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***Plasmodium falciparum* Merozoite Surface Protein 2 is Unstructured and Forms Amyloid-Like Fibrils**

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Abstract

Several merozoite surface proteins are being assessed as potential components of a vaccine against *Plasmodium falciparum*, the cause of the most serious form of human malaria. One of these proteins, merozoite surface protein 2 (MSP2), is unusually hydrophilic and contains tandem sequence repeats, characteristics of intrinsically unstructured proteins. A range of physicochemical studies have confirmed that recombinant forms of MSP2 are largely unstructured. Both dimorphic types of MSP2 (3D7 and FC27) are equivalently extended in solution and form amyloid-like fibrils although with different kinetics and structural characteristics. These fibrils have a regular underlying β -sheet structure and both fibril types stain with Congo Red, but only the FC27 fibrils stain with Thioflavin T. 3D7 MSP2 fibrils seeded the growth of fibrils from 3D7 or FC27 MSP2 monomer indicating the involvement of a conserved region of MSP2 in fibril formation. Consistent with this, digestion of fibrils with proteinase K generated resistant peptides, which included the N-terminal conserved region of MSP2. A monoclonal antibody that reacted preferentially with monomeric recombinant MSP2 did not react with the antigen *in situ* on the merozoite surface. Glutaraldehyde cross-linking of infected erythrocytes generated MSP2 oligomers similar to those formed by polymeric recombinant MSP2. We conclude that MSP2 oligomers containing intermolecular β -strand interactions similar to those in amyloid fibrils may be a component of the fibrillar surface coat on *P. falciparum* merozoites.

Keywords

amyloid; intrinsically unstructured protein; malaria; MSP2; vaccine

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1. Introduction

Merozoite surface antigens are important potential components of a vaccine against *Plasmodium falciparum*, the cause of the most serious form of human malaria [1]. In producing recombinant forms of these proteins for testing in preclinical and clinical vaccine trials it has been important to consider protein conformation because of the need to induce antibody responses that recognize the antigens *in situ* on the parasite surface. For the two leading vaccine candidates, merozoite surface protein 1 (MSP1) and apical membrane antigen 1 (AMA1) a conformation stabilized by intramolecular disulphide bonds is necessary to induce antibody responses that block parasite development [2–4]. Consequently, vaccine trials are being undertaken or planned with recombinant forms of these antigens that have the intramolecular disulphide bonds generated either using a eukaryotic expression vector [5–7] or *in vitro* folding of antigens expressed in *E. coli* [8,9].

Merozoite surface protein 2 (MSP2) is another antigen under development as a potential component of a vaccine against the asexual blood-stages of *P. falciparum* (www.malariavaccine.org). MSP2 expressed in *E. coli* with an N-terminal hexa-His tag was one of three components in a combination vaccine that significantly reduced parasite densities in Papua New Guinean children living in an area where the transmission intensity of *P. falciparum* is high [10]. MSP2 is an ~30 kDa polypeptide, which like MSP1, is anchored into the plasma membrane of the merozoite by a C-terminal glycosylphosphatidylinositol (GPI) moiety [11]. However, MSP2 differs from both MSP1 and AMA1 in that it lacks multiple intramolecular disulphide bonds and there is no knowledge of the three-dimensional structural features of the protein that are important for inducing a protective immune response to MSP2. MSP2 is highly polymorphic with conserved N- and C-terminal domains flanking a central variable region, which contains tandemly arrayed repetitive sequences [12,13]. All MSP2 alleles have been categorized into two groups typified by the 3D7 and FC27 alleles, respectively, because of differences in the repeats and flanking variable sequences [12,14,15]. Despite very different central domains in the dimorphic forms of MSP2 both are characterized by sequences of low complexity and biased amino acid composition. The polypeptides of both types are unusually hydrophilic and are equally deficient in hydrophobic residues, characteristics that are consistent with MSP2 being intrinsically unstructured.

In recent years it has been realized that a large number of proteins are intrinsically unstructured and many of these proteins have been shown to adopt a more ordered conformation when they interact with receptors or other ligands (reviewed in [16–20]). The analysis of various genomes has shown that intrinsically unstructured proteins (IUP) are more common in higher eukaryotes but many such proteins have also been described in lower eukaryotes and prokaryotes [21–24], and they appear to be relatively abundant in *Plasmodium* species [22,24]. Some of the proteins of *P. falciparum* predicted to contain large regions of disorder are parasite surface antigens that contain tandem arrays of sequence repeats and other regions of low complexity typical of IUP [23]. The sequence repeats in many *P. falciparum* proteins encode immunodominant B-cell epitopes and, consequently, they were preferentially selected by antibody screening of expression libraries (reviewed in [25]).

Here we report that recombinant forms of 3D7 and FC27 MSP2, which are equivalently extended and lacking in secondary structure as monomers in solution [26], polymerize to form amyloid-like fibrils but with different kinetics and structural characteristics. As recombinant MSP2 forms amyloid-like fibrils under physiological conditions the intermolecular β -strand interactions responsible for amyloid formation may contribute to the formation of MSP2 oligomers on the merozoite surface. The unusual structural characteristics of MSP2 described here need to be addressed when evaluating MSP2 as a vaccine candidate.

2 Materials and methods

2.1 Parasites

P. falciparum strains 3D7 and D10 (a cloned line of the FC27 isolate) were cultured using the method of Trager and Jensen [27]. Percoll-purified [28] late-stage parasites from synchronized cultures were saponin lysed to isolate schizonts. Harvested parasites diluted to 3×10^6 parasites/ μ l were fractionated by SDS-PAGE under reducing conditions and transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore) for Western blotting. Similar late-stage parasites were also used for immunofluorescence microscopy and in glutaraldehyde cross-linking experiments.

