Definition of structural elements in *Plasmodium vivax* and *P. knowlesi* Duffy-binding domains necessary for erythrocyte invasion

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Plasmodium vivax and *P. knowlesi* use the Duffy antigen as a receptor to invade human erythrocytes. Duffy-binding ligands belong to a family of erythrocyte-binding proteins that bind erythrocyte receptors to mediate invasion. Receptor-binding domains in erythrocyte-binding proteins lie in conserved cysteine-rich regions called Duffy-binding-like domains. In the present study, we report an analysis of the overall three-dimensional architecture of *P. vivax* and *P. knowlesi* Duffy-binding domains based on mild proteolysis and supportive-functional assays. Our proteolysis experiments indicate that these domains are built of two distinct subdomains. The N-terminal region from Cys-1–4 (C1–C4) forms a stable non-functional subdomain. The region spanning C5–C12 forms another subdomain, which is capable of binding Duffy antigen. These subdomains are joined by a protease-sensitive linker. Results from deletion constructs, designed for expression of truncated proteins on COS cell surface, show that regions containing C5–C8 of the Duffy-binding domains are sufficient for the binding receptor. Therefore the central region of Duffy-binding domains, which is flanked by two non-functional regions, is responsible for receptor recognition. Moreover, the minimal Duffy-binding region identified here is capable of folding into a functionally competent module. These studies pave the way for understanding the architecture of Duffy-binding domains and their interactions with host receptors.

Key words: domain architecture, Duffy antigen, Duffy receptor, erythrocyte-binding protein, erythrocyte invasion, host–parasite interactions.

INTRODUCTION

An essential step in the life cycle of malaria parasites is the invasion of host erythrocytes by merozoites. This process is dependent on specific molecular interactions that occur between erythrocyte receptors and parasite-encoded ligands [1]. The human malaria parasite *Plasmodium vivax* and the related simian parasite *P. knowlesi* both use the Duffy blood group antigen as a receptor to invade human erythrocytes [2,3]. Duffy-negative erythrocytes are resistant to infection by *P. vivax* and *P. knowlesi*. The Duffy blood group antigen also serves as a receptor for the chemokine family, which includes interleukin-8, the melanoma-growth-stimulating activity, monocyte chemotactic peptide-1 and regulated-on-activation, normal T-cell expressed and secreted antigen [4].

The *P. vivax* and *P. knowlesi* Duffy-binding proteins belong to the erythrocyte-binding protein (EBP) family [5]. The highly virulent *P. falciparum* has several EBPs, including EBA-175 (where EBA stands for erythrocyte-binding antigen), which bind diverse receptors to mediate erythrocyte invasion by multiple pathways [6–12]. The receptor-binding domains in EBPs lie in conserved, N-terminal cysteine-rich regions known as Duffy-binding-like (DBL) domains [13–15]. DBL domains are also found in members of the *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) family, encoded by the *var* genes [16–18]. The DBL domains of PfEMP-1 bind to a variety of endothelial receptors to mediate cytoadherence [19–23]. Therefore DBL domains are the key players in two essential pathogenic mechanisms in malaria, namely erythrocyte invasion and cytoadherence.

The Duffy-binding domains from *P. vivax* and *P. knowlesi* EBPs are approx. 35 kDa modules with 12 conserved cysteine residues each. To date, there is no three-dimensional structure information on these domains, and it is expected that these will have novel folds. A molecular understanding of the interaction of these domains with their receptors can lead to novel strategies that block erythrocyte invasion and therefore provide protection against malaria.

We have identified an essential central region in the *P. vivax* and *P. knowlesi* domains that is sufficient for binding to the Duffy antigen on human and rhesus erythrocytes. The present study provides first insights into the complex multidomain architecture of DBL domains and establishes a framework for detailed structure–function dissection of their interactions with receptors.

