Defining the Minimal Domain of the *Plasmodium falciparum* Protein MESA Involved in the Interaction with the Red Cell Membrane Skeletal Protein 4.1*

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During part of its life cycle, the protozoan parasite *Plasmodium falciparum* lives within the human red blood cell and modifies both the structural and functional properties of the red cell. It does this by synthesizing a number of polypeptides that it transports into the red cell cytoplasm and to the red cell membrane. One of these transported proteins, MESA (mature parasite-infected erythrocyte surface antigen), is anchored to the red cell membrane by noncovalent interaction with erythrocyte protein 4.1. We have utilized a combination of *in vitro* transcription and translation and a membrane binding assay to identify the protein sequence involved in anchoring MESA to the membrane. Labeled fragments of different regions of the MESA protein were evaluated for their ability to bind to inside-out vesicle membrane preparations of human red cells. Binding was dependent on the presence of red cell membrane proteins and was abolished either by trypsin treatment or by selective depletion of membrane proteins. Binding was specific and could be inhibited by the addition of competing protein, with an IC50 of 6.3 ± 1.2 × 10^-7 M, indicative of a moderate affinity interaction. Fractionation studies demonstrated that binding fragments interacted most efficiently with membrane protein fractions that had been enriched in protein 4.1. Binding inhibition experiments using synthetic peptides identified the binding domain of MESA for protein 4.1 as a 19-residue sequence near the amino terminus of MESA, a region capable of forming an amphipathic helix.

The red blood cell has become one of the pre-eminent systems for the analysis of structure-function relationships in the membrane skeleton. It is probably the best understood eukaryotic cell, particularly in regard to the structural organization of the membrane skeleton and its role in regulating the mechanical properties of the cell (1–3). The ordered arrangement of spectrin tetramers, their interconnection at the ternary complex (with actin and protein 4.1), and the linkages to the overlying cell membrane via band 3 and glycophorin C provide the basis for the cell’s ability to deform during passage through the microcirculation (4, 5). The stability of the spectrin network is influenced by factors such as the primary sequence of the component proteins and levels of protein phosphorylation (6, 7). This understanding of the relationship of the protein network to properties of the whole cell has been advanced by the study of pathological states of red cells in which specific protein interactions have been altered. These include inherited disorders of red cells such as hemoglobinopathies, hereditary spherocytosis, elliptocytosis, and ovalocytosis. We now recognize that the maturation of the intracellular malaria parasite also results in a series of dramatic and extensive changes in the structural and functional properties of the red cell (reviewed in Refs. 8–15). These changes include loss of the normal discoid shape as the infected cell becomes spherocytic and its surface is punctuated by 5,000–10,000 localized, electron-dense elevations of the red cell membrane called knobs (16–18). There is increased permeability of the infected red cell to a wide variety of substrates including essential amino acids, phospholipid precursors (19, 20), nucleotides (21, 22), and lactate and iron (23). There are changes in the membrane mechanical properties of the cell with a marked increase in rigidity in red cells infected with mature parasites, and the state of phosphorylation of membrane skeletal proteins is also changed. Strikingly, the infected cells become adhesive for a number of other cells including endothelial cells, normal red cells, and lymphocytes. These changes are important for the survival of the parasite, and in their absence, the parasite dies, or parasitized red cells are rapidly eliminated (11, 24, 25).

Crucial to these changes are the export of a group of proteins of parasite origin into the red cell cytoplasm and to the red cell membrane. This latter group of parasite proteins associates with the red cell membrane skeleton either by deposition on the inner aspect of the membrane or by transient or more permanent insertion into the membrane. One such exported protein is MESA (mature parasite-infected erythrocyte surface antigen), a 250–300-kDa phosphoprotein produced early in the trophozoite stage and found in association with the red cell membrane skeleton (26–28). MESA interacts with the internal aspect of the host erythrocyte membrane and is not exposed on the external surface, although in late stage schizonts, it becomes accessible to external surface-labeling agents such as lactoperoxidase (28, 29). The nucleotide sequence of MESA has been reported, and MESA is composed of sets of extensive repeat regions interspersed with nonrepetitive sequences. Lustigman et al. (30) showed that MESA was coprecipitated with the functionally important erythrocyte skeletal component protein 4.1, suggesting that it was anchored in the membrane skeleton by noncovalent linkage to protein 4.1. Further evidence for this association was provided by studies of malaria infection in...
P. falciparum MESA Binding Domain for Red Cell Protein 4.1

In mutant red cells with complete deficiency of protein 4.1. In such cells, the distribution of MESA was altered in that it was no longer membrane-bound, but was randomly distributed throughout the red cell cytoplasm (31).