2.2 Recombinant proteins

Recombinant 3D7 MSP2 (Ag1624) used in the initial studies was material prepared for the Combination B vaccine [10,29] and had been stored at -70°C for ~9 years prior to the commencement of these studies. For new preparations of recombinant 3D7 MSP2 (N-terminally hexa-His tagged Ag1624, C-terminally hexa-His tagged 3D7-6H) and recombinant FC27 MSP2 (N-terminally hexa-His tagged Ag661, non-tagged (NT) FC27 and C-terminally hexa-His tagged FC27-6H), modified purification procedures were used (described in the Supplementary information). The sequences of all forms of MSP2 used in this study are shown in Fig. S1. Monomeric and polymeric MSP2 were prepared by size-exclusion chromatography (SEC) performed using a Superose 6 HR 10/30 column (GE Healthcare Biosciences) equilibrated in phosphate buffered saline (PBS) at a flow rate of 0.4 ml/min. For monomeric 3D7 MSP2, Ag1624 was dialysed into a buffer (0.01 M Tris-HCl and 0.1 M NaH_2PO_4 , pH 8.0) containing 6 M guanidine-HCl (GdnHCl) at 4°C for 16 h prior to SEC. To seed fibril (polymer) growth, 4.5 μ g or 22.5 μ g of 3D7 MSP2 polymer were combined with 117 μ g of monomeric 3D7 MSP2. Samples were taken over 72 h and examined by transmission electron microscopy (TEM) to assess fibril length. For heterologous seeding, 25 μ g of 3D7 MSP2 polymer were combined with 60 μ g of monomeric FC27 MSP2 and incubated at room temperature for 20 h before analysis by TEM.

2.3 Preparation of proteinase K-resistant fragments

Monomeric and polymeric samples of 3D7 MSP2 (Ag1624) prepared by SEC were incubated at 37°C with proteinase K (PK) (Sigma) at a MSP2:PK ratio of 40:1 (w/w) [30]. The digestions proceeded for 15 min and 10 μ l samples were taken at 0, 1, 2, 5, 10 & 15 min intervals. These were immediately placed in $2 \times$ reducing sample buffer (RSB), boiled for 2 min to stop the reaction and analysed by silver-stained SDS-PAGE (SilverQuest™ silver staining kit by Invitrogen). The remaining sample at the end of the incubation time was diluted 6-fold into a buffer (0.01 M Tris-HCl and 0.1 M NaH_2PO_4 , pH 8.0) containing 7 M GdnHCl to stop the reaction and stored at 4°C . For the PK digestion of C-terminally hexa-His tagged 3D7 and FC27 MSP2, fibrils at 2 mg/ml were incubated at a MSP2:PK ratio of 40:1 (w/w) for 30 min at 37°C .

Reverse phase high pressure liquid chromatography (RP-HPLC) was used to purify PK-resistant fragments from the digested MSP2. For 3D7 MSP2 (Ag1624), a ZORBAX Eclipse XDB-C8 column was pre-equilibrated with buffer A (0.1% trifluoroacetic acid (TFA)). The PK-digested samples were applied to the column and eluted with a linear gradient (25 min; 0–100%) of 60% acetonitrile in 0.1% TFA, at a flow rate of 1 ml/min. For the C-terminally hexa-His tagged 3D7-6H and FC27-6H MSP2, a Vydac 0.21 $\times$ 25 cm C18 column was pre-equilibrated with buffer A. The PK-digested samples were applied to the column and eluted with a linear gradient (30 min; 4–45%) of 80% acetonitrile in 0.085% TFA, at a flow rate of 200 μ l/min. Protein peaks were collected and concentrated by rotary evaporation (SpeedyVac)

and analysed by SDS-PAGE. Fractions which contained the PK-resistant core were pooled and stored at -70°C for mass spectrometric (MS) analysis and N-terminal sequencing.

MS analysis of the peptides derived from 3D7 MSP2 (Ag1624) was carried out using an Electro Spray Ionisation-Quadrupole Time of Flight, Mass Spectrometer (Applied Biosystems Q-Star). N-terminal protein sequencing was performed by sequential Edman degradation using a Hewlett-Packard G1005A automated protein sequencing system [31] to identify the first ten amino acids of the 3D7 MSP2 PK-resistant peptide.

The peptides derived from C-terminally hexa-His tagged 3D7-6H and FC27-6H MSP2 were also analysed by MS and N-terminal sequencing. Eluates from gel spots were generated by electrophoretically separating RP-HPLC-purified peptides on Coomassie-stained 16% Tricine gels (NuSep Ltd), excising the peptide bands from the gel and eluting the peptides with 50% acetonitrile in 1% TFA. RP-HPLC fractions and gel spot eluates containing the N-terminal peptides were directly spotted onto a steel MALDI-MS target plate by mixing 0.5 μl of sample with 0.5 μl of 2,5-dihydroxybenzoic acid matrix. The samples were analysed using a Bruker Ultraflex mass spectrometer (Bruker-Daltonics, Bremen FRG) in reflector and linear mode. The peptide sequences were confirmed by in source dissociation (ISD) MS analysis [32]. N-terminal sequencing was conducted by the Australian Proteome Analysis Facility on RP-HPLC-purified peptide bands that were Western blotted onto PVDF membrane.

2.4 Generation of monoclonal antibodies

Female CBA mice were immunized with polymeric or monomeric 3D7 MSP2 (Ag1624) formulated as a water-in-oil emulsion in the adjuvant Montanide[®] ISA720 (SEPPIC, Paris). Mice were immunized by intraperitoneal injection with 10 μg of antigen twice, 30 days apart. Subsequently, 30 days after the second immunization mice were boosted intravenously with 10 μg 3D7 MSP2 (Ag1624) in PBS without adjuvant. Four days after the intravenous immunization mice were killed by cervical dislocation and their spleens removed for cell fusion and hybridoma selection at The Walter and Eliza Hall Institute of Medical Research hybridoma facility. Hybridoma supernatants were screened by ELISA for reactivity against 3D7 MSP2 (Ag1624). Monoclonal antibodies (mAb) 11E1 and 6D8 were derived from mice immunized with monomeric and polymeric MSP2, respectively.

2.5 Immunofluorescence microscopy

Smears of infected erythrocytes were dried, fixed with acetone : methanol (1:1, v/v) and probed with monoclonal antibody supernatants diluted in 1% BSA in PBS (BSA/PBS) followed by the secondary antibody (goat anti-mouse IgG conjugated to AlexaFluor[®] 568 also diluted 1:200 in BSA/PBS). Fluorescence was detected by excitation at 568 nm and images were captured using a SPOT-RT digital camera.

2.6 Electron microscopy

Protein samples (10 μl) were applied to 400 mesh copper grids coated with a thin layer of carbon for 2 min. In some cases, the grids were glow discharged in nitrogen prior to the application of sample. Excess material was removed by blotting and samples were negatively stained twice with 10 μl of a 2% uranyl acetate solution (w/v; Electron Microscopy Services). The grids were air dried and viewed using a transmission electron microscope, either a JEOL 2000FX operated at 120 kV or a JEOL JEM-2010 operated at 80 kV.

