PHAGE DISPLAY PEPTIDES BIND TO THE MALARIAL PROTEIN APICAL MEMBRANE ANTIGEN-1 AND INHIBIT THE INVASION OF MEROZOITES INTO HOST ERYTHROCYTES.

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SUMMARY

AMA1 is a transmembrane protein present on the surface of merozoites that is thought to be involved in the process of parasite invasion into the host erythrocytes. Although it is the target of a natural immune response that can inhibit invasion, little is known about the molecular mechanisms by which AMA1 could facilitate the invasion process. In an attempt to identify peptides that specifically interact with, and block the function of AMA1, a random peptide library displayed on the surface of filamentous phage was panned on recombinant AMA1 from *Plasmodium falciparum*. Three peptides with affinity for AMA1 were isolated and a characterisation of their fine binding specificities indicated that they bind to a similar region on the surface of AMA1. One of these peptides was found to be a potent inhibitor of the invasion of *P. falciparum* merozoites into human erythrocytes. It is proposed that this peptide blocks an interaction between AMA1 and a ligand on the erythrocyte surface that is involved in a critical step in malaria invasion. The identification and characterisation of these peptide inhibitors now permit an evaluation of the essential requirements that are necessary for efficient neutralisation of merozoite invasion by blocking AMA1 function.
INTRODUCTION

According to WHO reports malaria infects 300-500 million people per year worldwide and causes 2-3 million deaths annually, mainly in children less than 5 years of age. Currently significant efforts are directed towards the development of a vaccine based on recombinant apical membrane antigen (AMA1), a surface exposed integral membrane protein which is thought to play a crucial role in invasion of erythrocytes by malaria parasites (1). Vaccine strategies that target molecules on the surface of the invasive merozoite, such as AMA1, are a high priority in the search for an effective malaria vaccine. Our lack of understanding of the molecular mechanisms associated with the invasion process may hinder the achievement of this goal. A comprehensive ultrastructural description has emerged of a highly organised series of steps of attachment, reorientation, and junction formation leading to the complete encapsulation of the parasite within the erythrocyte (2,3). Constituents of organelles at the apical end of the merozoite have been implicated in the cascade of events leading to invasion and post invasion events (4,5). For example, rhoptries and micronemes, flask-shaped organelles at the apical end of the merozoite, have been implicated in invasion while dense granules, also part of the apical complex, appear to be involved in events immediately following invasion (6,7). Molecules that mediate the invasion process have been found to be located within apical organelles and also on the merozoite surface. Indeed some molecules such as apical membrane antigen (AMA1) may be initially localised in micronemes and later migrate to the rhoptries (reviewed 8). They are then relocated to the merozoite surface around the time of invasion. It was the timing of this redistribution that first suggested a potential role for AMA1 in the invasion process (9-11).

Evidence that AMA1 plays an important role in invasion comes from vaccine studies in monkey and mouse models, which showed that immunisation with either purified or
recombinant AMA1 could induce a protective immune response when immunised animals were challenged with the corresponding species of *Plasmodium* (12-15). Reports that monoclonal antibodies directed against AMA1 could also inhibit merozoite invasion provided further evidence that AMA1 has a central role in the invasion process (14-19).

Other important vaccine candidates that have been shown to induce antibodies that inhibit or block merozoite invasion *in vitro* include the rhoptry associated proteins (RAP1 and RAP2) (20-22). However, targeted gene disruption studies of the RAP1 gene performed by Baldi and coworkers revealed normal parasite growth and invasion into human erythrocytes *in vitro* suggesting that RAP1 does not play a crucial role in merozoite invasion (23). It has been suggested that the inhibitory activity of RAP1 antibodies is a result of steric hindrance of the invasion process rather than a direct inhibition of the function of this protein. In contrast, attempts to "knock-out" the *Pf*AMA-1 gene not been successful (24), suggesting that unlike other apically located proteins, AMA-1 is essential for erythrocyte invasion.

Although the precise steps involved in merozoite invasion are not well understood, Chitnis and Blackman have put forward some suggestions for the possible roles of various merozoite surface antigens in the overall invasion process. A possible scenario is that MSP-1 mediates the initial attachment of merozoites to the surface of the erythrocyte, a process that may be mediated by relatively low affinity interactions between MSP-1 and components of the erythrocyte membranes. The role of AMA1 may be to facilitate the reorientation of the merozoite after initial attachment so that the apical complex, consisting of rhoptries and micronemes, is closely apposed to the erythrocyte surface (25). It is feasible that AMA1, which gradually redistributes from the apical organelles to the merozoite plasma membrane, might form a concentration gradient on the merozoite surface that could mediate this
reorientation of the parasite. Although there is circumstantial evidence for these suppositions, a clearer understanding of the structure and function of AMA1 relies on further detailed molecular studies.