Protein 4.1 plays a critical role in maintaining the mechanical integrity of the red cell membrane through its interaction with spectrin and actin (32–34). Protein 4.1 also plays a role in attachment of the skeleton to the membrane by anchoring the spectrin network to the integral protein glycoporphin C (35). The role of the MESA-protein 4.1 interaction in parasite biology has not been clearly delineated, although it appears to be essential for the survival of the parasite in that malaria parasites cannot survive if MESA is unable to bind protein 4.1 and remains free in the red cell cytoplasm (31).

To further our understanding of the interaction between protein 4.1 and MESA, we set out to determine the precise molecular domains involved in this interaction. In this paper, we describe the identification of a sequence in MESA that we describe the identification of a sequence in MESA that

**EXPERIMENTAL PROCEDURES**

**Cell Culture of Plasmodium falciparum—**P. falciparum asexual stage parasites were grown in vitro as described by Trager and Jensen (36). P. falciparum-infected erythrocytes were grown in a 2–5% hematocrit to a maximum parasitemia of 20%. Cultured parasites were metabolically labeled with [35S]methionine, harvested, and immunoprecipitated as described (30). Rabbit antibodies against the hexapeptide amino-terminal repeat of the MESA sequence were generated as described (28).

**Triton X-100 Extraction of the Red Blood Cell Pellet—**Erythrocyte cell pellets were incubated for 30 min at 4 °C with a 10-pellet volume of TNET (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, and 0.05% Triton X-100) containing protease inhibitors (10 μg/ml phenylmethylsulfonyl fluoride, 25 μg/ml chymostatin, and 2 μg/ml leupeptin). Triton X-100-insoluble material was pelleted by centrifugation at 15,000 × g for 10 min at 4 °C, and the supernatant was kept as the Triton X-100-insoluble fraction. Triton X-100-insoluble pellets were solubilized in 50 μl of 2% SDS in PBS at 4 °C and incubated at room temperature for 30 min. Afterward, 50 μl of 2% Triton X-100 and 400 μl of TNET were added (final concentrations of 0.2% SDS and 0.7% Triton X-100). Immunoprecipitations were performed as described (30).

**Preparation of Inside-out Vesicles—**To 1 ml of fresh washed red blood cells, 25 ml of ice-cold 5 mM phosphate buffer, pH 8.0, was added and mixed by inversion. The cells were spun in a Sorvall centrifuge at 8,000 × g for 10 min. This procedure was repeated until the supernatant was free of hemoglobin (two to three times). The cell pellet, composed of red blood cells, was resuspended in 1.5 ml of incubation buffer (138 mM NaCl, 5 mM KCl, 6.1 mM NaH2PO4, 1.4 mM NaH2PO4, and 5 mM glucose). To make inside-out vesicles, 20 ml of 0.5 mM phosphate buffer, pH 8.0, was mixed with the cell ghosts, incubated on ice for 30 min, and pelleted by centrifugation at 8,000 × g. 500 μl of 0.5 mM phosphate buffer, pH 8.0, was added to the pellet, and the suspension was passed through a 26-gauge needle seven times. The vesicles were resuspended in 10 ml of 0.5 mM phosphate buffer, pH 8.0, and the mixture was centrifuged in a Sorvall centrifuge at 8,000 × g. The vesicles were resuspended in 1.5 ml of incubation buffer and stored at 4 °C until used.

**Binding Assay—**The binding assay involved coating the wells of a polyclinidine trichlo red buffer with IOVs by loading the IOV preparation into individual wells and incubating the tray at 4 °C overnight, followed by a 2-h incubation with 1% BSA in phosphate-buffered saline at 4 °C. An aliquot of TnT 35S-labeled MESA fragment in 50 μl of incubation buffer supplemented with 10 mM t-hydroxyamine was added to each well and incubated overnight at 4 °C. The wells were then washed three times with incubation buffer, and the bound MESA fragments were removed using heated (>70 °C) SDS sample buffer.

**Construction of MESA Clones in the pRSET Vector—**MESA DNA fragments were amplified by PCR amplification methods and directly cloned in frame into the PcoII site of the pRSET vectors. Conditions were adjusted to maximize fidelity and minimize numbers of cycles and a low concentration of enzyme and deoxyoligonucleotides. The oligonucleotide primers used in each reaction are listed in Table I. Orientation of the clone was determined by PCR DNA amplification and restriction enzyme digestion of DNA and was confirmed by automated double-stranded DNA sequencing. pRSET/MESA constructs were sequenced using the PRISM Ready Reaction Dye Deoxy Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA) and the ABI Model 373A DNA sequencing system (Applied Biosystems, Inc.) according to the manufacturer’s instructions. Sequencing studies on the recombinant clones did not reveal any PCR-generated changes in nucleotide sequence. Positive recombinants were purified by alkaline lysis and CsCl gradient centrifugation.