EXPERIMENTAL

Production of recombinant *P. knowlesi* Duffy-binding domain

P. knowlesi Duffy-binding domain was expressed in *Escherichia coli*, purified from inclusion bodies and refolded into its native conformation using methods similar to those described previously for the production of a recombinant Duffy-binding domain from *P. vivax* [24]. DNA fragments encoding the binding domain of *P. knowlesi* Duffy-binding protein (amino acid residues 200–536) fused to a C-terminal His$_6$ tag were amplified by PCR using specific primers (5′-ACTGAT**CCATGG**TTATTAATCAAACT-TTTCTTC-3′ and 5′-ATAGTTTA**GCGGCCGC**TCAGAGATG-ATGATGATGATGTTCAGTTATCGGATTAA-3′; bold residues indicate *Nco*I and *Not*I restriction sites respectively) and plasmid pGP67BKADR2.1 [25], which contains DNA encoding the *P. knowlesi* Duffy-binding domain as template. The PCR product was digested with *Nco*I and *Not*I restriction enzymes and cloned into expression vector pET28a + (Novagen, Darmstadt, Germany) to yield plasmid pET28a-pkαRII. *E. coli* BL21(DE3) cells transformed with plasmid pET28a-pkαRII were used for the expression of the *P. knowlesi* Duffy-binding domain. *E. coli*

BL21(DE3) pET28a-pkαRII was grown in shake flask cultures to an A_{600} of 0.6–0.8 and expression of *P. knowlesi* Duffy-binding domain was induced with 1 mM isopropyl β-D-thiogalactoside for 3–4 h at 37 °C. Following induction, the cells were lysed by sonication and inclusion bodies were collected by centrifugation at 12 000 *g* for 45 min and solubilized in 6 M guanidinium chloride. Recombinant *P. knowlesi* Duffy-binding domain was refolded by the method of rapid dilution, as described before for refolding of recombinant Duffy-binding domain from *P. vivax* [24]. Briefly, recombinant *P. knowlesi* Duffy-binding domain purified from inclusion bodies under denaturing conditions was diluted 50-fold in refolding buffer containing 50 mM phosphate buffer (pH 7.2), 1 mM GSH, 0.1 mM GSSG, 1 M urea and 0.5 M arginine so that the final protein concentration was 20 mg·l⁻¹. Refolding was allowed to proceed for 36 h at 10 °C. After removal of arginine by dialysis, refolded *P. knowlesi* Duffy-binding domain was purified by ion-exchange chromatography on a SP-Sepharose FF column (Amersham Biosciences, Bucks, U.K.). The protein was eluted from the SP-Sepharose FF column using a salt gradient and was purified further to homogeneity by gel-exclusion chromatography using a Superdex 75 column (Amersham Biosciences).

The recombinant *P. knowlesi* Duffy-binding domain was tested for the presence of free thiols by Ellman's assay using 5,5′-dithiobis-(2-nitrobenzoic acid) for the recombinant *P. vivax* Duffy-binding domain as described previously [24]. A known amount of free cysteine was used for determining the sensitivity of the assay and the detection limit was 30 μM. Guanidinium chloride (6 M) was added to the purified Duffy-binding domain to promote side-chain availability.

Erythrocytes and their treatment with chymotrypsin

Human and rhesus blood was collected in 10 % (v/v) citrate phosphate dextrose, stored at 4 °C for up to 2 weeks and washed three times in RPMI 1640 (Invitrogen, CA, U.S.A.) before use in EBAs. Chymotrypsin cleaves the Duffy blood group antigen. Washed human and rhesus erythrocytes were treated with chymotrypsin as described previously and used in EBAs [6]. Duffy phenotypes were determined by standard methods using two antisera, anti-Fya and anti-Fyb (Ortho Diagnostics Systems, Rantan, NJ, U.S.A.).

Proteolysis and EBAs of *P. knowlesi* Duffy-binding domain fragments

Trypsin was used for mild proteolysis of the bacterially expressed and refolded *P. knowlesi* Duffy-binding domain. In the protease-digestion reactions, 6 μg of purified protein was digested with 0.01, 0.04, 0.08 and 0.1 μg of trypsin in 10 μl of cleavage buffer [50 mM Tris/HCl (pH 7.5), 100 mM NaCl and 0.02 % sodium azide] for 1 h at 20 °C. The reactions were stopped by the addition of 1 mM PMSF and the digestion products were resolved by SDS/PAGE. For N-terminal sequencing of fragments, the proteins were transferred to a PVDF membrane, stained with Ponceau and sequenced at a commercial facility (Michigan State University, East Lansing, MI, U.S.A.). The digested fragments were also tested for functional activity in an EBA as described earlier [24]. A total of 10 μg of the Duffy-binding domain was cleaved and the reaction mix was incubated with 200 μl of normal or enzyme-treated rhesus erythrocytes at 50 % (v/v) haematocrit, 60 μl of foetal bovine serum and 300 μl of incomplete RPMI medium (Invitrogen, Carlsbad, CA, U.S.A.) at 37 °C for 1 h. As control, 10 μg of uncleaved Duffy-binding domain was also used. Following binding at 37 °C for 1 h, the reaction mix was layered over 600 μl of dibutyl phthalate and centrifuged at 16 000 *g* for