We chose to apply the powerful phage display technology to identify novel peptides with affinity for AMA1. Random peptide libraries displayed on phage have been used to isolate mimotopes against clinically important antibodies (26), peptides that recognise DNA sequences (27), peptides that mimic carbohydrate structures (28) and peptides that target organ specific molecules (29-30).  By panning peptide libraries on the receptors for erythropoietin and thrombopoietin, peptides have been isolated that are able to act as both agonists and antagonists (31-32).  After modification by mutagenesis these peptides were found to perform most of the functions of the native hormones such as receptor binding, dimerisation, and downstream signalling leading to biological activity. These peptides exhibited high potency, in some cases as potent as the natural cytokine (32). Furthermore, analysis of peptides selected on natural ligands has provided insights into the natural binding partners of these ligands (33-35). In view of the broad success of this approach, we panned a 15-residue random peptide library expressed on the gpIII protein of filamentous phage, against recombinant AMA1 ectodomain. One of the peptides that we have isolated specifically binds to recombinant AMA1 and recognises the native protein in malaria parasites. Binding of this peptide to AMA1 was found to inhibit the invasion of merozoites into host erythrocytes and alanine scanning has defined a small set of amino acid side chains that are essential for AMA1 binding. These peptides represent defined reagents that will help explore the structure of AMA1 and illuminate its function within the parasite life cycle, and may provide lead compounds for future therapies based on inhibition of AMA1 function.
EXPERIMENTAL PROCEDURES

Parasites.

The *P. falciparum* cloned lines 3D7, D10, FAC-8, K1, and HB3 were continuously cultured, essentially using the method of Trager and Jensen (36) except that the human serum supplement to the culture medium was substituted with 0.5 % Albumax, and the gas conditions were 1% O₂, 5% CO₂, and 94% N₂. Late stage parasites were purified from synchronized cultures on a cushion of Percoll (37).

Phage library preparation.

The 15-mer phage peptide library was kindly provided by George Smith, University of Missouri, Columbia, Mo. (38). Phage were amplified by infecting a log-phase culture of *E. coli* K91 and shaking overnight at 37°C in Luria-Bertani (LB) medium containing 25 μg/ml tetracycline (39). The supernatant was twice clarified by pelleting the cells at 8,000 X g for 15 min, and a 20% volume of PEG solution (20% polyethylene glycol 8000, 2.5 M NaCl) was added to precipitate the phage. The sample was incubated on ice at 4°C for at least 2 h before being centrifuged at 10,000 X g for 50 min. The phage pellet was resuspended in 1 ml of PBS (3mM KCl, 1.5mM KH₂PO₄, 137mM NaCl, 8mM Na₂HPO₄, pH 7.5) and stored at -20°C with 0.02% NaN₃.

Panning the phage library

The panning technique adopted by Parmley and Smith (40) was modified and used to screen the phage peptide library on *Plasmodium falciparum* apical membrane antigen-1 (*PfAMA1*). Four rounds of panning were performed on *E.coli* expressed and refolded AMA1 from the 3D7 strain of *P.falciparum*, (41). The wells of a 96-well enzyme-linked immunosorbent assay (ELISA) plate (Maxisorp; Nunc International) were coated with AMA1 (1 μg) in 100 μl of
coating buffer (0.1 M NaHCO₃, pH 8.5), sealed, and incubated overnight at 4°C. The wells were blocked for at least 2 h at room temperature with 300 µl of blocking solution (0.5% bovine serum albumin (BSA), 0.1 M NaHCO₃, pH 8.5). Following blocking, wells were washed three times with PBS. Phage (approximately 10¹¹ particles) were added to the wells in 100 µl of probing solution (0.5% BSA in PBS) and left for 2 h at room temperature with gentle agitation. After incubation, the wells were washed twice in the first round, four times in the second round, and eight times in subsequent rounds of panning with PBS-T (0.5% Tween 20 in PBS) to remove nonbinding phage. Phage that bound to PfAMA1 were eluted with 100 µl of elution solution (0.1 M glycine-HCl, pH 2.2) for 15 min at room temperature and neutralized with 7 µl of 2 M Tris. The titre of eluted phage was estimated, and an aliquot of the eluted fraction was used to infect E. coli K91 cells for amplification. The amplified phage was titred and 10¹¹ particles were used in the next round of panning.

*Phage titre determinations.*

Phage were subjected to serial 10-fold dilutions with 90 µl of LB medium and 10 µl of phage suspension in a 96-well microtiter plate (Nunc International). To each of the phage dilutions, 90 µl of log-phase E. coli K91 cells was added, and the mixture was incubated at room temperature for 20 min to allow the phage to infect the E. coli cells. A 50-µl aliquot of each dilution was spread onto LB agar plates containing 25 µg/ml tetracycline and incubated overnight at 37°C. Phage infection of bacteria confers resistance to tetracycline, and such colonies were counted and expressed as CFU per milliliter.

