An Erythrocyte Cytoskeleton-Binding Motif in Exported
*Plasmodium falciparum* Proteins†‡

Geoffrey K. Kilili and Douglas J. LaCount*

Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, Indiana 47907

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Binding of exported malaria parasite proteins to the host cell membrane and cytoskeleton contributes to the morphological, functional, and antigenic changes seen in *Plasmodium falciparum*-infected erythrocytes. One such exported protein that targets the erythrocyte cytoskeleton is the mature parasite-infected erythrocyte surface antigen (MESA), which interacts with the N-terminal 30-kDa domain of protein 4.1R via a 19-residue sequence. We report here that the MESA erythrocyte cytoskeleton-binding (MEC) domain is present in at least 13 other *P. falciparum* proteins predicted to be exported to the host cell. An alignment of the putative cytoskeleton-binding sequences revealed a conserved aspartic acid at the C terminus that was omitted from the originally reported binding domain. Mutagenesis experiments demonstrated that this aspartic acid was required for the optimal binding of MESA to inside-out vesicles (IOVs) prepared from erythrocytes. Using pulldown assays, we characterized the binding of fragments encoding the MEC domains from PFE0040c/MESA and six other proteins (PF10_0378, PFA0675w, PFB0925w, PFD0095c, PFF1510w, and PF11170w) to IOVs. All seven proteins bound to IOVs, with MESA showing the strongest affinity in saturation binding experiments. We further examined the interaction of the MEC domain proteins with components of the erythrocyte cytoskeleton and showed that MESA, PF10_0378, and PFA0675w coprecipitated full-length 4.1R from lysates prepared from IOVs. These data demonstrated that the MEC motif is present and functional in at least six other *P. falciparum* proteins that are exported to the host cell cytoplasm.

The pathology of the malaria parasite *Plasmodium falciparum* is associated with its ability to remodel the red blood cells (RBCs) it infects. Among the most dramatic changes induced by *P. falciparum* is the formation of thousands of protrusions (knobs) on the RBC surface, into which the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) is inserted (11, 12, 17). PfEMP1 is an antigenically variant protein that acts as a receptor for host ligands located on the endothelial lining of the vasculature or on other RBCs. Binding to endothelial cells sequesters infected RBCs in the microvasculature, which prevents the clearance of the infected RBC by the spleen and contributes to the pathogenesis of severe malaria (54). Other changes to the infected RBC include the elaboration of Golgi membrane-like vesicle stacks called Maurer’s clefts and a membrane-bound tubovesicular network that together promote trafficking of molecules to and from the RBC surface (27, 29). In addition to the ultrastructural changes, the functional properties of the infected RBC membrane also are affected. A new permeability pathway, which enables the transport of a range of nutrients and small molecules, is established through the insertion of parasite proteins into the RBC membrane (39, 50). The mechanical properties of the RBC membrane are altered, leading to decreased deformability and increased rigidity (37). Even the geometry of the RBC is affected, as indicated by an increase in the minimal cylindrical diameter, which is the smallest pore through which a cell may pass through (23). As the parasite develops further, the infected RBC completely loses its normal discoid shape.

These and other changes in the infected RBC are wrought by the actions of *P. falciparum* proteins that are secreted into the erythrocyte cytosol. Two pathways for transporting the secreted proteins to the host cell have been described. The majority of these proteins are targeted to the host cell by a sequence element (RxLxEx, where x is any amino acid) referred to as the *Plasmodium* export element (PEXEL) or host-targeting (HT) motif (24, 36). A signal peptide directs the PEXEL-HT motif proteins to the secretory system (24, 36), where they are cleaved by plasmepsin V after the leucine in position 3 (8, 9, 42, 47). The processed proteins then are transported across the parasitophorous membrane through a parasite-derived pore complex (15). Some exported proteins, including REX1 and skeleton binding protein 1, lack PEXEL-HT motifs and are transported by an independent pathway (21, 49). Several algorithms have been developed to predict which *P. falciparum* proteins are exported to the host cytosol (24, 36, 48, 55). Although the lists generated by these algorithms are only partially overlapping, comparisons of the data sets suggest approximately 400 proteins are likely to be exported, about half of which belong to the rinf, stever, and PfEMP1 families of RBC surface proteins. The number of PEXEL-HT-negative proteins secreted by the alternative pathway is not known.