2.7 SDS-PAGE and Western blotting

Recombinant proteins (0.25 μg) were separated under reducing conditions on 4–12% Bis-Tris NuPAGE (Invitrogen) gels and then transferred to PVDF membranes. The membranes were

probed with purified monoclonal antibodies (1 µg/ml in blotto [5% skim milk powder in PBS]), hybridoma culture supernatants (diluted 1:4 in blotto), sheep or rabbit antisera (1:1000 in blotto). After washing, membranes were incubated (1 h) with the appropriate horseradish peroxidase-conjugated secondary antibody (Chemicon) (diluted 1:1000 in blotto). Binding was detected by enhanced chemiluminescence using the SuperSignal West Pico Chemiluminescent Substrate (Pierce).

2.8 ELISA

96 well microtitre plates (F96 Maxisorb Nunc-Immuno plates) were coated overnight at 4°C with 4 µg/ml of 3D7 MSP2 monomer or polymer (prepared using SEC) diluted in 0.1 M NaHCO₃, pH 8.6. Monoclonal antibody supernatants were serially diluted two-fold in blotto from a 1:3 dilution and 100 µl applied to the plate in triplicate for each coating antigen. Where purified monoclonal antibodies were used, half log serial dilutions were prepared from 2 µg/ml in blotto. 100 µl of the peroxidase-conjugated affinity-purified sheep anti-mouse antibodies, diluted 1:1000 in blotto, were used as the secondary antibody. Antibodies were allowed to bind for 1 h. 150 µl of ABTS substrate (0.98 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), 0.98 mM citric acid and 0.003% H₂O₂) was added to each well and incubated for 45 min before spectrophotometric analysis at 414 nm.

2.9 Glutaraldehyde cross-linking

The glutaraldehyde cross-linking of recombinant MSP2 and parasites proteins was carried out using a procedure similar to one described previously [33]. For recombinant MSP2, monomeric and polymeric samples were prepared by SEC then incubated at 0.1 mg/ml for 20 min at 23°C in 0.005% to 0.5% (w/v) glutaraldehyde (Sigma). The reactions were quenched by the addition of 1 M Tris to a final concentration of 0.1 M. Samples were analysed by SDS-PAGE and the proteins silver stained (SilverQuest™ silver staining kit by Invitrogen). For the cross-linking of parasite proteins, Percoll-purified and saponin-lysed 3D7 and D10 strain parasites were prepared with parasite yields of 6.1×10^6 and 7.9×10^6 cells/µl, respectively. For each parasite strain, 70 µl of the parasites were diluted to 500 µl in PBS and 50 µl aliquots were prepared containing various concentrations of glutaraldehyde. The cross-linking reactions were incubated and quenched as described for the recombinant MSP2. The proteins were extracted by resuspending the samples in an equal volume of RSB and boiling them for 5 min. The cross-linked parasite extracts were separated by SDS-PAGE and analysed by Western blotting. Rabbit sera raised against 3D7 MSP2 and sheep sera raised against FC27 MSP2 were used to detect MSP2 in the Western blotted 3D7 and FC27 strain parasite extracts, respectively.

2.10 Congo Red and Thioflavin T assays

For the Congo Red assay, samples containing 200 µg/ml protein and 2.5 µM Congo Red in PBS were incubated for 5 min before being scanned at wavelengths between 200 and 600 nm using a Cary 1E UV-Visible Spectrophotometer.

For the Thioflavin T (ThT) assay, samples containing 30 µg/ml protein and 25 µM Th T in PBS were scanned at wavelengths between 200 and 600 nm. Emission spectra were collected with an excitation wavelength of 417 nm using a Perkin Elmer Luminescence Spectrometer model LS 50 B. For kinetics experiments, MSP2 was first filtered through a 0.02 µm membrane to remove any aggregates. 200 µl reactions were carried out in black 96 well microtitre plates (Nunc) containing various amounts of FC27 MSP2 monomer and 30 µM ThT in PBS. ThT fluorescence was assayed at 10 min intervals following agitation (20 sec) of the plate, using a SpectroMax M2 plate reader (Molecular Devices) at excitation and emission wavelengths of 443 nm and 484 nm, respectively.

2.11 Circular dichroism

Monomeric and polymeric protein samples were buffer exchanged into 10 mM phosphate, pH 7.4 using SEC and circular dichroism (CD) measurements performed essentially as described previously [34].

2.12 X-ray fibre diffraction

Droplets of solution containing fibrils were suspended between the ends of two wax-filled capillaries and allowed to air-dry at room temperature to promote fibre alignment. X-ray diffraction patterns were collected using a Cu K α Rigaku rotating anode source (wavelength 1.5418 Å) and MAR-Research image plate detector. Images were examined using Mosflm (A. Leslie, Laboratory of Molecular Biology, Cambridge, UK) or marView (Mar Research, Hamburg, Germany) and reflections were measured.

3 Results

3.1 MSP2 is intrinsically unstructured

MSP2 is predicted to be largely disordered by computational methods developed for identifying intrinsically unstructured regions of proteins (see Table S1). These predictors suggest that 3D7 and FC27 MSP2, representatives of both dimorphic forms of MSP2, are equivalently disordered, and more disordered than other *P. falciparum* antigens with extensive tandemly arrayed sequence repeats such as the circumsporozoite protein [35,36] and the S-antigen [37].

Analytical SEC and dynamic light scattering (DLS) experiments showed that the two forms of MSP2 had equivalently highly extended conformations in solution (Table SII), consistent with the previously published results of analytical ultracentrifuge studies and NMR diffusion measurements [26]. Both dimorphic forms of recombinant MSP2, after being incubated overnight in a buffer containing 6 M GdnHCl, with or without added reducing agent (10 mM dithiothreitol), eluted from a SEC column equilibrated with phosphate buffer with relative molecular masses of ~100 kDa (Fig. 1A). This is approximately 4-fold greater than the molecular mass of ~25 kDa calculated from the sequence of the hexa-His-tagged proteins expressed in *E. coli*. The relatively monodisperse species eluting from the SEC column was confirmed to be monomeric MSP2 by sedimentation equilibrium analyses in the analytical ultracentrifuge (data not shown). Native or recombinant MSP2 from different lines of *P. falciparum* exhibit limited size polymorphisms when analysed by SDS-PAGE but the relative molecular masses are in the range of 45,000 – 55,000, which is close to 100% higher than the molecular mass calculated from the sequence (Fig. 1B). This characteristic is common to many protein antigens of *P. falciparum* and is assumed to reflect low binding of SDS because of the extreme hydrophilicity of these proteins. IUP have the additional characteristic of being heat stable. This has long been known for the *P. falciparum* S-antigen [38] and is also a characteristic of both forms of MSP2 (Fig S3 and data not shown).