*Western blotting.*

The harvested parasites were diluted in sample buffer (10% glycerol, 63 mM Tris, pH 6.8, 2% SDS, 0.0025% bromophenol blue), and incubated at 100°C for 5 min. The parasite extracts
were then centrifuged for 10 min at high speed to remove insoluble material. 3μg recombinant PfAMA1 or *Plasmodium chabaudi* AMA1 (PcAMA1) was diluted in sample buffer and incubated at 100°C for 5 minutes. Parasite-derived and recombinant material was separated by SDS-PAGE gels (8% acrylamide) under non-reducing conditions. Separated proteins were then transferred to a polyvinylidene difluoride transfer membrane (PVDF-Plus; Millipore, Bedford, Mass.) and the membrane was blocked overnight in 5% Blotto (5% skim milk powder in PBS), rinsed for 5 min in PBS, and probed with phage (10^{10} particles/ml) or a rabbit polyclonal serum to PfAMA1 (1:1000 dilution in 5% Blotto) for 1 h at room temperature with gentle agitation. The membrane was washed every 10 min for 4 h in PBS-T (PBS 0.5% Tween 20). Horseradish peroxidase (HRP)-conjugated anti-M13 immunoglobulin G (IgG) (Pharmacia Biotech, Quarry Bay, Hong Kong) and anti-rabbit IgG-HRP antibody (Amersham Australia Pty Ltd.) were used as secondary antibodies, and binding was detected by chemiluminescence (Pierce Chemical Co. Rockford, Ill).

**Microtitre plate binding assays.**

Binding assays were carried out using a similar process to that described by Harlow and Lane (1988). Briefly, 96-well Maxisorp microwell plates(Nunc) were coated with PfAMA1, MAb 18/2, BSA, or PcAMA1 (all 1 μg in 100 μl of 0.1 M Na₂HCO₃, pH 8.5, per well) overnight at 4°C. Wells were blocked for at least 2 h at room temperature with 300 μl of 0.5% BSA in PBS then washed three times with PBS. Phage, diluted in PBS-0.5% BSA, were added to the wells and incubated for 1 h at room temperature. The wells were washed five times with PBS-T, and bound phage were detected with a peroxidase conjugated anti-M13 antibody (1:3000 dilution in PBS) using *o*-phenylenediamine as colour reagent. For competition experiments, 10^{10} phage particles were added to the PfAMA1-coated wells (1 μg/well) in the presence of increasing amounts of synthetic peptide or MAb 4G2 and the phage detected with
anti-M13-HRP antibody. In binding assays involving the detection of synthetic peptides, 96-well plates were coated with 10 μg of synthetic peptide in 100 μl coating buffer (15mM Na₂CO₃, 35mM NaHCO₃, pH 9.6). AMA1 (1 μg in 100 μl of probing solution), was added to the wells and bound AMA1 detected with rabbit polyclonal antiserum raised against AMA1, followed by an anti-rabbit IgG-HRP antibody as described above.

**PCR amplification.**

The region of the phage genome encoding the displayed peptide sequence was amplified using the following primers: 5’ primer (GAT AAA CCG ATA CAA TTA AAG) and 3’ primer (CAC AGA CAA CCC TCA TAG). In a 50 μl reaction volume, 2 U of Taq polymerase (Promega) was used to amplify 2 μl of template phage DNA solution (released from *E. coli* K91 cells by boiling) using 250 nM primers, 200 μM dNTPs and 2 mM MgSO₄. After an initial 1 min denaturation, the reaction was cycled at 95°C for 30 sec, 45°C for 30 sec and 72°C for 30 sec for 30 cycles. A final elongation was carried out at 72°C for 7 min. The resultant PCR product was purified using QIAquick 8 PCR Purification Kit (QIAGEN Pty Ltd).

**DNA sequence analysis.**

DNA was sequenced by automated dye terminator cycle sequencing (SUPAMAC, Centre for Proteome Research and Gene Product Mapping, Eveleigh, NSW). Sequences were analyzed with DNASIS V2.1 (Hitachi Software Engineering Co., Ltd.) computer software.

**Peptide synthesis.**

Peptides were synthesized by AUSPEP Pty Ltd., Parkville, Victoria, Australia and Jerini Bio Tools GMBH, Berlin Germany
**Alanine scanning mutagenesis of F1 peptide.**

Fifteen derivatives of the F1 peptide (GWRLLGFGPASSFSM), where each residue was replaced by an L-alanine (alanine was replaced by an L-glycine), were synthesized cleavable pepspots. These fifteen mutated peptides were solubilized in DMSO, followed by PBS, to a final concentration of 4% DMSO, and analysed for binding to PfAMA-1 using the competition assay with F1 phage described above.

**Indirect immunofluorescence assay.**

Indirect immunofluorescence microscopy was performed essentially as described previously by Bianco et al. (41) with phage displaying peptide (F1 or F2) as a primary reagent followed by rabbit anti-M13 antibodies. After incubation and washing a fluorescein isothiocyanate-labelled anti-rabbit IgG was used as a final detection step (Sigma Chemical Co., St. Louis, Mo.).