1 min at room temperature (25 °C). The supernatant containing unbound protein was removed. Bound proteins were eluted by adding 20 μl of 1.5 M NaCl to the erythrocyte pellet, mixed gently, and the sample was kept for an additional 15 min at room temperature. The samples were centrifuged at 16 000 *g* for 1 min and the supernatant containing bound proteins was collected. These samples along with appropriate controls were then resolved by SDS/PAGE. The resolved proteins were transferred on to a nitrocellulose membrane and used for Western-blot analysis. Antibodies raised against the *P. knowlesi* Duffy-binding domain in rabbit [25] and alkaline-phosphatase-linked anti-rabbit IgG were used as primary and secondary antibodies respectively for Western blotting. The protease chymotrypsin cleaves the Duffy antigen from the surface of the erythrocytes. Erythrocytes treated with chymotrypsin were used as negative controls in the EBAs.

Expression of *P. vivax* and *P. knowlesi* Duffy-binding domain-deletion constructs on the surface of COS cells

The plasmid constructs pHVDR22 and pHKADR22 which were designed for expression of the binding domains of *P. vivax* and *P. knowlesi* Duffy-binding proteins (amino acids 198–522, SWISS-PROT accession no. P22290; amino acids 189–525, SWISS-PROT accession no. P22545) on the surface of COS cells have been described previously [13]. The Duffy-binding domains were fused to the signal sequence and transmembrane domain of herpes simplex virus glycoprotein D (HSVgD) in these constructs to allow targeting to the COS cell surface. Similar constructs were used to express deletion constructs of the *P. vivax* and *P. knowlesi* Duffy-binding domains on the surface of COS cells. DNA fragments encoding truncated regions of the *P. knowlesi* and *P. vivax* Duffy-binding domains were amplified by PCR using *Pyrococcus furiosus* DNA polymerase (Stratagene, La Jolla, CA, U.S.A.). The plasmid pRE4, which contains the gene for HSVgD [26], was digested with restriction enzymes *Pvu*II and *Apa*I to excise the central region of HSVgD encoding amino acids 33–248. The PCR-amplified fragments were digested with *Pvu*II and *Apa*I and cloned in the *Pvu*II and *Apa*I sites of the pRE4 vector to yield the following constructs (the restriction sites in the oligonucleotides are shown in bold).

pkC5–C12 (C5–C12 stand for Cys-5–12): This construct contains DNA sequences encoding amino acids 256–506 of the *P. knowlesi* Duffy-binding domain. The primers 5′-GAC**CAGCTG**AATACAAGGACACATTCTCA-3′ and 5′-AAC**GGGCCC**CACGACACAAGGGCATAAAT-3′ were used for PCR amplification.

pkC5–C10: This construct contains DNA sequences encoding amino acids 256–455 of the *P. knowlesi* Duffy-binding domain. The 5′-GAC**CAGCTG**AATACAAGGACACATTCTCA-3′ and 5′-AAC**GGGCCC**TGAAAATTTTGTTGACAAAACATC-3′ primers were used for PCR amplification.

pkC5–C8: This construct contains DNA sequences encoding amino acids 256–426 of the *P. knowlesi* Duffy-binding domain. The primers 5′-GAC**CAGCTG**AATACAAGGACACATTCTCA-3′ and 5′-AAC**GGGCCC**CAACATACATATTGCCATATTATT-3′ were used for PCR amplification.

pkC1–C4: This construct contains DNA sequences encoding amino acids 202–245 of the *P. knowlesi* Duffy-binding domain. The primers 5′-AAC**CAGCTG**AATCAAACTTTTCTTCAAA-ACAATG-3′ and 5′-AAC**GGGCCC**CTTCATACATAATTGAT-ATCTTCG-3′ were used for PCR amplification.

pkC9–C12: This construct contains DNA sequences encoding amino acids 428–506 of the *P. knowlesi* Duffy-binding domain. The primers 5′-**CTG**TGCCATGATGCCTGTAAATCATCA-3′

and 5′-AAC**GGGCCC**CACGACACAAGGGCATAAAT-3′ were used for PCR amplification.

pvC5–C8: This construct contains DNA sequences encoding amino acids 258–429 of the *P. vivax* Duffy-binding domain. The primers 5′-**CTG**AATACAGACACAAATTTTCATAG-3′ and 5′-ATCACA**GGGCCC**TACCTTACATACTTTTTTATCAGT-3′ were used for PCR amplification.