3.2 MSP2 forms amyloid-like fibrils

The Combination B vaccine, which significantly reduced parasite densities in Papua New Guinean children [10], contained only the 3D7 form of MSP2. The surplus vaccine antigen (Ag1624), which had been stored long-term at –70°C, was found to be a soluble polymer eluting in the void volume of a Superose 6 SEC column (Fig. S2). This indicated a molecular mass of at least several million Daltons suggesting a polymer comprised of at least 100 ~25 kDa monomers. When the polymer was dialysed against a buffer containing 6 M GdnHCl, subsequent analyses by SEC showed more than 95% of the antigen to be monomeric (Fig. 1A). This was also found to be the case when the polymer was dialysed against high concentrations of urea (data not shown). Removal of the chaotrope by dialysis regenerated the polymer (Fig.

S2B). Similar results were obtained with freshly prepared recombinant 3D7 and FC27 MSP2, thus polymer formation appears to be an intrinsic property of recombinant MSP2 and not an artefact of long-term storage of one particular MSP2 construct.

The reproducible and reversible characteristics of the MSP2 polymer identified by SEC suggested an ordered interaction of MSP2 monomers rather than random aggregation. This was confirmed by the detection of fibrils in negatively-stained preparations of the MSP2 polymer examined by TEM (Fig. 2). Initial TEM studies were carried out with polymer isolated by SEC from the Ag1624 form of 3D7 MSP2 that had been stored long-term at -70°C . These fibrils had a diameter of ~ 13 nm and varied in length in the range of ~ 30 – ~ 400 nm (Fig. 2A). Subsequent studies were carried out with the same MSP2 that was dissociated in a buffer containing 6 M GdnHCl to generate monomer and then reconstituted into polymer by dialysis (for 96 h) against 0.02 M Na_2HPO_4 (pH 7.4) and 0.5 M NaCl. This reconstituted polymer also appeared fibrillar and had a more regular appearance. The average length of the fibrils was much longer, in some cases well in excess of 500 nm (Fig. 2B). A new preparation of MSP2 was also shown by SEC to form a polymer, which was comprised of short, less regular fibrils, but when this material was dialysed against a buffer containing 6 M GdnHCl and polymer was reconstituted by removal of the GdnHCl, longer, regular fibrils were observed when examined by TEM (data not shown). Many of the fibrils were observed to contain twists and hence had a ribbon-like appearance (Fig. 2C). Much longer fibrils were generated when monomeric MSP2 isolated by SEC was incubated at room temperature for several days (Fig. 2D).

Recombinant FC27 MSP2, representative of the other dimorphic form of MSP2, also formed fibrils. TEM examination of negatively-stained preparations of fibrils formed by two different FC27 MSP2 constructs, one of which lacked any hexa-His tag revealed twisted fibrils, which were similar in appearance to those formed by 3D7 MSP2 with a diameter of ~ 13 nm and lengths that varied but frequently exceeded $1\mu\text{m}$ (Fig. 2E and F).

Although the MSP2 fibrils had the general appearance of amyloid fibrils, including a possible protofilament ultrastructure (Fig. 2C & 2E), they were unusual in their instability when heated. Incubation of fibrils of either dimorphic form for 10 min at 80°C converted the fibrils to $> 95\%$ monomeric MSP2 (Fig. S3).

3.3 Fibril growth can be seeded

Preformed MSP2 fibrils seeded fibril growth when added to monomeric MSP2 at room temperature. By varying the ratio of seed to monomer, fibrils of varying length could be produced, with low seed to monomer ratios producing the longer fibrils (Fig. S4). Thus, the growth of MSP2 fibrils appeared to follow a nucleation and seeding pathway as has been described for a variety of amyloid or amyloid-like fibrils. In these first seeding experiments both the seeding fibrils and monomer were 3D7 MSP2 (Fig. 2G). In subsequent experiments 3D7 fibrils also effectively seeded fibril growth when added to FC27 MSP2 monomer (Fig. 2H). This result indicated that sequences conserved between the dimorphic forms of MSP2 were critical for the intermolecular interactions responsible for fibril formation.

3.4 3D7 MSP2 fibrils have some characteristics of amyloid

When Congo Red was added to polymeric but not monomeric 3D7 MSP2 (Ag1624) there was a significant increase in the molar extinction coefficient of Congo Red and a shift in maximum absorbance from 484 to 540 nm; the red shift typical of amyloid (Fig. 3A). Consistent with this, MSP2 fibrils stained with Congo Red exhibited apple-green birefringence when examined by microscopy under polarized light (data not shown). In contrast, the 3D7 MSP2 fibrils did not bind ThT. Thus, in this respect the 3D7 MSP2 fibrils differed from typical amyloid fibrils such as those formed from A β peptide (Fig. 3B).

The CD spectra of both monomeric and polymeric 3D7 MSP2 are dominated by an ellipticity minimum at ~210 nm, indicating a high content of random coil, but the CD spectrum for the polymer has a broad minimum at 215–220 nm indicating an increase in the content of β -sheet secondary structure when MSP2 polymerizes (Fig. 3C). The presence of β -sheet in the MSP2 fibrils was confirmed by X-ray fibre diffraction studies carried out on partially aligned 3D7 MSP2 fibrils. The diffraction pattern exhibited a strong 4.7 Å meridional reflection indicative of a structure composed of β -sheet in which the constituent strands are aligned at 90° to the long axis of the fibril (Fig. 3D). X-ray fibre diffraction patterns of amyloid fibrils are usually also characterized by an equatorial reflection arising from a regular spacing between β -sheets in the protofilaments. However, no equatorial reflection was seen in the 3D7 MSP2 (Ag1624) diffraction pattern even though the 4.7 Å reflection shows a strong meridional orientation, indicating significant alignment of the fibrils within the sample.

Amyloid fibrils are notable for their resistance to proteolysis and digestion with PK has been used to define the regions of a polypeptide chain that are involved in the β -strand interactions that lead to fibril formation [39]. Monomeric 3D7 MSP2 (Ag1624) was readily cleaved by PK with no remaining fragments of MSP2 identifiable by SDS-PAGE after 10 min of digestion (Fig. 4A). In contrast, digestion of polymeric 3D7 MSP2 (Ag1624) generated an ~6 kDa fragment, which was relatively resistant to further digestion with PK. This fragment, which was isolated by RP-HPLC (Fig. 4B) and subjected to mass-spectrometric analysis (Fig. 4C) and N-terminal sequencing by Edman degradation (data not shown), corresponded to the N-terminal conserved region of 3D7 MSP2 and included the hexa-His tag on the Ag1624 recombinant protein used for these studies. Although there appeared to be heterogeneity at both the N-terminus and C-terminus of the peptide generated by PK cleavage, all species identified by the MS analysis contained the entire N-terminal region that is conserved in the two forms of MSP2.