**Peptide inhibition of merozoite invasion into erythrocytes.**

*P. falciparum* cloned lines 3D7 and HB3 were grown to a parasitaemia of approximately 10% late stage (schizont). Following Percoll purification (36D1uzewski et al., 1984), schizonts were mixed with uninfected erythrocytes and aliquoted into microwells containing the test/control solutions. A reference smear was examined and retained (approximately 3-4% initial parasitaemia). After approximately 20 h in culture, smears were made to determine the number of invaded erythrocytes (cells containing ring stage parasites). Parasitaemias were determined by counting 1000 cells from methanol-fixed, Giemsa-stained thin blood films.

**RESULTS**
Isolation of AMA1-binding peptides from a random peptide library.

In order to identify peptides that have affinity for AMA1 a phage library displaying random 15-residue peptides was panned against immobilised AMA1. A dramatic enrichment of phage with affinity for the antigen was observed after the third round of panning (Fig. 1 A). These pools of phage showed no binding to the irrelevant proteins BSA and the ring infected erythrocyte surface antigen (RESA) (Fig. 1 B) but did bind to PcAMA1 which shares 52% amino acid sequence identity with PfAMA1.

Individual phage clones were examined for their ability to bind either PfAMA1 or PcAMA1. It is clear that some clones are able to bind only to PfAMA1 (Fig. 2 A, clones 2,4,5,6,8,9), whereas other clones bound for both PfAMA1 and PcAMA1 (Fig. 2 A, clones 1,3,7). The binding activity was conferred by the displayed peptides since phage lacking peptide (C) and two phage clones picked at random from the unpanned peptide library (lib1 and lib2) were unable to bind to either PfAMA1 or PcAMA1 (Fig. 2A). None of the clones examined displayed any binding to the irrelevant RESA protein.

Sequencing the DNA inserts of over 30 phage clones that bound PfAMA1 and translation of the corresponding peptide sequences allowed the classification of all binding clones into one of three groups (Fig. 2 B). The majority of binding clones consisted of the sequence GWRLLGFGPASSFSM (F1), while the remainder consisted of either TRLFRVPVLPSGVTS (F2), or PFARAPVEHHDVVGL (F3). Realignment of the latter two sequences revealed a common motif, ΦRXPVXXXXV, where Φ represents a hydrophobic residue and X represents any residue.
Representative clones of each group were selected and their binding properties examined further. Phage displaying a peptide of the F1 class (Fig 2 A, clones; 2,4,5,6,8 and 9) recognised only PfAMA1 and had no reactivity with PcAMA1. Phage expressing peptides from the F2 and F3 groups (Fig 2 A, clones; 1,3 and 7) bound to recombinant PfAMA1 and PcAMA1. Phage clones displaying each of the three peptides bound to PfAMA1 in a dose-dependant manner, although F1 and F3 appeared to have an approximately 10-fold higher relative affinity compared to F2 (Fig 2 C). Absolute affinities are difficult to estimate from these data because the presence of up to five copies of peptide on each phage particle may impart avidity effects that are difficult to predict. Phage containing a peptide picked at random from the unpanned library and consisting of the sequence GDVWLFKTSTSHFAR (F5) was unable to bind to PfAMA1 even at phage concentrations of $10^{11}$ cfu/ml (Fig 2 B).

**Phage displaying F1 peptide recognise native antigen expressed in parasites.**

To examine whether the isolated peptides could recognise native as well as recombinant AMA1 we used the phage displaying the peptides as reagents in fluorescence microscopy and Western blotting assays. The presence of the phage particle attached to the peptide enabled the use of an anti-phage antibody followed by a secondary antibody conjugated to FITC or HRP to assess the binding of peptides to the AMA1, as shown schematically in Figure 3 A. When phage displaying F1 peptide were incubated on thin blood films of the *P. falciparum* 3D7 strain a distinct merozoite apical fluorescence was observed in trophozoite and schizont stage parasites and was indistinguishable from that obtained with a rabbit antiserum raised against PfAMA1 (data not shown), and similar to that found previously by other workers (1,44,45). These data are consistent with the reported apical location of AMA1 in mature parasites followed by a reorganisation of AMA1 to the merozoite surface. As expected, when
this assay was carried out using phage displaying an irrelevant 15-residue peptide no fluorescence was seen (data not shown).