The erythrocyte cytoskeleton is a prominent target of exported *P. falciparum* proteins. At least 12 *P. falciparum* proteins have been found to interact with erythrocyte actin (5, 26, 41, 58, 59), spectrin (26, 41, 43, 44, 59), or ankaryn (33, 53). Multiple cytoskeletal proteins are phosphorylated during in-
fection, and at least some of these phosphorylation events are mediated by the exported FIKK family kinases (14, 31, 33, 38, 40, 60). Late during infection, cytoskeletal proteins are cleaved by parasite proteases that are exported to the host cell (22). A large-scale deletion analysis of genes encoding exported proteins identified additional *P. falciparum* proteins that likely target the RBC cytoskeleton (34, 35). Eleven of the deletion strains (20% of those tested) caused either increased or reduced rigidity of the infected RBC relative to that of the parental strain (35). Given that only about one-quarter of the genes encoding exported proteins have been targeted for deletion, this study suggests that there are many more that affect the RBC cytoskeleton.

The mature parasite-infected erythrocyte surface antigen (MESA; also known as PFEMP2) was the first exported *P. falciparum* protein whose cytokinesis-binding partner was identified (31). MESA originally was discovered as a surface antigen in infected RBCs but subsequently was shown not to be surface exposed; rather, it was associated with the RBC cytoskeleton (13, 14). MESA is exported to the erythrocyte cytosol via a PEXEL-HT motif (7) and colocalizes with knobs, but it is not required for knob formation or cytoadherence (45). Immunoprecipitation of MESA from infected cells coprecipitated a phosphorylated form of protein 4.1 (4.1R; also known as band 4.1 or EPB41) (31). Deletion-mapping studies and competition binding experiments defined a 19-amino-acid region within the 30-kDa domain, although it was suggested that the domain could be further narrowed to the 51 amino acids encoded by exon 10 (57). The binding site of MESA identified on 4.1R partially overlaps that of the cellular protein p55 (57). The two proteins compete for binding to 4.1R, suggesting that MESA may alter RBC cytoskeletal properties by displacing p55 from 4.1R, although this has not been experimentally confirmed (57).

We report here that the amino acid sequence from MESA that mediates binding to the RBC cytoskeleton is present in at least 13 other exported proteins. We tested seven fragments encompassing this sequence from six *P. falciparum* proteins and found that all were competent to bind to RBC proteins on IOVs, but that their relative affinities vary. From a multiple sequence alignment of the domains, we identified a critical amino acid as part of the erythrocyte cytoskeleton binding motif that was not included as part of the binding domain reported for MESA (6). The identification of this motif suggests that at least 14 exported proteins use a common mechanism to bind to the erythrocyte cytoskeleton.

**MATERIALS AND METHODS**

** Constructs and site-directed mutagenesis.** The sequences of the oligonucleotide primers used in this study are provided in Table S1 in the supplemental material. Gene fragments encoding the MEC domains from pFPE0040c, pFPE0099c, pPF10_0378, pPF10_0378, pPF10_0376, pPF10_0375, and pPF10_0377c plus 45 to 75 bp of 5′ and 3′ flanking sequences were PCR amplified with gene-specific primers and cloned in frame with the maltose-binding protein (MBP) gene in the bacterial expression vector pMal-C4e (New England Biolabs). To mutate leucine 114 and aspartic acid 115 of pFPE0040c to alanine in the pMal-C4e plasmid described above, complementary site-directed mutagenic primers targeting the indicated codons were used to PCR amplify the entire plasmid. After the digestion of the parental plasmid with DpnI, 50% of the reaction was transformed into *Escherichia coli*. Plasmids carrying the desired mutations were identified by sequencing. The inserts then were PCR amplified, cloned into pMal-C4e, and sequenced.