3.5 3D7 and FC27 MSP2 fibrils have different characteristics

The characteristics of the amyloid-like fibrils formed by MSP2 were examined further with highly purified forms of 3D7 and FC27 MSP2 with C-terminal hexa-His tags. As for the Ag1624 fibrils, fibrils prepared using the C-terminally hexa-His tagged construct of 3D7 MSP2 bound Congo Red but not ThT. As expected, fibrils prepared from the C-terminally hexa-His tagged construct of FC27 MSP2 also bound Congo Red but surprisingly, also bound ThT as indicated by a strong fluorescence emission spectrum with a peak at 484 nm (Fig. 5A). Thus, in this respect the FC27 MSP2 fibrils are more similar to typical amyloid fibrils such as those formed by the A β peptide (Fig. 3).

The finding that the FC27 fibrils bound ThT enabled us to use a ThT binding assay to examine the kinetics of formation of fibrils by this form of MSP2 (Fig. 5B). When monomeric FC27 was incubated at room temperature with occasional agitation there was a lag phase followed by an exponential increase in fibril formation indicative of a nucleation-dependent polymerization pathway, which is well documented for numerous forms of amyloid or amyloid-like aggregates [40–42]. The length of the lag phase was found to be markedly concentration-dependant; the lag phase was halved by doubling the MSP2 concentration from 10 μ M to 20 μ M (Fig. 5B). TEM studies on the reaction mixtures confirmed that the increase in ThT binding reflected the formation of amyloid-like fibrils (data not shown). Because the 3D7 fibrils did not bind ThT the kinetics of formation of fibrils by the two types of MSP2 were compared by using SEC to assess the time-dependent loss of monomeric MSP2. This experiment showed that FC27 MSP2 formed fibrils with much faster kinetics than did 3D7 MSP2 (Fig. S5). Again TEM was used to confirm that the loss of monomeric MSP2 was associated with the generation of amyloid-like fibrils (data not shown).

A structural difference between the two types of fibrils suggested by the differences in kinetics and ThT binding was confirmed by X-ray fibre diffraction studies on fibrils formed from C-terminally hexa-His tagged proteins (Fig. 6). The X-ray fibre diffraction pattern collected from 3D7 fibrils was consistent with the earlier results with 3D7 MSP2 (Ag1624) (Fig. 3). The meridional reflection at ~ 4.7 Å is relatively well-aligned, indicating that the constituent β -strands are at right angles to the long axis but again, there is no distinct equatorial reflection (Fig. 6). In contrast, the pattern from FC27 fibrils displays the cross- β reflections that are more characteristic of amyloid fibrils. In addition to the strong and sharp reflection at ~ 4.7 Å on the meridian of the pattern that arises from the inter-strand spacing in the direction of hydrogen-bonding, i.e. along the fibril long axis, there is also a weaker and more diffuse reflection centered at ~ 8.9 Å arising from the inter-sheet spacing.

Structural differences between the fibrils formed by the two types of MSP2 were further confirmed by PK digestion (Fig. 7). PK digestion of the 3D7 MSP2 (3D7-6H) generated a single resistant peptide with a relative molecular mass less than 5,000 on SDS-PAGE (Fig. 7A, lane 4). MS analysis of RP-HPLC fractions identified a dominant peptide with a mass of 3,840 Da (Fig. 7C). The mass and N-terminal sequencing (data not shown) established that this peptide corresponded to the N-terminal 34 residues of the protein. Thus, the major resistant peptides generated by PK digestion of the fibrils formed by the two forms of 3D7 MSP2, both contained the entire N-terminal region of MSP2 and the first six (Ag1624) or seven (3D7-6H) residues of the central variable region (Fig. 4C and 7C).

PK digestion of FC27 MSP2 (FC27-6H) generated resistant peptides also containing the N-terminal conserved region, but three peptides identified by silver stained SDS-PAGE had relative molecular masses approximately twice the size of the 3D7 MSP2 peptides (Fig. 7A, lane 2). MS analysis and N-terminal sequencing of RP-HPLC fractions and gel-spot eluates established that the two most abundant peptides extended to near the end of the first 32-mer repeat, which characterizes the FC27 allele of MSP2 (Fig. 7D). The mass of the peptides corresponded to the N-terminal peptides and this was confirmed by ISD-MS sequencing of approximately 50% of the sequence (data not shown).

3.6 MSP2 oligomers may be a component of the merozoite surface coat

The conformation of MSP2 on the merozoite surface was examined with mAbs produced to recombinant 3D7 MSP2 and an existing mAb to the MSP2 expressed by FC27 parasites [43]. The two variant-specific mAbs (11E1 and 8G10 specific for 3D7 and FC27 MSP2, respectively) reacted with the homologous but not the heterologous forms of recombinant and parasite MSP2 by ELISA and on Western blots (Fig. 8 and S6). Both of these mAbs, which reacted by ELISA with monomeric and polymeric recombinant MSP2, gave a strong signal on the homologous parasite when examined by immunofluorescence microscopy (Fig. 8D and data not shown). The third mAb (6D8) used in these analyses reacted with both 3D7 and FC27 recombinant MSP2, and the epitope was localized to the conserved N-terminal region by the reactivity of mAb 6D8 with the PK-resistant peptide derived from fibrils (Fig. 8C). Consistent with this later observation, fibril formation by recombinant MSP2 was associated with partial loss of the 6D8 epitope although this was more marked for 3D7 MSP2 than FC27 MSP2 (Fig. 8 and S6). Immunofluorescence microscopy showed a more marked loss of the 6D8 epitope in MSP2 on the merozoite surface. We found no evidence of a processing event that could remove the 6D8 epitope (Fig. S7), and the variable reactivity of parasite MSP2 with 6D8 on Western blots (Fig. 8C and S8) suggests that a conformational difference between monomeric recombinant MSP2 and the GPI-anchored parasite antigen may be responsible for the loss of the 6D8 epitope.