Further evidence that the F1 peptide is binding specifically to AMA1 was obtained by western analysis of parasite extracts using phage displaying F1 peptide as the primary reagent. Two polypeptides of approximately 80kDa and 60kDa were strongly recognised by the F1 phage probe (Fig. 3 B; right hand panel), Polypeptides of identical sizes were recognised by a rabbit antisera raised against recombinant AMA1 (Fig. 3 B; left hand panel). The molecular masses of the two polypeptides are in agreement with the previously reported masses of the full length AMA1 gene product (80-83 kDa) and the 62-63 kDa processed form (1,44,46). F1 phage and anti-AMA1 rabbit antisera also recognised a polypeptide of ~40kDa in size from parasite material, which is likely to result from a secondary processing event within the parasite (47). Both anti-AMA1 rabbit antisera and F1 phage recognised *E.coli* expressed AMA1 ectodomain (Fig. 3 B). Consistent with previous ELISA experiments, F1 phage did not bind to *PcAMA1* although the anti-*PfAMA1* polyclonal antisera did recognise this orthologue (Fig. 3 B). F1 phage recognised AMA1 from all parasite strains examined except HB3 (Fig. 3 B; right hand panel). In contrast, rabbit antisera raised against *PfAMA1* bound to AMA1 from all strains examined including HB3 (Fig. 3 B; left hand panel). No binding of F1 phage to AMA1 was observed when Western blots were carried out under reducing conditions (data not shown) indicating that F1 binds to a conformation dependent epitope on the intramolecular disulfide bonds in AMA1. An examination of the deduced amino acid sequence of AMA1 from all strains revealed only 7 positions that had a residue unique to HB3 (Fig. 3 C). Specificity controls showed that phage expressing an irrelevant peptide (F5) did not bind to recombinant AMA1 or AMA1 from parasites by Western analysis (data not shown).
**Synthetic peptides bind to AMA1 in close proximity to each other.**

The displayed peptides are fused to the N-terminus of the gpIII (38,48,49) and it is conceivable that the gpIII protein could influence the AMA1 binding characteristics of the phage-displayed peptide. In order to address this question F1, F2 and F3 peptides were synthesised and their respective AMA1-binding qualities assessed. When F1 peptide was immobilised onto wells of a microtitre plate it was able to capture AMA1 from solution as determined by secondary capture of an anti-AMA1 antibody (Fig. 4 A). By contrast, wells coated with a peptide consisting of a scrambled F1 sequence (F1(s); AMSPWFRSLGFGSLG) did not capture AMA1 (Fig. 4 A). F2 and F3 were also able to capture PfAMA1 in this assay (data not shown). This demonstrates that sufficient information for binding AMA1 is contained within the peptide sequences identified by panning and the phage framework plays a negligible role in the binding affinity.

To explore further the ability of the three peptides to bind AMA1, a competition assay using F1 phage as the capture moiety was performed. As expected, the F1 peptide in solution was able to inhibit the binding of phage displaying F1 peptide to AMA1 almost completely, having an IC50 of 100nM (Fig. 4 B). The dependence of the linear sequence of the F1 peptide in conferring AMA1 binding was evidenced by the inability of the scrambled peptide to inhibit binding (Fig. 4 B). Importantly, synthetic peptides corresponding to the F2 and F3 AMA1 binding sequences were able to inhibit the interaction between F1 phage and AMA1, albeit with a lower apparent affinity (IC50 of 100μM and 10μM respectively) (Fig. 4 B). Thus although the three peptides have very different sequences and there is no obvious homology between the F1 peptide and the other two, they appear to be able to bind to a
similar region on the AMA1 surface. Clearly the footprints of the three peptides, although not identical, do overlap sufficiently to allow cross competition.

**Critical binding residues revealed by alanine scanning.**

In an effort to identify amino acids within the F1 peptide that are critical for binding to AMA1 we performed an alanine scan of the F1 sequence. The extent of AMA1-binding by peptides, with each residue in turn replaced with alanine, indicates that residues 5-9 are important for binding. When any of these residues (LGFGP) were replaced with alanine the binding of the resulting peptide to AMA1 was dramatically reduced as assessed by the inability of these peptides to inhibit authentic F1 displaying phage from binding to immobilised AMA1 (Fig. 5). In contrast, substitution of residues N-terminal or C-terminal to this central motif had no effect on the ability of the F1 phage peptide to bind to AMA1. In order to confirm that the C-terminal 5 residues (SSFSM) were not required for AMA1 binding, the binding to AMA1 and invasion inhibitory activity of a truncated F1 peptide (F1[trunc]) lacking the last 5 residues was determined. As predicted the binding of this 10-residue peptide to AMA1 was virtually indistinguishable from the full length F1 peptide (data not shown).

**Synthetic peptides can inhibit P. falciparum merozoite invasion.**

It has been reported that the AMA1 reactive monoclonal antibody, MAb4G2, is an efficient inhibitor of *P. falciparum* merozoite invasion into host erythrocytes (17Kocken et al., 1998) and *P. reichenowi* (48Kocken et al., 2000). To determine whether this inhibitory monoclonal and the F1 peptide bind to a similar region on the AMA1 surface, phage displaying F1 peptide were incubated with immobilised recombinant AMA1 in the presence of increasing concentrations of MAb4G2. In this assay, MAb4G2 was able to inhibit the binding of F1
phage to AMA1 in a dose-dependant manner and the extent of this inhibition was similar to that produced when soluble F1 peptide was included in the assay (Fig. 6 A). To exclude the possibility that MAb4G2 inhibits binding of the F1 peptide by steric hindrance, we examined the ability of soluble F1 peptide to block the binding of MAb4G2 to AMA1. Figure 6B demonstrates that, of the peptides tested, only F1 was able to inhibit MAb4G2 binding to any extent. It is therefore likely that both the antibody MAb4G2 and the F1 peptide bind to a similar if not identical site on AMA1.