COS cell culture, transfection experiments and immunofluorescence assays

COS cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 10 % heat-inactivated foetal bovine serum in a humidified CO_2 (5 %) incubator at 37 °C. Fresh monolayers of COS cells (40–60 % confluent) growing in 35 mm diameter wells were transfected with 3 μg of plasmid DNA using LIPOFECTAMINE™ PLUS reagent as described by the manufacturer (Invitrogen). The transfected cells were used for erythrocyte binding and immunofluorescence assays 36–40 h post-transfection. Immunofluorescence assays were performed as described earlier using mouse monoclonal antibody DL6 (gift from Roselyn Eisenberg and Gary Cohen, University of Pennsylvania, Philadelphia, PA, U.S.A.) that reacts with amino acids 272–279 of HSVgD to detect expression of the fusion proteins in transfected COS cells and determine transfection efficiencies [13,26].

EBAs with COS cells expressed Duffy-binding domain fragments

Transfected COS cells expressing various deletion constructs of the *P. knowlesi* and *P. vivax* Duffy-binding domains were tested for binding to erythrocytes. COS cells were washed twice with incomplete DMEM and used for EBAs as described earlier [13]. Normal human Duffy-positive and rhesus erythrocytes, Duffy-negative human erythrocytes as well as chymotrypsin-treated Duffy-positive human and rhesus erythrocytes were used for EBAs. To 0.9 ml of complete medium in wells containing transfected cells, 100 μl of a 1 % erythrocyte suspension was added and incubated at 37 °C for 2 h. Unbound erythrocytes were removed by washing the cells three times with incomplete DMEM. Binding was scored as negative when no rosettes were seen in the entire well. The number of transfected COS cells with rosettes of erythrocytes was scored in 30 fields at a magnification of × 200.

RESULTS

Production of the *P. knowlesi* Duffy-binding domain in a soluble and functional form

The recombinant *P. knowlesi* Duffy-binding domain expressed in *E. coli* accumulated in inclusion bodies. The protein was extracted and refolded into its functional form using methods similar to those described previously for the homologous *P. vivax* Duffy-binding domain [24]. The final yield of refolded and purified protein from shake flask cultures was approx. 1 mg/l. As expected, this protein migrates on SDS/PAGE as a single band of approx. 40 kDa (Figure 1). The recombinant *P. knowlesi* Duffy-binding domain is monomeric, based on its migration in gel-filtration columns (results not shown). Ellman's method was used to assay the presence of free thiols in the recombinant protein [24,27]. The lower detection limit of this assay was 30 μM. No free thiols in the recombinant protein could be detected up to a protein concentration of approx. 50 μM. This is consistent with the results of the recombinant *P. vivax* Duffy-binding domain [24], which suggests that all 12 cysteine residues in these domains are disulphide-linked.
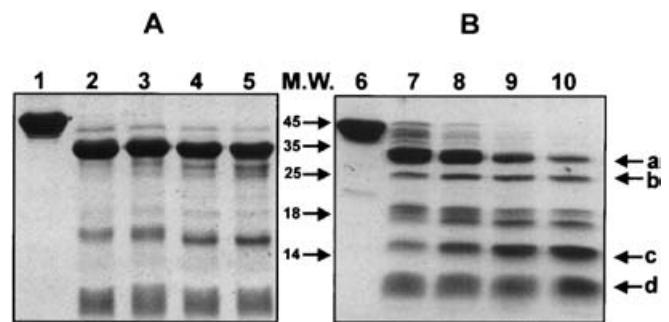

Figure 1   Proteolysis of the *P. knowlesi* Duffy-binding domain with trypsin

Samples of *P. knowlesi* Duffy-binding domain were analysed on (**A**) non-reducing and (**B**) reducing SDS/PAGE [12 % (w/v) gel]. Lanes 1 and 6 show the full-length *P. knowlesi* Duffy-binding domain (6 μg). Samples of *P. knowlesi* Duffy-binding domain (6 μg) were treated with different amounts of trypsin before SDS/PAGE. Lanes 2 and 7, 0.01 μg of trypsin; lanes 3 and 8, 0.04 μg trypsin; lanes 4 and 9, 0.08 μg of trypsin; lanes 5 and 10, 0.1 μg of trypsin. The approximate molecular mass of the proteolytic products a, b, c and d are approx. 32, 22, 15 and 5 kDa protein respectively. MW denotes the molecular-mass marker in kDa.