**In Vitro Transcription and Translation Methods—**In vitro transcription and translation were performed in a TnT rabbit reticulocyte lysate expression system (Promega, Madison, WI) according to the manufacturer’s protocols. A typical reaction mixture included 25 μl of rabbit reticulocyte lysate, 2 μl of TnT reaction buffer, 1 μl of 77 RNA polymerase, 1 μl of amino acid mixture minus methionine, 4 μl of Trans-S-100 μCi/ml [35S]methionine, 1 μCi/ml [32P]orthophosphate, 1,000 mCi/ml [35S]methionine, (ICN Amersham, Buckinghamshire, United Kingdom), 1 μl of RNAse inhibitor (1,000 units/μl), and 0.2–2 μg of DNA template, to a final volume of 50 μl in nucleoside-free water. The mixture was incubated for 120 min at 30 °C, and the translation products were analyzed by SDS-PAGE.

**Purification of the pGEX Fusion Protein—**Overnight cultures (60 ml) of Escherichia coli (JM109) transformed with recombinant pGEX plasmids were diluted 1:10 in fresh Luria broth containing 200 μg/ml ampicillin and grown for 1 h at 37 °C. Isopropyl-1-thio-β-D-galactopyranoside was added to 1 mM, and the culture was grown for a further 3–4 h. After centrifugation, the pellet was resuspended in PBS, lysed by sonication, and centrifuged at 10,000 × g for 5 min at 4 °C. The supernatant was then passed through a column containing 2 ml of preswollen glutathione-agarose beads equilibrated with PBS. The column was washed with 100 ml of PBS. Fusion protein was eluted by competition with 5 mM reduced glutathione (Sigma) in 50 mM Tris-HCl, pH 8.0.

**Purification of pRSET Fusion Proteins—**Overnight cultures (60 ml) of E. coli (JM109DE3) transformed with the recombinant pRSET construct were diluted 1:10 in fresh Luria broth containing 200 μg/ml ampicillin and grown at 37 °C to A600 nm = 0.6–0.8. Isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM and further incubated with shaking for 3–8 h at 37 °C. After centrifugation, the cell culture was pelleted, snap-frozen in dry ice, and stored at −70 °C until purification. The pellet was thawed at room temperature, resuspended in 20 ml of guanidine lysis buffer (6 μM guanidine HCl and 0.1 mM KPO4, pH 7.4), and mixed by rotation for 1–2 h at room temperature. The lysate was sonicated and transferred to a 30-ml Corex tube and centrifuged at 8,000 × g for 1 h. The supernatant was carefully transferred to a small 40-ml tube and centrifuged (12,000 × g, 15 min, 4 °C). The supernatant was transferred to a 40-ml tube and stored at −70 °C or used immediately for affinity purification of the recombinant peptides. A 2-ml bed volume column of Ni2+-NTA-agarose absorbent was prepared and equilibrated with guanidine lysis buffer. A 20-ml supernatant sample was diluted to 100 ml with guanidine lysis buffer and passed twice over the column. Four washes followed: 50 ml of guanidine lysis buffer; 50 ml of 1 mM NaCl and 50 mM KPO4, pH 7.4; and 50 ml of 1 mM NaCl and 50 mM KPO4, pH 7.4; and 100 ml of low salt buffer (30 mM NaCl and 50 mM KPO4, pH 7.4). The column was washed with additional low salt buffer until the A280 nm of the wash was <0.005. To elute the bound recombinant protein from the column, 5 ml of 100 mM EDTA, pH 8.0, was added; 1-ml fractions were collected; and the A280 nm, reading was measured. The Ni2+ was removed by extensive dialysis against PBS. The proteins were then analyzed by SDS-PAGE and Coomassie Blue staining.