Since these results raised the possibility that the peptides may block the invasion of merozoites into host erythrocytes, we assessed the invasion efficiencies of *P. falciparum* parasites cultured in the presence of the corresponding synthetic peptides. Concentrations of 25 μg/ml of F1 peptide resulted in approximately 50% inhibition of invasion, while 50 μg/ml of F1 peptide showed close to 90% inhibition (Fig. 7 A). By contrast F2 and F3 were much less effective inhibitors of invasion requiring 10-fold higher concentrations to give an effect (Fig. 7 A), with F2 peptide being a more efficient inhibitor of invasion than F3. These experiments were performed on a number of occasions with slight variations in parasitaemias and haematocrits, however the dose-dependant trend was always consistent with F1 being more active at lower concentrations than F2 or F3. The synthetic peptide corresponding to the scrambled sequence of F1 peptide (F1[s]) showed little inhibitory activity even at concentrations of 500 μg/ml. In addition two irrelevant synthetic 15-mer peptides (P1; CFDYAPYVSAVDDIC and P2; GWLSPSWFEPGLASM) were found to have little effect on merozoite invasion at similar concentrations (Fig. 7 A). A peptide corresponding to a scrambled version of the F2 sequence, (F2(s); VDAPHVFGVPHREA), also showed little inhibitory activity at 500 μg/ml (data not shown). Significantly, the parasites that were able to invade despite the presence of inhibitory peptide appeared to develop normally and
progressed from ring trophozoite through schizogeny normally. Further evidence for the specificity of the mechanism of inhibition was obtained by noting that when peptides F1, F2 and F3 were added to parasite cultures immediately after invasion had occurred, no observable effects on parasite development were seen (data not shown), ruling out a general toxic effect of the peptide on the parasitised erythrocytes.

The observation that the F1 peptide did not bind to AMA1 from parasites of the HB3 strain suggested that it should not inhibit the invasion of erythrocytes by HB3 parasites. As predicted F1 peptide was unable to block the invasion of HB3 parasites, but did reduce the invasion of 3D7 parasites grown under the same conditions (Fig. 7 B). This result strongly supports the proposition that it is the binding of F1 to AMA1 that is the critical event that mediates the inhibition of merozoite invasion of host erythrocytes.
DISCUSSION

In this study we have identified a set of peptides, from a random peptide library, that bind to AMA1. Low micromolar concentrations of one of these peptides effectively blocked the invasion of merozoites into human erythrocytes in vitro. The three peptides with affinity for *P. falciparum* AMA1 were obtained by panning a phage-displayed library containing hundreds of millions of peptides on bacterially expressed, refolded, AMA1 followed by deconvolution of the final round pool. Interestingly, although they were panned on *P. falciparum* AMA1, two of these peptides (F2 and F3) were also able to recognise recombinant AMA1 from the rodent malaria *P. chabaudi*, thus distinguishing them from the F1 peptide, which recognised only PfAMA1. These peptides did not recognise a variety of other proteins (Fig. 1B, Fig. 3C). When used in fluorescence microscopic analysis to probe the location of AMA1 in schizont-infected erythrocytes, the punctate pattern obtained with the F1 peptide was indistinguishable from that obtained with an anti-AMA1 antiserum. Moreover, the peptides were specifically reactive with AMA1 in Western blots of asexual parasite culture material subjected to electrophoresis under non-reducing conditions. We did not observe binding of phage displaying F1 peptide to AMA1 in Western blots carried out under reducing conditions indicating that the F1 peptide recognises a binding site that is dependent on the intra-molecular disulfide bonds in AMA1. Thus, although the F1 peptide was isolated by panning on recombinant protein, it is capable of recognising authentic, parasite-derived, AMA1.

The observation that peptides F2 and F3 bind to AMA1 from *P. chabaudi* (DS strain), whereas F1 peptide binds only to *P. falciparum* AMA-1 suggests that F1 peptide makes different molecular contacts with AMA1 or binds to a different location on AMA1 than F2 or F3. There is 52% amino acid identity between AMA1 from these two species and the sixteen-
cysteine residues present are absolutely conserved in both polypeptides. It therefore seems reasonable to postulate that these molecules share a similar folded structure and that F2 and F3 peptides bind to a common feature between PfAMA1 and PcAMA1. It may therefore be expected that panning on AMA1 from one source will identify not only peptides that are specific for AMA1 from that species (eg F1), but also peptides that react more broadly across AMA1 molecules from different species (eg F2 and F3). When all three soluble synthetic peptides were examined for their ability to inhibit F1 phage from binding to AMA1, F1 peptide was the most potent inhibitor, however F2 and F3 were both able to inhibit the binding of phage displaying F1. Taken together, this data suggests that despite the sequence diversity, all three peptides bind in close proximity on the AMA-1 polypeptide, possibly making overlapping but not identical molecular contacts with the surface of the protein.