Direct evidence that MSP2 forms oligomeric complexes on the merozoite surface has been obtained from cross-linking experiments. Cross-linking polymeric recombinant MSP2 with a

glutaraldehyde concentration of 0.005% generated a ladder of MSP2 oligomers (Fig. 9A). Similar higher-order forms of MSP2 were seen when glutaraldehyde was used to cross-link late-stage D10 erythrocytic (schizont) parasites. 0.005% and 0.01% glutaraldehyde generated cross-linked forms of MSP2 that corresponded closely to the oligomers generated by cross-linking polymeric recombinant MSP2 (Fig. 9B and Fig. S9). Glutaraldehyde cross-linking of 3D7 parasites also generated higher molecular weight species that reacted with anti-MSP2 antibodies (Fig. 9C). Some low abundance bands had the same relative mobilities as the smaller cross-linked oligomers formed by recombinant polymeric MSP2 (Fig. 9A) but much of the material, particularly at the higher glutaraldehyde concentrations, was in the form of unresolved high molecular weight complexes. At higher concentrations of glutaraldehyde, monomeric recombinant and parasite MSP2 had increased mobility on SDS-PAGE presumably due to an increased number of intra-molecular cross-links reducing the hydrodynamic radius of the protein.

4 Discussion

Many of the first *P. falciparum* asexual blood-stage antigens to be cloned contained extensive tandem repeat sequences or other sequences of low complexity, which are now recognized as structural features of IUP. The first asexual blood-stage antigen of *P. falciparum* to be cloned was the S-antigen of the FC27 isolate, which contained about 100 copies of an 11-residue sequence repeat [37,44]. This antigen was the protein most highly predicted to be intrinsically unstructured by Romero *et al.* [21] in their early survey of proteins with long disordered regions in the Swiss Protein Database. More recent studies have indicated that IUP are unusually abundant in *P. falciparum* and some other malaria parasites [23]. MSP2, one of several merozoite surface antigens being developed as components of a vaccine against *P. falciparum*, contains a central variable domain in part composed of sequence repeats although these are far less extensive than those seen in S-antigens [12,13]. MSP2, irrespective of the allelic form, is unusually hydrophilic and is predicted by the three computational methods used, PONDR [45], GlobPlot [46] and disEMBL [47], to be largely unstructured (Table S1). Consistent with these predictions several physicochemical approaches, including SEC and DLS used in this study, have shown recombinant 3D7 and FC27 MSP2, representatives of the two families of MSP2 alleles, to be highly extended in solution. CD studies indicated that both forms of MSP2 contained little secondary structure but NMR studies on FC27 MSP2 have shown that there is motional restriction in three limited regions of the polypeptide [26]. Two of these regions, one in the conserved N-terminal sequence and one towards the C-terminal end of the central variable region show some helical propensity. The third constrained region in the vicinity of the intramolecular disulphide bond in the conserved C-terminal sequence shows no propensity for helical or strand secondary structure.

One function of the conformational flexibility of intrinsically unstructured regions of proteins is to facilitate protein-protein interactions [48–51]. This may be a function of some of the unstructured proteins of *P. falciparum* [24] but no interacting partners have been experimentally identified for MSP2 in contrast to what has been reported for the other merozoite surface proteins, for example, MSP1 [52,53] and AMA1 [54]. Therefore, it was of considerable interest to find that recombinant MSP2 had a propensity to form amyloid-like fibrils under physiological conditions. The ultrastructure of negatively stained MSP2 fibrils viewed in the electron microscope closely resembled that reported for amyloid fibrils formed by diverse proteins. Periodic twists were apparent in some fibrils and in some images the 13 nm diameter fibrils appeared to be composed of two protofilaments. Because of the variability of fibril morphology in different preparations a more detailed study will be required to establish if there are consistent morphological differences between the fibrils formed by 3D7 and FC27 MSP2.

The X-ray fibre diffraction and CD studies indicated that fibril formation by both forms of MSP2 is associated with the acquisition of β -sheet secondary structure, as is well documented for amyloid formation by a variety of proteins [55]. The presence of the 4.7 Å meridional reflection in the X-ray fibre diffraction patterns indicated that in both forms of MSP2 fibrils the β -strands were oriented perpendicular to the long axis of the fibrils, consistent with the characteristic cross- β structure of amyloid fibrils. The cross- β structure of amyloid is also characterized by an equatorial reflection due to the inter-sheet spacing. A weak diffuse equatorial reflection at ~8.9 Å was seen with the FC27 fibrils but no equatorial reflection was seen with either of the two forms of 3D7 MSP2 fibrils examined. Given the presence of the strong and oriented inter-strand spacing in the diffraction patterns from both FC27 and 3D7 samples and the similar dimensions of the fibrils composed of the two MSP2 forms, it is likely that the absence of the inter-sheet spacing reflects some variation in the way the constituent sheets pack together within the 3D7 fibrils or in the way 3D7 fibrils associate with each other in the sample. The MSP2 sequences are more hydrophilic than other proteins with a propensity to form amyloid and, unlike conventional forms of amyloid, the MSP2 fibrils melt at high temperatures. These characteristics are consistent with a structure in which between-sheet-packing of hydrophobic side-chains is of relatively minor importance. Both types of MSP2 fibrils, like amyloid, bound Congo Red but the 3D7 fibrils were also atypical in that they did not stain with ThT. It is not known how ThT binds to amyloid but this may also reflect a difference in the way the constituent sheets pack in the two types of MSP2 fibrils.

The failure of the 3D7 fibrils to bind ThT prevented the use of the ThT-binding assay to measure the kinetics of fibril formation but SEC analyses showed that FC27 MSP2 assembles into fibrils more rapidly than 3D7 MSP2. The lag phase, which could be reduced by agitation (data not shown), and the effect of seeding on MSP2 fibril formation demonstrated the nucleation dependence, which is typical of amyloid formation. Also typical for amyloid was the demonstration of PK-resistant core peptides [56,57]. The PK-resistant peptide derived from 3D7 fibrils contained the entire N-terminal conserved region of MSP2 and extended a few residues into the central variable region of the polypeptide. The dominant PK-resistant peptides from FC27 fibrils, which also contained the N-terminal conserved region, were much larger, extending C-terminally to within one or two residues of the C-terminal end of the first of the 32-mer repeats that characterize members of the FC27 MSP2 allelic family. If the assumption that these peptides are PK-resistant because they are the core of the cross- β structure is correct, fibril formation must be associated with significant conformational changes in both the helical region of the N-terminal conserved sequence and the unstructured 32-mer in FC27 MSP2 [26]. As fibrils can be readily generated from isolated N-terminal sequences [58] and from an MSP2 construct lacking the central variable region (data not shown) the role of the FC27 variable sequences (including the 32-mer repeat) in fibril formation is not clear. Although the PK experiments indicate that these sequences pack into the fibril core, it is possible that the decreased propensity of 3D7 to form fibrils reflects different conformational constraints imposed by the very different variable sequences in this type of MSP2. It is not clear why PK did not degrade the N-terminal hexa-His tag in the Ag1624 polymer. This may reflect the proximity of the His tag to the core of the fibril but the possibility that the His tag contributes to β -sheet formation in this MSP2 construct cannot be excluded.