**Purification of the Cytoskeletal Proteins of Erythrocytes—**Fresh blood (50 ml) was used to make IOVs as described above, and equal amounts of IOVs were treated with high ionic strength buffer (5 mM phosphate buffer, pH 8.0, and 1 M NaCl) to elute the proteins from the vesicle membrane. The eluate was dialyzed to 20 mM KCl and 5 mM phosphate buffer and passed over a DEAE-Septadex column (Pharmacia, Uppsala). Increasingly higher ionic strength KCl solutions (50, 80, and 165 mM KCl) in 5 mM phosphate buffer were used to elute bound protein fractions from the column. Each of the fractions was dialyzed against PBS over 2 days and stored at −70 °C. Samples were separated...
uninfected red blood cells were labeled with \([^{32}P]\)orthophosphate 4.1. Lanes 1–4, Triton X-100-soluble fractions (TX-S); lanes 5–8, Triton X-100-insoluble fractions (TX-I). Lanes 1 and 5, D6-3 (+MESA, +KAHRP); lanes 2 and 6, D6-1 (+MESA, +KAHRP); lanes 3 and 7, D10 (+MESA, +KAHRP); lanes 4 and 8, uninfected erythrocytes (urinephed red blood cells (URBC)). Note the biepin solubility in Triton X-100 of D10 (lanes 3 and 7) and, to lesser extent, D6-3 (lanes 1 and 5). Immunoprecipitation with anti-GESKET antiserum precipitated MESA and protein 4.1 proteins (lane 7). B, Immunoprecipitation with [\(^{32}\)P]orthophosphate-labeled P. falciparum cell cultures using the polyclonal rabbit anti-protein 4.1 antiserum. Mid-trophozoite stage (+32 h) cell cultures were grown in the presence of [\(^{32}\)P]orthophosphate in phosphate-free medium for 4 h, and the cell pellets were extracted with Triton X-100.

**FIG. 1.** A, immunoprecipitation of [\(^{32}\)P]orthophosphate-labeled P. falciparum cell cultures using the polyclonal rabbit anti-GESKET antiserum. Mid-trophozoite stage (+32 h) cell cultures were grown in the presence of [\(^{32}\)P]orthophosphate in phosphate-free medium for 4 h, and the cell pellets were extracted with Triton X-100. Lanes 1–4, Triton X-100-soluble fractions (TX-S); lanes 5–8, Triton X-100-insoluble fractions (TX-I). Lanes 1 and 5, D6-3 (+MESA, +KAHRP); lanes 2 and 6, D6-1 (+MESA, +KAHRP); lanes 3 and 7, D10 (+MESA, +KAHRP); lanes 4 and 8, uninfected erythrocytes (uninfected red blood cells (URBC)). Note the biepin solubility in Triton X-100 of D10 (lanes 3 and 7) and, to lesser extent, D6-3 (lanes 1 and 5). Immunoprecipitation with anti-GESKET antiserum precipitated MESA and protein 4.1 proteins (lane 7). B, Immunoprecipitation with [\(^{32}\)P]orthophosphate-labeled P. falciparum cell cultures using the polyclonal rabbit anti-protein 4.1 antiserum. Mid-trophozoite stage (+32 h) cell cultures were grown in the presence of [\(^{32}\)P]orthophosphate in phosphate-free medium for 4 h, and the cell pellets were extracted with Triton X-100. Lanes 1, 3, and 5, Triton X-100-soluble fractions; lanes 2, 4, and 6, Triton X-100-insoluble fractions. Lanes 1 and 2, D6-1 (+MESA, +KAHRP); lanes 3 and 4, uninfected erythrocytes (uninfected red blood cells (URBC)); lanes 5 and 6, D6-3 (+MESA, +KAHRP). Note the increase in phosphorylation of the red cell membrane proteins upon infection with P. falciparum. Immunoprecipitation with anti-protein 4.1 antiserum precipitated protein 4.1 and MESA (lane 6).

by denaturing SDS-PAGE, and the gels were stained with silver nitrate (37).

**Figure 1**

**A**

**B**

**RESULTS**

**Association of MESA and Protein 4.1**—Previous work demonstrated that rabbit antibodies raised against the repeat region of MESA were capable of immunoprecipitating two proteins from infected red cells that had been biosynthetically labeled with [\(^{32}\)P]orthophosphate (30). The two coprecipitated proteins were identified as MESA and the red cell membrane skeletal protein protein 4.1 on the basis of molecular mass determination and characteristic peptide mapping profiles (30). We set out to determine whether MESA would be coprecipitated from labeled lysates of infected cells by an antiserum to protein 4.1. *P. falciparum* lines D6-1, D6-3, and D10 and uninfected red blood cells were labeled with [\(^{32}\)P]orthophosphate. Both D6-3 and D10 express MESA; however, D6-1 is a laboratory-derived, mutant parasite line that has undergone a spontaneous deletion of a region of chromosome 5 that encompasses the MESA-coding sequence and consequently does not express MESA. Parasite lysates were successively solubilized in Triton X-100 and SDS to yield fractions of Triton X-100-soluble and Triton X-100-insoluble proteins. These fractions were then immunoprecipitated with various antisera, including a polyclonal rabbit anti-MESA antiserum, a polyclonal rabbit anti-protein 4.1 antiserum, and normal rabbit serum. The MESA antiserum was raised against the hexapeptide sequence GESKET, a highly conserved repeating unit found in the most NH₂-terminal repeat domain of MESA (38). MESA in isolates D10 and D6-3 was found predominantly in the Triton X-100-insoluble phase, due to its association with the erythrocyte membrane (Fig. 1A). No phosphorylated proteins in uninfected red blood cells or D6-1 were immunoprecipitable with the polyclonal rabbit anti-GESKET antiserum. Phosphorylated protein 4.1 was coprecipitated with MESA in D10 and, to a lesser extent, in D6-3 (Fig. 1A). In parallel experiments, lysates of [\(^{32}\)P]labeled D6-1 (+MESA) and D6-3 (+MESA) cultures were solubilized in Triton X-100 and immunoprecipitated with anti-protein 4.1 antibodies. A number of proteins of varying molecular mass were coprecipitated. These included the red cell protein spectrin (240 and 220 kDa), actin (43 kDa), and protein 4.1 (80 kDa). In addition, there was no phosphenoprotein of >250 kDa precipitated in D6-3 (+MESA) parasites, but not in D6-1 (−MESA) parasites, that comigrated with MESA (Fig. 1B). We conclude that there is an association between MESA and protein 4.1 in infected red cells that results in coprecipitation of the two proteins by antiserum to either MESA or protein 4.1.