**Bacterial expression and batch purification of MBP fusion proteins.** MBP fusion proteins were overexpressed in the *Escherichia coli* Rosetta (DE3) pLysS strain (Novagen). Overnight cultures were diluted 1:40 in fresh Luria broth (LB) containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol and grown at 37°C to an optical density at 600 nm (OD600) of 0.8 to 1.0. The expression of the fusion proteins was induced by adding isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 0.5 mM. After 8 h at 28 to 25°C, cells were collected by centrifugation, resuspended in cold phosphate-buffered saline (PBS) (1.05 mM KH2PO4, 154 mM NaCl, 5.48 mM Na2HPO4) supplemented with 100 μM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml lysozyme (Sigma), subjected to one freeze-thaw cycle, and incubated for 30 min at room temperature (RT) with mixing. The crude bacterial lysates were sonicated to shear genomic DNA and centrifuged for 10 min at 48,000 × g at 4°C. Cleared supernatants were incubated with prewashed amylase resin (New England BioLabs) at a ratio of 1 ml of 50% (shurry) per 100 ml of culture pellet. After 1 h at RT, the beads and bound fusion proteins were collected in a chromatography column by gravity flow and washed with 3 column volumes of cold PBS. Bound fusion proteins were eluted with 50 mM Tris–HCl, pH 8, 0.5 M NaCl, and stored at 4°C. After elution, the column fractions were pooled and dialyzed overnight (RT, 1000 ml) into 1× PBS supplemented with 10% glycerol. The dialyzed fusion proteins were stored at −20°C in 1× PBS plus 50% glycerol.

**Determination of protein concentration.** Because some of the purified MBP fusion proteins also contained a contaminating band equal in size to the recombinant MBP protein expressed by the empty MBP expression vector, the concentration of purified MBP-P. falciparum proteins was determined by comparing the intensity of Coomassie blue-stained bands on SDS-PAGE gels. Purified MBP fusion protein and serial dilutions of bovine serum albumin (BSA) were subjected to electrophoresis on a 14% SDS-PAGE gel, stained with Coomassie blue, and destained until all bands were clearly visible. The intensities of the sample and BSA bands were quantified with NIH Image 1.44 (1). For purified samples with contaminating bands, only the band that matched the expected molecular size was quantified. Densitometry values obtained from BSA bands were plotted against the μg quantities to establish a standard curve, which was used to determine the μg BSA equivalent of each sample. Protein concentrations were obtained by dividing the μg BSA equivalent by the volume loaded on the gel.

**SDS-PAGE and Western blot analysis.** Samples were boiled in 3× sample loading buffer, separated on 12% SDS-PAGE gels, and transferred to a nitrocellulose membrane (Bio-Rad). Membranes were blocked for 1 h in 1× PBS supplemented with 0.5% Triton X-100 and 5% nonfat dried milk powder. Blots then were incubated overnight at 4°C with antisera against MBP (New England BioLabs) or 4.1R (generously provided by P. Low, Purdue University) in 1× PBS, 0.5% Triton X-100, 1% nonfat dry milk powder; washed with 1× PBS, 0.5% Triton X-100; and probed for 1 h at RT with horseradish peroxidase (HRP)-conjugated anti-IgG secondary antibodies. After washing, protein bands were visualized using enhanced chemiluminescence (ECL) Western blotting reagent (Pierce) followed by exposure to autoradiography film (Denville Scientific).