Proteolytic cleavage of the *P. knowlesi* Duffy-binding domain yields two functional fragments

To identify potential subdomains of the *P. knowlesi* Duffy-binding domain that retain binding activity, we employed the technique of mild proteolysis. The recombinant protein was digested with trypsin and the cleavage mix was resolved by SDS/PAGE. A striking pattern of fragments was observed (Figure 1). A prominent protein fragment of approx. 32 kDa (labelled a) was observed on both reducing and non-reducing gels (Figures 1A and 1B). In reducing gels, in addition to band a, other bands of approx. 22 kDa (labelled b) and approx. 15 kDa (labelled c) were also seen (Figure 1B). A small fragment of approx. 5 kDa (labelled d) was present on both reducing and non-reducing gels (Figures 1A and 1B).

The trypsin cleavage reaction containing bands a–d was allowed to bind to rhesus erythrocytes in a binding experiment. Results indicate that both bands a and b are bound to rhesus erythrocytes (Figure 2). Erythrocytes treated with chymotrypsin (which specifically removes the Duffy antigen) did not bind to the protein (Figure 2). The proteolysis products c and d did not bind to erythrocytes and were therefore considered non-functional. The antibodies against the *P. knowlesi* Duffy-binding domain recognize all proteolytic fragments a–d. We obtained the N-terminal amino acid sequence of proteolysis fragments a–d. These results revealed that both bands a and b started at Thr-256 of the mature protein, which lies between C4 and C5. Molecular-mass considerations suggested that band a was likely to span the region containing C5–C12 (calculated molecular mass 32.1 kDa). Similarly, band b was likely to span the region containing either C5–C8 (calculated molecular mass 20.3 kDa) or C5–C10 (calculated molecular mass 21.2 kDa). The C5–C10 region contains nine residues more than that in the C5–C8 region. It was not possible to determine the C-terminal boundary of band b (C5–C8 or C5–C10) based on the mobility on denaturing acrylamide gels. However, since there were no lysine or arginine residues between C8 and C10, it was quite likely that trypsin cleavage had occurred immediately after C10, where there was an abundance of basic amino acid residues. Protein sequencing of band c revealed its start site as Asn-260. The estimated molecular mass of band c is approx. 15 kDa, suggesting that this non-functional fragment ends between C6 and C7. Band d started at Leu-206 and therefore was most likely to correspond to the region containing C1–C4 (calculated molecular mass approx. 5 kDa).
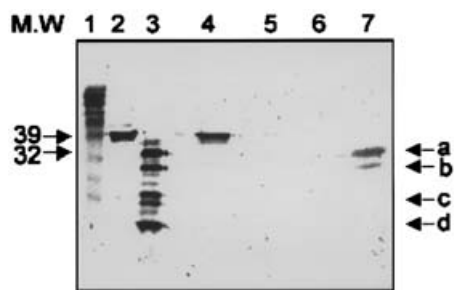
Figure 2   EBA with trypsin-treated _P. knowlesi_ Duffy-binding domain

Recombinant _P. knowlesi_ Duffy-binding domain was used for EBAs before and after treatment with trypsin. Normal rhesus erythrocytes and chymotrypsin-treated rhesus erythrocytes were used for EBA. Proteins bound to erythrocytes in the binding assay were eluted with 300 mM NaCl and detected by Western blotting with mouse serum raised against _P. knowlesi_ Duffy-binding domain. Lane 1, molecular-mass markers (MW; kDa); lane 2, full-length _P. knowlesi_ Duffy-binding domain used for EBA; lane 3, trypsin-treated _P. knowlesi_ Duffy-binding domain used for EBA; lane 4, EBA with full-length _P. knowlesi_ Duffy-binding domain and normal rhesus erythrocytes; lane 5, EBA with full-length _P. knowlesi_ Duffy-binding domain with chymotrypsin-treated rhesus erythrocytes; lane 6, EBA with trypsin-treated Duffy-binding domain and chymotrypsin-treated rhesus erythrocytes; lane 7, EBA with trypsin-treated _P. knowlesi_ Duffy-binding domain and normal rhesus erythrocytes. Note that the antibodies reacted against all the major proteolytic fragments a–d.