**Expression of MESA in the Prokaryotic System**—We set out to identify sequences in MESA responsible for its association with the red cell membrane skeleton. The pRSET expression system (Invitrogen, San Diego, CA) was chosen because it offered several advantages including easy purification of the fusion protein under native or denaturing conditions and in vitro labeling and expression of the very same construct. The coupled transcription/translation TnT rabbit reticulocyte lysate system from Promega was used to express MESA constructs termed IVTT (in vitro transcription and translation). This system provided us with a simple and reliable cell-free transcription and translation system for the expression of
MESA polypeptides. Certain experimental approaches were not available to us due to peculiarities of the MESA protein. It was not feasible to purify native MESA from infected red cells due to the relatively low amounts of this protein in infected cells. Furthermore, the insolubility of membrane-bound MESA in non-ionic detergents precluded the use of lysates of infected cells in direct binding assays. The full-length mesa gene is extremely unstable in prokaryotic cloning vectors, and we were therefore unable to undertake studies in which the full-length protein could be used in binding studies.

Accordingly, we adopted an approach in which stable fragments of the MESA protein were cloned into the RNA transcription vector pRSET. RNA produced in this manner was translated in an in vitro translation system in the presence of \[^{35}S\]methionine, and the labeled protein was used for binding assays. Although there was some variability in the yield of any particular MESA fragment, all fragments gave readily detectable amounts of radiolabeled product (data not shown). Variation in strength of labeling between fragments appears to be due both to differences in the number of methionine residues in particular sequences and to intrinsic differences in the efficiency of transcription and translation of individual constructs. Translation products consisted of the full-length product and, in some constructs, a series of bands of lower molecular mass (data not shown), which we interpret to be fragments that resulted from premature termination, internal initiation, or perhaps proteolytic degradation. All translated fragments exhibited a lower mobility than expected for their molecular mass, presumably reflecting their highly charged sequences, a finding that is very common for malaria proteins as a group (39). For the binding assay, we chose to vary the amount of added translation products so that approximately equal amounts of labeled material (as judged from autoradiographs of gels of electrophoretically separated translation products) were added to each aliquot of red cell membrane preparation (data not shown).

**Binding of MESA Fragments to IOVs**—We elected to study the binding of MESA to the red cell membrane skeleton by examining the interaction of labeled fragments with IOVs prepared from uninfected red cells. We chose IOVs as they provide a convenient and accessible preparation of erythrocyte membrane and skeletal proteins that are present in combinations that approximate those found in vivo. Thus, there may be particular constraints on the conformation or accessibility of regions of the skeletal proteins that are better approximated in IOV preparations than in isolated proteins, and these would provide a more suitable binding target for our initial studies. To assess which part of the MESA protein contained a binding domain for IOVs, six pRSET/MESA polypeptide constructs encompassing the whole second exon of the mesa gene sequence were radiolabeled by IVTT and added to aliquots of IOVs. The first exon, which encodes 50 residues, is believed to contain a signal sequence that is removed from the mature MESA polypeptide (38). Bound fragments (F1–F6) from parallel reactions were eluted, pooled, and subjected to SDS-PAGE (Fig. 2). Autoradiographs of the recovered products revealed that MESA F3 (residues 51–496) and its subfragment F1 showed specific affinity for IOVs (Fig. 2, lanes 1 and 3). This suggested that the MESA F1 polypeptide contained a domain that mediates the interaction of MESA with the erythrocyte membrane or skeleton. No MESA fragments that lacked MESA F1 sequence (i.e. MESA F2 and F4–F6) showed detectable binding, suggesting that at least by this assay, the only membrane-binding sequences were in MESA F3 (Fig. 2). The amount of binding of MESA F1 and F3 to IOVs increased in a linear fashion, but became saturated when larger amounts were added (data not shown). All further binding experiments were performed using amounts of MESA fragments that were within the linear range of binding.