**Preparation of IOVs.** IOVs were prepared from normal human red blood cells (RBCs) using previously published methods with slight modifications (6, 51). Briefly, RBCs from 40 ml of whole blood (BioReclamation LLC, NY) were collected by centrifugation and washed at least three times in five volumes of cold RPMI medium without calcium or magnesium. After each wash, cells were pelleted and the supernatant, along with any visible buffy coat, was removed. After the final wash, RBCs were resuspended in RPMI at 50% hematocrit and stored at 4°C. RBC ghosts were prepared from 1 ml of packed RBCs by adding 30 ml of ice-cold lysis buffer (5 mM phosphate buffer, pH 6.0, protease inhibitor cocktail, and 0.1 mM EDTA), mixing several times by inversion, and incubating on ice for 10 min. Lysed cells were spun at 48,000 × g for 10 min. After the removal of approximately 95% of the supernatant, the pellet was dislodged by slowly swirling in the remaining buffer and transferred to a fresh tube, leaving behind the contaminating unlysed RBC pellet. Fresh buffer was added, and the centrifugation and pellet transfer steps were repeated two to three times until the pellet appeared creamy white and the supernatant was free of hemoglobin. To prepare IOVs, the pellet was resuspended in 30 ml of vesiculation buffer (0.5 M phosphate buffer, pH 8.5), incubated on ice for 1 h, and spun at 48,000 × g for 15 min. The pellet was resuspended in 1 ml of vesiculation buffer, and the suspension was passed through a 27-gauge needle at least 10 times. Vesiculation buffer was added to adjust the volume to 30 ml, and the suspension was subjected
to centrifugation as described above. The pellet, which contained the IOVs, was resuspended in 1.5 to 2 ml of IOV storage buffer (138 mM NaCl, 5 mM KCl, 6.1 mM Na2HPO4, 1.4 mM NaH2PO4, 5 mM glucose) and stored at 4°C for less than 1 week until use (6).

**IOV pulldown assays.** IOVs (specific amounts used are indicated in the figure legends) were incubated overnight without mixing at 4°C in 250 l of cold IOV blocking buffer (1× PBS, 2% BSA, protease inhibitor [PI] cocktail). The IOVs were collected by centrifugation at 16,000 g for 10 min at 4°C, and the supernatant was removed by aspiration. The pellet was resuspended in 250 l of 50 mM Tris-HCl buffer, pH 8.0, and the native proteins were subjected to SDS-PAGE analysis as described above and in the relevant figure legend. (C) Domain organization of the MEC motif proteins. Proteins are represented as black bars whose length is proportional to the size in amino acids, as indicated by the scale bar at the bottom of the figure. The MEC motif, PHIST-b domain, and DNAJ domain are shown as white, red, and blue boxes, respectively. Gray boxes indicate a second motif in PFF1510w and PFF0075c with lower homology to the MEC motif. (D) Purification of MEC domains from seven *P. falciparum* proteins. The MEC domains from the indicated proteins were expressed in E. coli as C-terminal fusions to MBP and were purified with amylose resin. A sample of each protein was subjected to SDS-PAGE and stained with Coomassie blue.

**Protease K digestion of IOVs.** IOVs at a protein concentration of ~0.5 mg/ml were digested with 20 μg/ml of protease K in 1× PBS supplemented with PI cocktail and 40 μg/ml PMSF before the addition of protease K. After 1 h at 37°C, the reaction was stopped by the addition of 40 μg/ml PMSF, and the IOVs were washed three times with excess 1× PBS supplemented with PI cocktail.

**Extraction of IOVs with LIS.** IOVs (120 μg) were collected by centrifugation and resuspended in 250 μl of cold IOV blocking buffer (1× PBS, 2% BSA, protease inhibitor [PI] cocktail). The IOVs were collected by centrifugation at 16,000 g for 10 min at 4°C, and the supernatant was removed by aspiration. The pellet was resuspended in 250 μl of freshly made ice-cold IOV binding buffer (1× PBS, 1% BSA, PI cocktail). Equal amounts of purified P. falciparum MBP fusion proteins (see the figure legends for details) were added to the IOVs, mixed briefly, and incubated on ice overnight at 4°C with occasional mixing during the first 2 to 3 h. The IOVs then were washed twice with cold IOV wash buffer (1× PBS, 0.25 mM KCl) by completely resuspending the pellet and incubating it for 5 min on ice. After the last wash, the pellet was resuspended in 3× SDS sample loading buffer and processed for SDS-PAGE analysis as described above and in the relevant figure legend. For the saturation binding curves, the same procedure was used, except that the assay was performed in 150-μl reaction mixtures in a 96-well plate with tapered wells (VWR).