Phage displaying F1 peptide proved to be a robust reagent in both fluorescence microscopy and blotting assays, giving comparable patterns to those observed using serum from a rabbit immunised with purified AMA1 (Fig. 3). This suggests phage-displayed peptide libraries may be a source of affinity reagents that can be assessed rapidly without the need for animal immunisation. The surface features of the binding site on AMA that make contact with the F1 peptide appear to be present on AMA1 molecules from most strains tested in this study, but interestingly, are absent from AMA1 from HB3 parasites. Analysis of the sequences of AMA1 from the different strains used in this assay revealed that there are only seven positions that are unique to HB3. These polymorphisms are clustered at the N- and C-termini of AMA1 and four of the seven polymorphisms result in changes of charge, suggesting that the residues at these positions could have a large influence on the binding energy of the peptide. Mutational analysis could be used to define the relative contributions of each of these seven residues to the binding site of F1. If it is assumed that more than one of
these residues is involved in forming the F1 binding site, then the distribution of these residues along the AMA1 sequence implies that the binding site is formed by regions of AMA1 that are distant in the primary sequence but brought into close proximity in the folded structure. This is consistent with the observation that F1 displaying phage were unable to bind AMA1 that had been treated with a reducing agent prior to SDS-PAGE and Western blotting.

It was not possible to identify a motif responsible for AMA1 binding by comparison with other peptide sequences, as F1 was the only peptide isolated which binds solely to PfAMA1. In order to address the possibility of a subdomain or motif contained within the F1 peptide alanine scanning of the whole peptide sequence was performed. The small size of F1 makes it particularly amenable for assessing how specific mutations affect AMA1 binding. Alanine replacement at each position in the central LGFGP sequence reduced AMA1 binding compared with the wild type F1 sequence. The mutants that had the greatest effect on binding activity were G6A and F7A indicating that these residues may be critical for binding. In the absence of structural information, it is difficult to conclude whether these residues contact the AMA1 directly or are important in maintaining the peptide in the correctly folded state. It is likely however that the phenylalanine at position 7 in F1 binds to a hydrophobic pocket on AMA1.

It is interesting to note that the central residues FGP in F1 are also present in a peptide described by Wrighton and colleagues (31) that is able to interact with the erythropoietin receptor. Structural studies on this peptide, which acts as a dimer to stimulate erythropoiesis, reveals that the GP dipeptide forms a β-turn on the peptide backbone. It was noticed that the residues in this β-turn (the GP dipeptide and the adjacent leucine), made several hydrogen
bond contact with the receptor and were important for overall binding activity. While the F1 peptide and the peptides described by Wrighton and colleagues (31) are clearly different, it might be predicted that the GP dipeptide in F1 induces a turn that is important for binding to AMA1 and ultimately in inhibiting merozoite invasion.

The observation that the F1 peptide does not bind to AMA1 from HB3 parasites and is incapable of inhibiting the invasion of erythrocytes by HB3 merozoites, together with the identification of residues on F1 that are important for AMA1 binding, provides the basis of examining in molecular detail the structure and function of AMA1. Besides mutating the amino acid residues that are unique to AMA1 from HB3, it is also possible to create libraries of F1 peptides with mutations flanking the conserved LGFGP region in order to improve the affinity of interaction with AMA1. Thus, libraries of different peptide sequences can, for example, be panned to isolate peptides with higher affinity binding to AMA1 from 3D7 as well as for peptides that bind to AMA1 from HB3. Sequence information from these peptides coupled with an investigation of whether these peptides inhibit merozoite invasion will enable a delineation of the features necessary for inhibition of invasion due to inactivation of AMA1 and possible rational design of a non-peptide inhibitor of the invasion process.

Examination of the primary sequence of F2 and F3 peptides reveals a potential common motif. The core of this motif consists of an arginine followed by a small hydrophobic residue (either alanine or valine) then a proline and valine. There is also a valine at a similar position in both peptides several residues C-terminal to this cluster. Furthermore, the two positions immediately preceding the arginine are hydrophobic in both F2 and F3. This RXPVXXXXV motif would be predicted to be important for the binding of these peptides to AMA1 and may explain why both F2 and F3 are able to cross-react with AMA-1.
from different species. It is tempting to postulate that this F2/F3 binding site common to AMA1 from different parasite species is located close to the site on PfAMA1 occupied by the F1 peptide. This is evidenced by the ability of F2 and F3 to prevent F1 from reacting with PfAMA1.

Although the peptides isolated in this study would be unlikely to be therapeutic agents in themselves they do provide a set of tools with which to probe the structure and function of AMA1. Identification of important functional regions of AMA1 will enhance the possibility of developing ‘second generation’ vaccines based on domains or sub-domains of AMA1 rather than on the highly disulphide-bonded ectodomain. Furthermore, the interactions of the chemical groups on these peptides may provide a starting point for the screening of non-peptide drugs by for example, the method recently described by Qureshi and colleagues, (50), that will bind AMA1 and inhibit invasion in a similar manner to F1 peptide.