The migration pattern of band d on reducing and non-reducing acrylamide gels (Figure 1) suggested the possibility that the four cysteine residues in this fragment were linked by two internal disulphide bonds. The non-functional intermediate proteolysis products between bands b and c (Figure 1) were not sequenced.

The binding site for Duffy antigen is contained in the central C5–C8 region of _P. knowlesi_ and _P. vivax_ Duffy-binding domains

Sequence and functional analysis of proteolysis products of the _P. knowlesi_ Duffy-binding domain suggested the N-terminal boundary of internal fragments that retain receptor-binding activity. To dissect further the region that contains the Duffy-antigen-binding site, a series of _P. knowlesi_ Duffy-binding domain deletion constructs were designed for expression on the surface of COS cells. These constructs started at Thr-256 and ended after C8, C10 or C12 of the _P. knowlesi_ Duffy-binding domain (Figure 3). Binding of human and rhesus erythrocytes to transfected COS cells expressing the various deletion constructs on their surface was tested. In control experiments, binding to chymotrypsin-treated human and rhesus erythrocytes or Duffy-negative human erythrocytes was examined. COS cell-binding experiments were also conducted with Duffy-negative human erythrocytes. Our results indicated that the deletion constructs pkC5–C8, pkC5–C10 and pkC5–C12 bound to erythrocytes with specificity (Table 1 and Figure 4). We also tested the competence of regions C1–C4 and C9–C12 to bind the Duffy antigen on erythrocytes. Results of EBA showed that these failed to bind human or rhesus erythrocytes and indicated the essentiality of the central C5–C8 region for interaction with the Duffy antigen (Table 1). These constructs also suggested that the extra eight residues between C8 and C10 did not contribute to the Duffy-binding site. To verify whether our observations were valid for the homologous _P. vivax_ Duffy-binding domain, an additional deletion construct was generated which contained the C5–C8 region (pvC5–C8; see Table 2 and Figure 4). This experiment indicated that the _P. vivax_ Duffy-binding domain fragment C5–C8 also bound to human erythrocytes with specificity. Both pkC5–C8 and pvC5–C8 did not bind Duffy-negative human erythrocytes. The constructs pkC5–C8 and pvC5–C8 encode for 174 and 172 amino acid
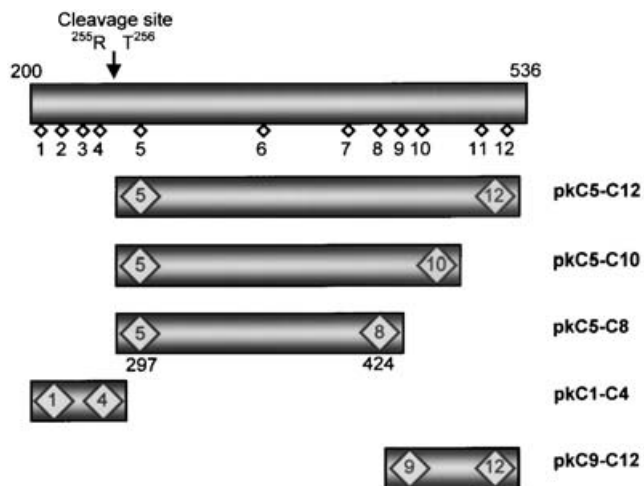

Figure 3   Overall architecture of _P. knowlesi_ Duffy-binding domain and deletion constructs used for EBAs

The _P. knowlesi_ Duffy-binding domain contains 12 conserved cysteine residues. Position of the putative trypsin cleavage site in the _P. knowlesi_ Duffy-binding domain is shown by an arrow. The various deletion constructs of _P. knowlesi_ and _P. vivax_ Duffy-binding domain used for EBAs in COS cells are also shown.

Table 1   Binding data for deletion constructs of the _P. knowlesi_ Duffy-binding domain

Rosettes were scored in 30 fields at a magnification of × 200. The number of rosettes reported for each construct was calculated after normalizing the transfection efficiency to 5 %. The average number of rosettes observed in three experiments is reported. Standard deviations were < 10 % of the average number of rosettes observed in each case. The average transfection efficiency for each construct is also shown. No rosettes were observed with chymotrypsin-treated erythrocytes in the entire well.