**FIG. 1.** MESA erythrocyte cytoskeleton-binding (MEC) domain is found in other exported *P. falciparum* proteins. (A) Alignment of the MEC domains found in 14 exported *P. falciparum* proteins. Numbers at the N terminus of the alignment indicate the position of the motif in the respective proteins. Asterisks indicate domains purified in panel D. (B) Sequence logo derived from the MEC domains shown in panel A. Amino acids are represented by one-letter abbreviations. Colors indicate groups of similar amino acids as follows: blue, hydrophobic; green, polar amino acids; purple, acidic; red, basic. Letter height is proportional to the frequency of the amino acid at that position. Total letter height reflects the amount of information contained at a given position. (C) Domain organization of the MEC motif proteins. Proteins are represented as black bars whose length is proportional to the size in amino acids, as indicated by the scale bar at the bottom of the figure. The MEC motif, PHIST-b domain, and DNAJ domain are shown as white, red, and blue boxes, respectively. Gray boxes indicate a second motif in PFF1510w and PFF0075c with lower homology to the MEC motif. (D) Purification of MEC domains from seven *P. falciparum* proteins. The MEC domains from the indicated proteins were expressed in E. coli as C-terminal fusions to MBP and were purified with amylose resin. A sample of each protein was subjected to SDS-PAGE and stained with Coomassie blue.
Coprecipitation of 4.1R. IOVs were extracted with 10 mM LJS, cleared by centrifugation at 48,000 × g for 30 min, and concentrated to ~6 ml using a Centricron Plus-70 column (Millipore). Triton X-100 was added to a final concentration of 0.5%, and the lysate was dialyzed against 1× PBS. Amylose resin bound to MBP-MEC domain fusion proteins was mixed with dialyzed extract, incubated overnight at 4°C with constant mixing, and washed three times with cold wash buffer A (1× PBS plus 0.5 to 1% Triton X-100 and 0.25 mM KCl). The final pellet was resuspended in 3× SDS sample loading buffer and subjected to SDS-PAGE, followed by Western blotting with anti-4.1R and anti-MBP antisera.

Statistical analysis. Western blot band intensities were determined by densitometry using Image J version 1.44 (1) and were expressed as the means from at least three independent experiments ± the standard errors of the means. An unpaired t test was performed to determine if the differences in band intensities were significant (P < 0.05). All statistical data analyses were performed using GraphPad Prism software (version 5.0b).