No part of the C-terminal conserved region was resistant to proteolysis when fibrils were incubated with PK and we assume that this region of MSP2 remains largely unstructured in the fibril. Disruption of the single intramolecular disulphide bond in the C-terminal conserved domain of MSP2, had no consistent effect on fibril formation (data not shown). Consequently, the conformational constraints imposed by this disulphide bond are not an important factor in fibril formation.

For many amyloid-forming proteins, fibril formation is promoted by solution conditions that destabilize an ordered native conformation, but other proteins that are largely or partially intrinsically unstructured are known to form amyloid, for example, α -synuclein [59], gelsolin [60] and PrPc [61]. In MSP2 and other IUP, fibril formation may reflect the existence of one or more conformations with a propensity to form intermolecular β -strand interactions within an ensemble of equilibrium conformations. Structural studies on peptides corresponding to conserved N-terminal sequences of MSP2 have identified an eight-residue sequence (SNTFINNA) that forms a turn-like structure in solution [62]. This sequence is contained within the PK-resistant core identified here and it seems likely that it may nucleate the intermolecular interactions of MSP2 that result in the formation of fibrillar aggregates with a cross β -structure scaffold.

The unusual structural features described here for recombinant forms of MSP2 need to be considered in the context of vaccine development programs with this antigen. To our knowledge no subunit vaccine has been developed that includes an IUP as one of the component antigens. It is clear that infected individuals develop high-titre antibody responses to MSP2 but, because MSP2 must undergo a disorder to order transition when binding an antibody or B-cell receptor [23], it seems likely that much of the anti-MSP2 antibody response will be of relatively low affinity [63,64]. Monomeric recombinant MSP2 will exist in solution as an ensemble of equilibrium conformations, which may not include the dominant conformation(s) adopted by MSP2 on the merozoite surface as a result of the constraints imposed by membrane-anchoring and the protein-protein interactions involved in the formation of the merozoite surface coat. This may be an additional constraint on the ability of an MSP2 subunit vaccine to induce a highly effective antibody response.

The results presented here raise the possibility that some of the MSP2 on the merozoite surface exists as a homo-oligomer. The failure to detect the mAb 6D8 epitope on the merozoite surface by immunofluorescence microscopy is indirect evidence of polymerization of native MSP2 *in situ*. MAb 6D8 reacts with the conserved N-terminal fragment derived from fibrils by PK digestion but the availability of this epitope is substantially reduced upon polymerization of recombinant 3D7 MSP2 although, less so for FC27 MSP2. This raises the intriguing possibility that regions of MSP2 that are conserved in sequence among different alleles may have different conformations and therefore differ antigenically. MAb 6D8 reacted strongly on immunoblots with both forms of recombinant MSP2 but poorly with the parasite antigen. One possible explanation for this unexpected result is that the GPI anchor modifies the conformation of MSP2, as has been described for other GPI-anchored proteins [65], so that it is more prone to polymerize. N-terminal proteolytic processing of MSP2 (other than removal of the secretion signal) has not been observed (Fig. S7) but attempts by us (data not shown) and others [66] to identify the N-terminus of the mature MSP2 polypeptide have failed because the N-terminus was blocked.

Direct evidence that parasite MSP2 can form homo-oligomers has come from glutaraldehyde cross-linking experiments, which generated an oligomeric series with the same relative mobilities as the oligomers generated by cross-linking polymeric recombinant MSP2. Although it is likely that MSP2 is also cross-linked with other proteins to produce some of the very large complexes seen on the Western blot (Fig. 9), our results provide evidence that at least some of the MSP2 in the infected erythrocytes exists as homo-oligomers.

Several other parasite proteins are attached to the merozoite surface by GPI anchors but there have been no reports of any forming homo-oligomers. In contrast, the GPI-anchored major merozoite surface protein MSP1 forms hetero-oligomers with MSP6 [53] and MSP7 [52], two peripheral membrane proteins. Any oligomers of MSP2 on the merozoite surface would be much smaller than those formed *in vitro* by recombinant MSP2 as the extent of the lateral

association of MSP2 monomers is likely to be restricted by the localization of the GPI anchor within lipid rafts in the merozoite membrane [67]. Irrespective of the size of the MSP2 oligomers, the aggregation of MSP2 would significantly increase the conformational order of the monomeric subunit with consequences for the protein's antigenicity. At the same time much of the protein, presumably including the immunodominant central variable region, must remain relatively disordered and this could potentially result in antibody responses to alternative conformers of MSP2, which vary in their ability to block MSP2 function. These considerations highlight the importance of gaining an increased understanding of how the conformation of MSP2 is constrained by the protein-protein interactions on the merozoite surface for optimising the design of an MSP2 vaccine.

The propensity for proteins to form amyloid-like fibrils appears to have been selected against during evolution [68] but there have been numerous reports in recent years of proteins in which the 'amyloid-fold' has been preserved for functional reasons (reviewed in [69,70]). The most well known examples of this are prions in yeast and fungi [71–73] but more relevant to this study are the amyloid-like protein aggregates associated with the surface of several microorganisms [74]. Curli [75] and chaplins [76] form amyloid-like fibrils on the surface of certain bacteria whereas hydrophobins form related protein aggregates on the surface of some fungi [77,78].

If MSP2 oligomers formed by β -strand interactions are a component of the fibrillar surface coat on *P. falciparum* merozoites, it would be the first example of a functional amyloid-related protein aggregate on the surface of a protozoan parasite. The amyloid-like protein aggregates on other microorganisms have roles in attachment and invasion of substrates but also may enable certain pathogens to evade host immune responses [74]. It will be of considerable interest to determine whether MSP2 aggregates on the surface of *P. falciparum* merozoite have similar functions, particularly given the reports of some forms of amyloid interacting with red blood cells [79,80].

If MSP2 exists as a homopolymer on the merozoite surface it might be an effective strategy to include a recombinant homopolymer of MSP2 in a vaccine. However, given the uncertain relationship between the amyloid-like fibrils formed by recombinant MSP2 and the merozoite surface protein, the variable and unstable nature of the MSP2 fibrils, and the association of amyloid with a variety of pathologies, it seems reasonable and prudent for current MSP2 vaccine development activities to focus on the monomeric protein.