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REFERENCES


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FIGURE LEGENDS

Figure 1. Selection of phage binding to AMA1. Equal numbers of phage (10^{11}cfu/ml) after successive rounds of panning on PfAMA1 were incubated with wells coated with PfAMA1 (A), or PcAMA1, RESA and BSA (B). For comparison, binding of round 4 phage to PfAMA1 is shown under these conditions.

Figure 2. A: Fine specificity of individual phage after panning on AMA1. Sixteen phage clones were isolated and each clone was examined for binding to PfAMA1 (dark bars), PcAMA1 (shaded bars) or RESA (white bars). The binding specificity of helper phage lacking a displayed foreign peptide (C) as well as two clones picked at random from the unpanned library (lib1, lib2) was also examined. B: Deduced amino acid sequences of phage peptides that bind AMA1 are boxed. Shading indicates amino acid residues common between F2 and F3. C: Binding of a representative clone from each sequence to PfAMA1 with increasing phage concentration. The binding of a phage expressing an irrelevant peptide (F5) to PfAMA1 was also examined.

Figure 3. Phage displaying F1 peptide binds to native AMA1. (A) Schematic of the assay used to detect the binding of phage displaying F1 peptide to AMA1 expressed in parasites cultured in vitro. The secondary or tertiary antibody was conjugated with either FITC or HRP for immunofluorescence or western blotting experiments (B) Phage displaying F1 peptide were incubated with nylon filters on which were immobilised mature parasite extracts after SDS-PAGE. Five parasite strains were used (HB3, K1, fac8, D10, 3D7) and recombinant PfAMA1 (Pf) and PcAMA1 (Pc) were also included. Bound phage were detected using HRP conjugated anti-phage antibodies (right hand panel). Similar blots were probed with antibodies to PfAMA1 and binding was detected using an HRP conjugated anti rabbit
secondary antibody (left hand panel). (C) Schematic showing the locations of polymorphisms in the ectodomain of PfAMA1 that are unique to HB3.

Figure 4. Synthetic F1 peptide binds to AMA1. (A) Synthetic peptides consisting of the F1 sequence and the scrambled F1 sequence were immobilised onto wells of a microtitre plate and incubated with PfAMA1. Binding of AMA1 to the immobilised peptides was detected firstly with rabbit anti-PfAMA1 antibodies followed by incubation with HRP conjugated anti-rabbit IgG, in an ELISA format. These data are the means of duplicate measurements (+ individual values). (B) Competition phage ELISA to determine the ability of various synthetic peptides to compete with phage displaying F1 peptide for binding to PfAMA1. Phage binding was detected by HRP conjugated anti phage antibodies.

Figure 5. Alanine scan of the F1 peptide sequence. Fifteen peptides (pep1 -pep15) corresponding to the F1 sequence but with a systematic replacement of a residue for alanine were synthesized. The residue replaced in each peptide is shown above the peptide on the histogram. Peptides were incubated with F1 phage and added to wells of a microtitre dish with immobilised AMA1. Binding of F1 phage was detected as previously described. The effect of each mutated peptides were compared to the control parental F1 peptide (F1), which abolishes binding of phage, and F1 scrambled (F1(s)) which has no effect on phage binding. If either AMA1 (no AMA1) or F1 displaying phage (no phage) were omitted from the assay there was no detectable binding.

Figure 6. F1 peptide and the invasion inhibitory MAb4G2 bind to a similar region of AMA1. (A) MAb4G2 can inhibit phage displaying F1 peptide from binding to immobilised AMA1 in a competition phage ELISA. These data are the means of duplicate measurements (+ individual values). (B) ELISA of MAb4G2 binding to immobilised AMA1 in the presence of increasing doses of peptides. Only F1 peptide was able to inhibit binding of MAb4G2 to AMA1. Binding of MAb4G2 was detected by HRP conjugated anti rat IgG.
Figure 7. F1 peptide can inhibit merozoite invasion of erythrocytes. (A) Synthetic peptides were incubated with synchronised *P. falciparum* parasites in vitro and invasion assessed by counting newly formed ring stage parasites. Of the peptides tested, F1 peptide was the most efficient at inhibiting invasion. At higher concentrations of peptide, F2 was also able to inhibit invasion however the other peptides tested were not found to have any inhibitory activity. All peptide concentrations are given in µg/ml. (B) F1 peptide inhibits *P. falciparum* of the 3D7 strain but not HB3 strain. F1 scrambled showed no inhibitory effect on either strain of parasites. These data are the means of duplicate measurements (± individual values).
Figure 3

A

AMA1

anti-phage Ab

FITC or HRP

Phage

B

anti-AMA1

F1-Phage

C

Domain I

Domain II

Domain III

3D7: E K G F

HB3: K K E L

R I R

K

H H K

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Figure 4

A

B
Figure 5
Phage display peptides bind to the malarial protein apical membrane antigen-1 and inhibit the invasion of merozoites into host erythrocytes
Felomena Li, Anton Dluzewski, Andrew M. Coley, Alan W. Thomas, Leann Tilley, Robin F. Anders and Michael Foley

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