RESULTS

Identification of the MESA erythrocyte cytoskeleton-binding domain in exported P. falciparum proteins. The interaction between MESA and the RBC cytoskeleton is perhaps the best characterized among the exported P. falciparum proteins. Deletion experiments and peptide mapping studies implicated a 19-amino-acid sequence in MESA as the binding site for the erythrocyte cytoskeleton (amino acids 96 to 114; DHLYSIRNYIECLRNAPYI) (6). Subsequent studies delineated a 131-amino-acid fragment of 4.1R as a binding partner of MESA (amino acids 133 to 263 of NCBI reference sequence NP_976217.1) (57). To determine if other P. falciparum proteins encoded sequences similar to those of the erythrocyte cytoskeleton-binding domain in MESA, we performed BlastP searches using the PlasmoDB database (3). The top six matches (PF06075w, PFL2540w, PFF1510w, PFF0075c, PFI1790w, and PFB0925w) all contained putative PEXEL-HT motifs, suggesting that these proteins are exported. Though the exact (e) values were quite high due to the short query sequence, none of these proteins were listed as orthologs of PFE0040c in OrthoMCL (30) and no significant similarity was detected in the flanking sequences using pairwise BlastP searches. Thus, the homology appeared to be neither random nor part of a larger domain. To determine if this putative motif could be independently identified, we submitted the sequences of MESA and the top six BlastP matches for analysis with the Multiple Expectation Maximization for Motif Elicitation (MEME) suite of motif discovery programs (http://meme.nbcr.net) (4). A sequence motif that nearly exactly matched the RBC cytoskeleton-binding domain in MESA was returned with an e value of 3.3 × 10−32. Surprisingly, the consensus sequence identified by MEME extended beyond the previously reported motif to include an aspartic acid that was present in all eight proteins submitted for analysis. Using this extended sequence from MESA (HLYSI RNYIECLRNAPYI), we repeated the BlastP search using PlasmoDB and submitted the sequences of the top 100 matches to MEME for motif discovery. From this set of proteins, MEME identified sequences similar to those of the MESA erythrocyte cytoskeleton-binding domain in 16 proteins, 14 of which have putative protein export motifs (e-value ≥ 5 × 10−35). The two other proteins (PF11_0058-a and PF11_0058-b) were annotated as RNA polymerase subunits and most likely are intracellular, since they lack predicted export motifs, signal peptides, or transmembrane domains; they appear to be splice variants that differ from each other by only three amino acids. We here refer to this sequence element as the MESA erythrocyte cytoskeleton-binding (MEC) motif. An alignment of the MEC domains from the 14 exported proteins is shown in Fig. 1A (20, 28).

A consensus sequence for the MEC motif was developed using MEME after excluding the two nonexported proteins and two proteins whose motifs were identical to others in the list (Fig. 1B). The core of the consensus sequence is 13 residues in length (NYx[E/K][C/L][I/K][R][N/T]APYID, where x is any amino acid) and extends one amino acid beyond the previously reported motif (6) to include an aspartic acid residue. This aspartic acid is the only amino acid that was absolutely conserved. All proteins except PF11790w and PFB0925w are predicted in PlasmoBD to contain export motifs. However, both are predicted to contain signal peptides at their N termini, have sequences resembling an export motif (RNME and RNLYT, respectively), and were predicted by Sargeant et al. to be exported (48). All proteins with the MEC motif also contain either a DNAJ domain, a Plasmodium helical interpersed subtelomeric family b (PHIST-b) domain, or both (Fig. 1C). In the five proteins that contain only a DNAJ domain, the MEC motif is present within the first 400 amino acids and is to the N-terminal side of the DNAJ domain. In proteins with PHIST-b domains, the MEC motif is always found on the C-terminal side, but it is N terminal to the DNAJ domain, if present. Two pairs of proteins in the list have nearly identical sequences. PFF1510w and PFF0075c differ by a single amino acid. They are also the only proteins with two putative MEC motifs, one at the C terminus that closely matches the consensus and a second more divergent sequence (amino acids 243 to 257, IFINGINNVPYI DGI, indicated in protein designations as “-a”) near the PHIST-b domain (Fig. 1C). PF11_0034 differed from PF10_0381 at two positions and contains a C-terminal extension identical to the sequence of PF10_0382.