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Abbreviations

MSP2	merozoite surface protein 2
AMA1	apical membrane antigen 1
MSP1	

	merozoite surface protein 1
GPI	glycosylphosphatidylinositol
SEC	size-exclusion chromatography
DLS	dynamic light scattering
RSB	reducing sample buffer
GdnHCl	guanidine-HCl
mAb	monoclonal antibody
TEM	transmission electron microscopy
PK	proteinase K
RP-HPLC	reverse phase high pressure liquid chromatography
TFA	trifluoroacetic acid
PVDF	polyvinylidene fluoride
ThT	Thioflavin T
IUP	intrinsically unstructured proteins
MS	mass spectrometry

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Fig. 1.

MSP2 has the characteristics of an IUP. (A) 3D7 MSP2 stored long term at -70°C was solubilized in a buffer containing 6 M GdnHCl to form monomer and analysed by SEC. A similar profile was obtained (not shown) for recombinant FC27 MSP2 and for MSP2 in the presence of 10 mM dithiothreitol. (B) SDS-PAGE analysis of recombinant 3D7 and FC27 MSP2 stained with Coomassie Blue (left panel) and an extract of 3D7 and FC27 *P. falciparum* proteins probed with sheep antibodies raised against 3D7 MSP2 in a Western blot analysis (right panel).

Fig. 2.

Fibril morphology of MSP2. (A) 3D7 MSP2 stored for ~9 years at -70°C that was solubilized in a buffer containing 6 M GdnHCl to form monomer and (B) then dialysed against 0.02 M Na_2HPO_4 (pH 7.4) and 0.5 M NaCl to reconstitute the polymer. (C) Arrows indicate the presence of twists in the fibrils. (D) Monomeric 3D7 MSP2 in PBS incubated at room temperature for 3 days. (E) Freshly prepared recombinant FC27 MSP2 incubated in PBS at room temperature for several weeks. (F) Freshly prepared recombinant FC27 MSP2 lacking the hexa-His tag incubated in PBS at room temperature for 3 days. 3D7 MSP2 can seed the growth of (G) 3D7 and (H) FC27 MSP2 fibrils. Samples were negatively stained and viewed using transmission electron microscopy.

Fig. 3.

MSP2 has some characteristics of amyloid. (A) Absorbance spectrum of Congo Red alone (closed diamonds) and in the presence of monomeric 3D7 MSP2 (open squares). Polymeric 3D7 MSP2 (closed triangles) bound Congo Red with the maximum absorbance red-shifting from 488 nm to 540 nm as did amyloid formed from apolipoprotein CII (open circles). (B) Neither monomeric (open triangles) or polymeric (open squares) 3D7 MSP2 bound ThT in contrast to A β peptide 1–42 (closed squares) and apolipoprotein CII (open circles) amyloid which exhibited a fluorescence emission spectrum typical of amyloid stained with ThT. (C) Circular dichroism spectra of monomeric (squares) and polymeric (triangles) FC27 MSP2. (D) X-ray fibre diffraction pattern of polymeric 3D7 MSP2.

Fig. 4.

Digestion of 3D7 MSP2 polymer results in a PK-resistant core. (A) Silver-stained SDS-PAGE gel illustrates the results of the digestion of polymeric and monomeric 3D7 MSP2 with PK. (B) The chromatograph of polymeric 3D7 MSP2 digested by PK and separated by reverse phase chromatography. The peak eluting at 17.4 min was subjected to Edman sequencing and MS analysis, which indicated that the core corresponded closely to the conserved N-terminal region of MSP2. (C) The four most abundant peptides, based on intensity (listed), were found to be methylated. Refer to Fig. S1 for the complete primary sequence of the MSP2 fragments. (* most abundant peptide)

Fig. 5.

FC27 MSP2 binds ThT. (A) Three concentrations of 3D7 and FC27 monomeric and polymeric MSP2 were analysed for ThT binding. Error bars represent the standard error of the mean for triplicate assays. (B) Kinetics of fibril formation by various concentrations of FC27 MSP2. Triplicate reactions were established with monomeric FC27 MSP2 and the average values for the measured ThT fluorescence were plotted against time.

Fig. 6. X-ray fibre diffraction pattern of polymeric C-terminally hexa-His-tagged 3D7-6H and FC27-6H MSP2.

Fig. 7.

Digestion of C-terminally hexa-His-tagged 3D7-6H and FC27-6H MSP2 polymer results in the generation of PK-resistant peptides. (A) Silver-stained SDS-PAGE gel before (lanes 1 and 3) and after (lanes 2 and 4) the digestion of FC27-6H (lanes 1 and 2) and 3D7-6H (lanes 3 and 4) MSP2 with PK. (B) The chromatograph of polymeric 3D7-6H and FC27-6H MSP2 digested by PK and separated by reverse phase chromatography. The peaks eluting at ~35 min (boxed) were collected, subjected to Edman sequencing and MS analysis, which indicated that the core corresponded closely to the conserved N-terminal region of MSP2. The most abundant peptides for (C) 3D7-6H and (D) FC27-6H MSP2 are listed. The observed molecular weights include an additional proton. Refer to Fig. S1 for the complete primary sequence of the MSP2 fragments.

Fig. 8.

MSP2 may be polymeric on the merozoite surface. Binding of monoclonal antibodies (A) 6D8 and (B) 11E1 to monomeric (triangles) and polymeric (squares) 3D7 MSP2 as shown by ELISA. The error bars represent the SEM of three replicates on the same plate. (C) Western blotting experiments of recombinant 3D7 (lane 1) and FC27 MSP2 (lane 2), the PK-resistant core (lane 3), and 3D7 (lane 4) and D10 (lane 5) parasite lysates probed with mAbs 6D8 and 11E1 and polyclonal sheep sera raised against 3D7 and FC27 MSP2. (D) Immunofluorescence microscopy data showing mAb 11E1 binding to the 3D7 parasite strain only (f, fluorescence; n, nuclear stain; b, bright field).

Fig. 9.

MSP2 forms oligomeric complexes. (A) A silver-stained SDS-PAGE gel showing monomeric and polymeric 3D7 MSP2 that was isolated by SEC and cross-linked with various concentrations of glutaraldehyde. Western blotted extracts of (B) D10 and (C) 3D7 strains of *P. falciparum* from parasites that were cross-linked with various concentrations of glutaraldehyde. Sheep sera raised against FC27 MSP2 (B) and rabbit sera raised against 3D7 MSP2 (C) were used to detect the presence of the parasite antigen.