on coverslips for 24 h in 6 well plates. Cells were grown for an additional 24 h post-transfection before subsequent analysis. Expression and correct folding of PfAMA1 (and the mutants) at the host cell surface was verified by IFA performed with or without permeabilisation, using antibodies either specific to the cytoplasmic tail (anti-myc tag) or specific to the extracellular ectodomain of PfAMA1 (mouse mAb F8.12.19 [38]). For binding assays, coverslips from a same well were incubated with R1 or PfRON2sp1 peptides. Blood smears were collected 16 hours post-invasion and used for ring-stage parasites counting. The results presented are representative of three independent experiments, each performed in triplicate.

**Supporting Information**

**Figure S1 Detailed analysis of interactions at the PfAMA1-PfRON2sp1 interface.** (A). Open-book surface representation of PfAMA1 (left) and PfRON2sp1 (right) showing the extensive involvement of residues from both molecules in forming a complex interface. Residues involved in hydrogen bonding are coloured blue, while residues contributing significant buried surface area (BASA > 20 Å² for PfAMA1, > 5 Å² for PfRON2sp1) are coloured green. (B). Table of residues involved in hydrogen bonding at the PfAMA1- PfRON2sp1 interface (left) and residues contributing significant buried surface area (right), as calculated by PISA (http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html). Polymorphic residues of PfAMA1 are shown in blue. (PPTX)

**Figure S2 Isothermal titration calorimetry of peptide R1 binding to PfAMA1 3D7.** (PPTX)

**Table S1 Polar interactions and buried surface areas in the PfAMA1-R1 crystal structure.** (A). Polar contacts between PfAMA1 3D7 and R1-major (column 1), and buried surface areas of individual residues of PfAMA1 3D7 (column 2) and R1-major (column 3). Salt bridges are indicated in bold. (B). Polar contacts between PfAMA1 3D7 and R1-minor (column 1), and buried surface areas of individual residues of PfAMA1 3D7 (column 2) and R1-minor (column 3). (C). Polar contacts between R1-major and R1-minor (column 1), and buried surface areas of individual residues of R1-major (column 2) and R1-minor (column 3). Polymorphic residues of PfAMA1 are shown in blue. (PPTX)

**Table S2 Polymorphic residues of PfAMA1 contacting peptide R1.** (A). Polymorphic residues contacting R1-major showing the sequence for strains analyzed using ELISA (*) [14], SPR (†) [13] and in this study using SPR (‡). (B). Polymorphic residues contacting R1-minor, showing the sequence for strains as presented in (A). (C). Binding to PfAMA1, classified as strong (s) or weak (w) for the studies presented in (A) and (B). (PPTX)

**Table S3 Primers used in this study.** (PPTX)

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**Author Contributions**

Conceived and designed the experiments: ML MJB GAB. Performed the experiments: BVLN MLT MHL SL SH MR FAS MJB GAB. Analyzed the data: BVLN ML MHL MLT MJB GAB. Contributed reagents/materials/analysis tools: BWF. Wrote the paper: BVLN ML MLT MJB GAB.

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