The MEC domains bind to IOVs. To determine if the newly identified MEC domains were functional, we cloned the motifs from MESA and six other P. falciparum genes (PF06075w, PFB0925w, PFD0095c, PFF1510w, PFI1790w, and PF10_0378) into a maltose-binding protein (MBP) expression plasmid; both motifs from PFF1510w were cloned. Because the sequence alignments suggested a weak preference for particular amino acids at positions outside the core motif, 15 to 25 resi-
FIG. 3. MBP-MEC motif fusion proteins differ in their affinity for IOVs. The interaction of eight MEC motifs from seven *P. falciparum* proteins with IOVs was assessed in coprecipitation experiments. (A) The MBP-tagged MEC motifs differ in their ability to interact with IOVs. The experiment depicted in Fig. 2 was repeated with reduced amounts of IOVs and purified MBP-MEC domain fusion proteins to detect differences in the binding ability of the different motifs. The amounts of IOVs and MBP-MEC domain proteins used were empirically determined. IOVs (approximately 20 μg of total protein per reaction) were incubated with blocking buffer as described for Fig. 2, at which point ~1 μg of each MBP-tagged MEC motif was separately added. Reactions were processed as described in the legend to Fig. 2. The upper panel shows a Western blot with MBP antibody. To confirm equal loading, the Ponceau S-stained membrane was imaged prior to probing with the antibody (middle panel). To confirm that equal amounts of input were used, a separate gel was loaded with the exact amounts used in the IOV binding experiment and stained with Coomassie blue (lower panel). A representative blot from three independent experiments is shown. (B) Quantitation of the binding of the MBP-MEC domain fusion proteins shown in panel A. The intensity of the bands from the IOV pulldown experiments in panel A were quantitated using ImageJ. Each data point represents the average densitometry value from three independent experiments. Error bars indicate the standard errors of the means (SEM). (C) Saturation binding analysis of MBP-MEC motif fusion proteins with IOVs. IOVs were incubated with blocking buffer as described for panel A and distributed to wells in a 96-well plate (~5 μg/well). Serial dilutions of the indicated purified MBP-MEC domain fusion proteins were added (final concentrations, 0.16 to 0.005 μM), incubated overnight at 4°C without mixing, and processed as described for panel A. Binding was quantified as described for panel B and graphed as a function of the concentration of the MBP-MEC domain fusion protein. The entire experiment was repeated twice with similar results; the graph shows data from one representative experiment. Western blots from which the bands were quantitated are shown in Fig. S1 in the supplemental material. Closed circles, PFE0040c; open circles, PFA0675w; closed squares, PFB0925w; open squares, PFI1790w; closed triangles, PFF1510w-a; open triangles, PFI10_0378; closed diamonds, PFF1510w; open diamonds, PFD0095c.
and performed pulldown assays (Fig. 3C; also see Fig. S1 in the supplemental material). Consistent with the results from our initial experiment, the MEC motif from PFA0675w bound nearly as well as the motif from MESA, those from PFB0925w and PFI1790w showed intermediate binding, and the motifs from PF10_0378, PFF1510w, and PFD0095c bound at barely detectable levels.

The MEC motifs bind to a protein component of IOVs. To confirm that the association with IOVs was the result of binding to proteins present on IOVs, we treated the IOVs with proteinase K or 10 mM lithium 3,5-diiodosalicylate (LIS) to digest or selectively extract cytoskeletal proteins, respectively (10, 52). Proteinase K-treated IOVs did not bind to any MBP-MEC motif fusion proteins, in contrast to IOVs that were exposed to proteinase K in the presence of protease inhibitors (Fig. 4A). Similarly, LIS extraction reduced or completely eliminated binding to IOVs, with the exception of PFF1510w-a, which showed enhanced binding (Fig. 4B and C). Thus, we conclude that the MEC domains from PFA0675w, PFB0925w, PFD0095c, PFI1790w, and PFD0095c bound at barely detectable levels.

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The MEC domains coprecipitate 4.1R. PFE0040c has been reported to bind to full-length 4.1R from RBCs and to fragments of 4.1R expressed as glutathione S-transferase (GST) fusions in E. coli (31, 32, 57). To determine if the newly identified MEC domains also could coprecipitate 4.1R, MBP-MEC domain fusion proteins were incubated with lysates prepared from IOVs by LIS extraction and purified using amylase resin. 4.1R coprecipitated with all three MEC domains tested (MESA, PFA0675w, and PF10_0378) but not with the MBP (data not shown) or MBP-MF4 domain, confirming that the newly identified MEC domains form a complex with 4.1R (Fig. 5).