As the F3 fragment was more abundantly produced and more strongly labeled than the F1 fragment in our system, we used it in additional studies. To examine the specificity of binding of the F3 fragment to IOVs, we performed competition experiments in which unlabeled MESA proteins produced in a prokaryotic expression vector were used to inhibit the binding of labeled proteins to IOVs. In these experiments, a binding assay was performed in which (following the 1% BSA blocking step) a second blocking step was included, in which IOV-coated wells were preincubated with 500 \(\mu\)g/ml unlabeled MESA F2, F3, or F6 fusion protein. Labeled MESA F3 was then added to the wells, and the assay was performed and analyzed in the usual manner. Preincubation of IOV-coated wells with MESA F3 markedly reduced the binding of IVTT-expressed MESA F3 to IOVs (Fig. 3A, lane 1). However, preincubation of IOVs with MESA F2 or F6 had no effect on the binding of IVTT-expressed MESA F3 to IOVs (Fig. 3A, lanes 2 and 3). Wells coated with BSA, which lacked any IOV target, demonstrated the level of background binding in the assay and revealed that the addition of unlabeled MESA F3 reduced the binding of labeled MESA F3 to the background level (Fig. 3A, lane 4).

**Binding of MESA F3 to Various Components of the Erythrocyte Cytoskeleton**—We set out to examine the factors that influence the binding of the MESA F3 fragment to the membrane skeleton by treating IOVs with a number of agents and measuring the effect on binding. Pretreatment of IOVs with trypsin abolished binding of MESA F3 (Fig. 3A, lane 5), as did treatment with 1 M KCl solution (Fig. 4). This suggested that the MESA ligand was a protease-sensitive peripheral membrane protein or was stabilized at the red cell membrane by association with a peripheral membrane protein. This was confirmed by examining the binding of MESA F3 to IOVs stripped of peripheral proteins or the freed mixture of peripheral membrane proteins. MESA F3 bound strongly to wells coated with freed peripheral membrane proteins (Fig. 4). Silver staining of separated proteins found in the freed peripheral membrane

![Fig. 2. Binding of IVTT-expressed MESA fragments to IOVs.](image-url)
MESA F3 MINIMAL BOUND TO IOVs

- F3
- F2
- F1
- F0

PRE-DIACUBATION OF IOVs WITH
- MESA F3
- MESA F2
- MESA F1
- MESA F0

Fig. 3. A, trypsin sensitivity and competitive inhibition of the binding of radiolabeled MESA F3 to IOVs. Microtiter tray binding assays were carried out using IVTT-expressed and radiolabeled MESA F3. Triplicate samples were pooled, analyzed by SDS-PAGE on 10% acrylamide gel, and autoradiographed. Before the addition of radiolabeled MESA F3, the IOV-coated wells were preincubated with a molar excess (>200 \( \mu \)g/ml) of unlabeled purified MESA F3 (lane 1), MESA F2 (lane 2), and MESA F6 (lane 3). Lane 4 contains radiolabeled MESA F3 that bound to wells coated with BSA alone, and lane 5 contains radiolabeled MESA F3 that bound, in the presence of soybean trypsin inhibitor, to wells coated with trypsin-treated IOVs. Lane 6 contains MESA F3 bound to untreated IOVs. Note the competitive inhibition of MESA F3 binding to IOVs by MESA F3, but not MESA F2 or F4. The binding of MESA F3 to IOVs was trypsin-sensitive, indicating the importance of protein components of the membrane skeleton for MESA binding. B, titration of competitive inhibition of the binding of radiolabeled MESA F3 to IOVs. A standard microtiter binding assay was carried out using IVTT-expressed and radiolabeled MESA F3. Pooled triplicate wells were analyzed by SDS-PAGE on a 12% polyacrylamide gel and autoradiographed. Before the addition of radiolabeled MESA F3, the IOV-coated wells were preincubated with increasing concentrations of MESA F3.7, 0.2 (lane 1), 2 (lane 2), 20 (lane 3), 200 (lane 4), and 2,000 (lane 5) \( \mu \)g/ml. Note that MESA F3.7 competitively inhibited the binding of MESA F3 to IOVs and that this inhibition was concentration-dependent.

A series of fragments that encompassed the carboxyl terminus of the F3 fragment including the hexapeptide repeat region (MESA F3.3, F3.4, and F3.5) (Table I) all failed to show binding (data not shown). However, PCR-generated fragments that included the amino-terminal sequences of MESA F3 did show binding, including MESA F3.7 (residues 51–143) (data not shown).