	Number of rosettes				
	Human erythrocytes		Rhesus erythrocytes		
Construct	Normal	Chymotrypsin treated	Normal	Chymotrypsin treated	Transfection efficiency (%)
Untransfected	0	0	0	0	Nil
pHKADR22	52	0	67	0	1.2
pkC5–C12	47	0	25	0	3.5
pkC5–C10	39	0	22	0	3.0
pkC5–C8	59	0	17	0	2.7
pkC1–C4	0	0	0	0	1.5
pkC9–C12	0	0	0	0	2.2

residues respectively with seven conserved tryptophans each. Together, these transfection experiments therefore corroborate our proteolysis results and highlight the pivotal role of the central C5–C8 region in Duffy-binding domains.

DISCUSSION

Studies aimed at understanding molecular interactions between the malaria parasite and the host may enable the development of novel strategies to combat malaria. The identification of functionally important domains of parasite proteins that mediate crucial processes such as erythrocyte invasion and cytoadherence can form the basis for receptor-blocking approaches. Invasion of erythrocytes by _Plasmodium_ parasites is a complex process that
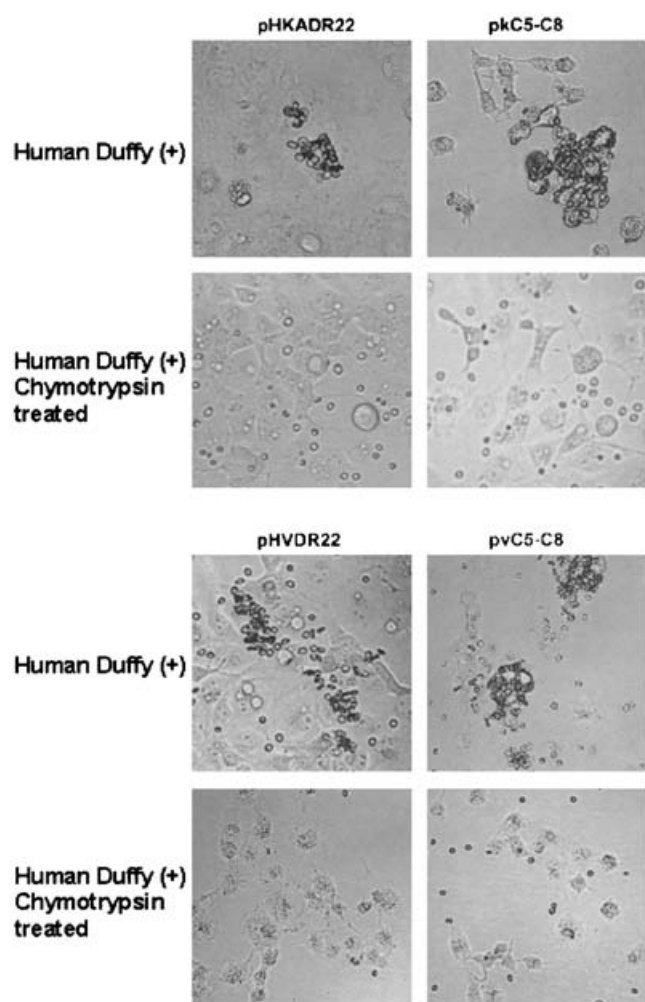
Figure 4   **EBAs with transfected COS cells expressing deletion constructs of *P. knowlesi* and *P. vivax* Duffy-binding domains**

Duffy-positive human erythrocytes and chymotrypsin-treated human erythrocytes were tested for binding to COS cells expressing the full-length *P. knowlesi* and *P. vivax* Duffy-binding domains (pHKADR22 and pHVDR22 respectively) as well as COS cells expressing the region spanning C5–C8 of the *P. knowlesi* and *P. vivax* Duffy-binding domains (pkC5–C8 and pvC5–C8 respectively). Erythrocytes bound to the surface of COS cells were visualized at a magnification of × 200.

Table 2   **Binding data for deletion constructs of the *P. vivax* Duffy-binding domain**

For legend see Table 1.

Construct	Number of rosettes		
	Human erythrocytes		
	Normal	Chymotrypsin treated	Transfection efficiency (%)
Untransfected	0	0	Nil
pHVDR22	61	0	2.5
pvC5–C8	42	0	3.0

involves multiple and specific receptor–ligand interactions [1]. Several studies have shown that DBL domains within the EBPs serve as the receptor-binding domains in erythrocyte invasion
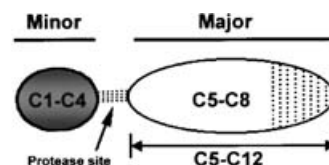

Figure 5   **Schematic representation of the proposed multidomain architecture for Duffy-binding domains**

Based on proteolysis and supportive functional results, we propose that the *P. vivax* and *P. knowlesi* Duffy-binding domains contain two subdomains, called minor and major domains. Each domain contains intra-linked disulphide bonds. The minor domain spans C1 to C4 and the major domain spans C5 to C12. Position of the putative trypsin cleavage site, which lies between the two subdomains, is denoted by an arrow. The minor domain is non-functional. The major domain contains the recognition site for the Duffy antigen. The region containing C5–C8 within the major domain is sufficient for receptor binding. The dotted area in the major domain corresponds to region containing C9–C12.