A competitive inhibition experiment was performed in which unlabeled MESA F3.7 was used to compete the binding of radiolabeled MESA F3 to IOVs. MESA F3.7 fusion protein at 200 \( \mu \)g/ml abrogated the binding of IVTT-expressed MESA F3 to IOVs (Fig. 3B, lane 4). Inhibition of binding was lost as the concentration of MESA F3.7 decreased (Fig. 3B, lane 1–3). Further experiments determined the IC \(_{50}\) of this interaction to be \((6.3 \pm 1.2) \times 10^{-7} \text{ M (data not shown)}, a value very similar to that determined for the association constant of protein 4.1 for spectrin in the red cell skeleton (40). This result suggests that MESA F3 binding to IOVs is specific and inhibitable in a concentration-dependent manner and that all binding sequences of MESA F3 are contained in sequences in the MESA F3.7 fragment.

In a complementary set of experiments, the binding of restriction digest-derived fragments yielding a series of fragments progressively truncated at the carboxyl terminus of MESA F3 was assayed. All COOH-terminally truncated MESA F3 fragments bound to IOVs (Fig. 5 and data not shown). The minimal binding region was found to be 64 residues at the NH\(_2\) terminus of MESA F3 flanked by an EcrRV site at the start of the second exon and a ClaI site (residues 51–114).

**Peptide Inhibition of the Binding of MESA F3 to IOVs—**
MESA fragment boundaries are defined by nucleotide and residue positions using the numbering given by Coppel (38).

| Fragment | Primers | Boundaries of fragment, nucleotides (residues) |
|----------|---------|---------------------------------------------|
| F1       | M5, ATGGATATCATTAAGGAAATTTGCAAGA | 502–1399 |
|           | M9, CTGTATATCAAGGTTTCTCGG      | (51–1395) |
| F2       | M10, CCAAAATAATACAGAAACATCAAAG | 1574–1837 |
|           | M11, CATTTTTTACAGATTACAGC       | (408–495) |
| F3       | M5, ATGGATATCATTAAGGAAATTTGCAAGA | 502–1399 |
|           | M11, CATTTTTTACAGATTACAGC       | (408–495) |
| F3.3     | M7, GGGTTGCGGTGGTGACAG         | 707–1399 |
|           | M9, CTTGTATACGCCGTTTTGTCG       | (119–349) |
| F3.4     | M8, GAAATCGATGGAAACATCAACATCAAAGA | 880–1399 |
|           | M9, CTTGTATACGCCGTTTTGTCG       | (176–349) |
| F3.5     | M7, GGGTTGCGGTGGTGACAG         | 707–1399 |
|           | M11, CATTTTTTACAGATTACAGC       | (119–495) |
| F3.7     | M5, ATGGATATCATTAAGGAAATTTGCAAGA | 502–781 |
|           | M6, TTTCCGATCTCTCCGATTTCAGTTTGCAC | (51–143) |
| F4       | M12, ATGGATATCATTAAGGAAATTTGCAAGA | 1819–2937 |
|           | M13, TACCTTTTTACCTGTGTGTGTTCAC | (490–862) |
| F5       | M14, ATGTTGACAAAAACAGGGAAAAAGTA | 2917–4465 |
|           | M15, GTACCAAAACCTGTATGTAT       | (856–1371) |
| F6       | M16, ATACATACGTTGGTGAC         | 4448–4995 |
|           | M17, CATTTTTTACAGATTACAGC       | (1350–1526) |

**DISCUSSION**

The successful interaction between malaria-encoded polypeptides and proteins of the red cell membrane skeleton is necessary for the parasite to modify the structural and functional properties of the red cell. We have shown that of the 1476 amino acids in the second exon of MESA (D10 strain), a remarkably short 19-amino acid sequence is involved in a specific interaction with the red cell membrane skeleton via protein 4.1. We do not believe that the 19 amino acids would confer all the strength of the interaction between MESA and protein 4.1. Other portions of the MESA molecule may assist in enhancing the strength of interaction of MESA with protein 4.1, probably elsewhere within the MESA F3 region.