[13–15]. Other studies have shown that antigenic variation and cytoadherence of *P. falciparum*-infected erythrocytes are modulated by the PfEMP-1 family of variant surface proteins encoded by the *var* multigene family [16–23]. Again, DBL domains of the PfEMP-1 family bind a wide array of host receptors and thereby mediate cytoadherence [19–23]. DBL domains are thus utilized by malaria parasites for a plethora of binding interactions that form the basis for many crucial pathogenic mechanisms. To date, there is no information on the three-dimensional structure of DBL domains. Therefore regions within these domains that contain ligand-binding sites have not been structurally mapped. Protein sequence alignments of DBL domains show that cysteine and tryptophan residues are amongst the most highly conserved residues, suggesting that DBL domains share conserved three-dimensional structures. The disulphide linkage pattern of conserved cysteine residues in these domains has not yet been elucidated.

The *P. vivax* and *P. knowlesi* Duffy-binding domains are highly homologous in sequence (approx. 70 % identity) and therefore are likely to have similar three-dimensional structures. Our studies with recombinant Duffy-binding domains from *P. vivax* [24] and *P. knowlesi* suggest that all 12 cysteine residues are intra-linked by disulphide bonds. Further, these domains exist as monomeric units in solution [24,25]. In an attempt to understand the overall architecture of these domains, we performed limited proteolysis on the *P. knowlesi* Duffy-binding domain. Gel electrophoresis and protein-sequencing analyses provided the first hint that these domains have a multidomain architecture. Our results show that Duffy-binding domains are built of a minor and a major domain, which are connected by a proteolytically sensitive linker region (Figure 5). The proteolysis experiments suggest that these two subdomains contain an even number of internally linked cysteine residues. The minor domain (approx. 5 kDa) contains C1–C4, whereas the major domain (approx. 32 kDa) contains C5–C12. Further dissection of the major domain shows that an approx. 22 kDa fragment (band b) is sufficient for interaction with the Duffy antigen on erythrocytes. This fragment spans the region from C5 to either C8 or C10 (C5–C8 or C5–C10). We verified these observations independently using COS cell-based-adhesion assays which provided additional evidence that the region C5–C8 of both *P. vivax* and *P. knowlesi* Duffy-binding domains is sufficient for binding to the Duffy antigen. These results are consistent with our previous studies that used EBAs with chimaeric DBL domains expressed on the surface of COS cells to demonstrate that binding residues map to the central region of *P. vivax* Duffy-binding domain [15]. In the present paper, we show that the central regions of both *P. vivax* and *P. knowlesi*

Duffy-binding domains (C5–C8) are independently folded functional modules that retain receptor-binding activity. It is possible that the non-functional regions (C1–C4 and C9–C12) that flank C5–C8 modulate affinity and fine specificity of the binding interaction, as suggested in our previous study [15]. Alternatively, these regions may play a predominantly structural role in the intact EBP, and allow easier access of the central C5–C8 region to the Duffy receptor on the erythrocyte surface. The present study cannot distinguish between these and related possibilities.

The Duffy-binding domains are part of the DBL family, which are responsible for interactions with a wide array of host receptors. So far, DBL domains have been found only in *Plasmodia*, and therefore their uniqueness offers an opportunity for rational design of anti-adhesive molecules that block invasion and cytoadherence. Based on our present analysis, we expect other members of this family to also have complex multidomain architectures. Significantly, our studies show that a minimal Duffy-binding module expressed in heterologous systems is capable of folding into a functionally competent structure. The present study will enable further localization of the Duffy-binding residues using the technique of site-directed mutagenesis. Our identification of a minimal functional Duffy-binding module paves the way for both X-ray crystallographic and NMR studies aimed at elucidating the atomic structure of these receptor-binding modules from malaria parasites. A detailed understanding of the structure–function relationships of these receptor–ligand interactions may enable development of receptor-blocking strategies, which target inhibition of erythrocyte invasion to control malaria.

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