The 19-residue sequence contains no repeats and shows no significant homologies to any known protein 4.1-binding proteins such as myosin and spectrin. MESA shows some low level homologies to chicken and mouse myosin, rat and mouse MAP-2 protein, chicken troponin T, mouse neurofilament L, rat and mouse actin, although the relative importance of the two interactions is not known (42). The RESA binding domain has been mapped to a 48-residue region (41), whereas the MSP-1 binding domain has been mapped to a 30-residue region (43). The studies on KAHRP are less advanced, and binding activity is shown by a 30-kDa fragment of the protein (42), but this may eventually be
demonstrated to be a considerably shorter sequence. Thus, in the case of two of the proteins that interact with the red cell membrane, the binding domain is a relatively short linear region. This is now also the case for MESA, the binding region of which includes a 19-residue linear sequence. This reliance on short linear sequences may arise from the necessity of the transported malaria protein to interact with a pre-existing complex network of proteins. The pathway by which malaria proteins are exported to the red cell membrane is not fully understood, but involves the passage of the protein through a series of membrane-bound compartments. This may involve sequential unfolding and refolding of the polypeptide chain, and it is not known whether the final interaction at the red cell membrane occurs with the protein in a folded or unfolded state.

Computer analysis of the 19-residue protein 4.1-binding domain of MESA revealed that when the 19-amino acid sequence was arranged in a helical wheel plot with an angle of rotation of 110°, the residues were arranged such that one face was predominantly hydrophilic and the other exclusively hydrophobic, suggesting that it may form an amphipathic α-helical structure in vivo (Fig. 7). This biphasic distribution of residues in the helical wheel is consistent with what would be expected of a linear surface domain. The positioning of the tyrosine residues on the hydrophilic face is consistent with tyrosine's nature as a polar amino acid, and it is unlikely that their presence would disrupt the helical structure. Analysis of the RESA binding domain did not predict the presence of a similar structure; however, the MSP-1 spectrin-binding domain also strongly predicts an amphipathic helical structure using an angle of rotation between residues of 110° (Fig. 7). Amphipathic helices have often been identified in proteins that interact with lipids or with DNA molecules (44, 45), but have also been found to be the anchoring domain in some multicomponent protein...
complexes such as those containing the type II cAMP-dependent protein kinase (46). It is not clear if the amphilic helical arrangement would be particularly important in the binding interactions of these malaria proteins, but this could be addressed by in vitro mutagenesis experiments. Examination of the putative spectrin-binding domain of RESA identified a shared motif of 7 residues between the binding domains of RESA and MESA. In MESA, this was SIRNYIE, and in RESA, 7TN. As RESA binds to spectrin, and MESA binds to protein 4.1, it is not clear what the significance of this observation is. A search of the SwissProt data base with this motif revealed its presence in only one additional protein, the human \( \text{cav} \) oncogene, a protein ubiquitously expressed in hemopoietic cells, but no data are available as to whether this region of the protein is involved in any protein-protein interactions (47).

Several roles for the inordinately common and sometimes complex repeat elements of \( P. falciparum \) antigens have been proposed (39, 48, 49). It had been suggested that repeat elements are responsible for protein-protein interactions, with some evidence that the repeats of the circumsporozoite protein increased the avidity of interaction of the protein with the hepatocyte receptor (50). However, Cerami et al. (51) found that the circumsporozoite repeats were not directly involved in the interaction of the circumsporozoite protein with the hepatocyte receptor. Furthermore, binding domains do not require the presence of the repeat regions in the proteins that interact with the membrane skeleton, at least for RESA and MSP-1. The binding domain of KAHRP has been mapped to a region of the molecule that includes a repeat element, although it is unclear if this lysine-rich repeat plays a role in the interaction of KAHRP with spectrin (42). The GESKET repeats found in MESA F3 did not seem to be directly involved in binding to IOVs since several regions of MESA F3 containing the repeats, but not the NH2-terminal sequences, failed to show any significant binding (data not shown). However, we did observe decreased binding of MESA F3 in the presence of antibodies directed against the repeats. The repeat array provides an extremely avid target for antibody, and several antibody molecules would be expected to bind. Perhaps these interfere with MESA F3 binding to IOVs by some form of spatial interference.

The critical role of protein 4.1 in the maintenance of the erythrocyte membrane skeleton and anchoring it to the erythrocyte membrane is well documented. Although MESA does not appear to be required for survival or cytoadherence of the parasite in \( \text{in vitro} \) culture (52), MESA is consistently detected in parasites isolated directly from patients.2 When MESA is present, it is essential for parasite survival that MESA be securely anchored to the membrane. If it is allowed to accumulate in the red cell cytoplasm, as occurs in the case of parasites infecting mutant red cells that lack protein 4.1, the parasite dies over the course of 36–48 h (31, 53). Molecules that are able to block the binding of MESA to protein 4.1 could provide a novel therapeutic approach to malaria. We hypothesize that MESA may be required for resistance of parasitized cells to hemodynamic forces in the circulation and perhaps also for increasing the strength of cytoadherence. Further studies will address the biophysical consequences of MESA binding both on the material properties of infected cells and on the cytoadherence process (54).