FIG. 5. MBP-MEC domain fusion proteins bind to 4.1R. Erythrocyte cytoskeleton proteins were extracted from IOVs using 10 mM LIS and incubated with MBP-MESA, MBP-PFA0675w, MBP-PF10_0378, or the MBP-MF4 negative control. MBP-MEC domain fusion and associated proteins were precipitated with amylose resin and subjected to Western blotting with anti-4.1R and anti-MBP antisera. Representative blots from three independent experiments are shown.
We report here that the erythrocyte cytoskeleton-binding motif in MESA is present in at least 13 other *P. falciparum* proteins predicted to be exported to the host cell. From ClustalW alignments, we defined a core 13-amino-acid consensus sequence that differed from the earlier reported motif (6). In particular, we identified a conserved aspartic acid residue not previously recognized as being part of the binding domain and showed that it was critical for the optimal binding of the MEC motif to IOVs. This amino acid likely was not included in the originally defined domain for technical reasons: the cloning procedure used to generate the fragments for mapping the IOV binding site used a ClaI site that cuts just before the codon encoding this aspartic acid residue in *MESA* (6). We further demonstrated that the MEC motifs from diverse proteins were able to bind to erythrocyte proteins present on IOVs and to 4.1R. The affinity of these motifs for the RBC cytoskeleton was related to their homology to the motif from MESA, with the most distantly related motifs (PFD0095c and PFF1510w) appearing to have the lowest affinity. Although this motif from MESA was demonstrated to be sufficient to direct exported proteins to the RBC cytoskeleton in infected RBCs (7), additional studies are required to demonstrate that the other motifs function in the same manner.

The true number of exported *P. falciparum* proteins with the MEC motif may be higher than that reported here. BlastP searches using the consensus sequence from Fig. 1B identified six additional exported proteins that shared homology with the MEC motif (see Fig. S2 in the supplemental material). The sequences from these proteins were more distantly related to the binding domain from MESA but nonetheless were recognized by the MEME program from a set of 100 *P. falciparum* proteins that also contained sequences similar to those of the MESA MEC domain. These additional proteins include PFA0110w (ring-expressed surface antigen; RESA), which binds to the erythrocyte cytoskeleton (18, 43), and four RESA-related proteins. However, the putative MEC motif from RESA does not appear to be responsible for binding to the RBC cytoskeleton (18, 43). Rather, domain-mapping studies demonstrated that fragments C terminal to the MEC-like sequence in RESA bound to IOVs via an interaction with spectrin, whereas a fragment including the MEC-like motif showed no binding (18, 43).

The molecular functions of the proteins that contain the MEC motif are not well characterized. MESA is the most extensively studied of the MEC motif proteins, having been discovered more than two decades ago (13). Based on its localization to the RBC membrane and the observation that MESA competes with p55 for binding to 4.1R, MESA has been proposed to alter RBC cytoskeletal functions (57). MESA RNA and protein were expressed at higher levels in parasites from children than from pregnant women (56). Similar expression differences also were observed in laboratory strains that adhered either to CD36 or chondroitin sulfate A (CSA) (46). The significance of this change in MESA expression is unclear, since MESA-negative parasites have no apparent differences in binding to endothelial cell receptors (32).

Six of the 13 genes encoding MEC motif proteins have been targeted in gene knockout experiments (Table 1) (35). Of these, *PF0095c* and *PF11_0034* could not be deleted, suggesting that they are essential for parasite growth (35). *PF11790w* is deleted from at least two laboratory strains (FCB and FCR3), suggesting that it too is nonessential for growth in cell culture (25). The deletion of *PF10_0381* resulted in parasites with reduced adherence to CSA and much smaller knobs that were fewer in number than those of wild-type parasites (35).
PIEMP1 transport to the surface was normal in ΔPF10_0381-infected erythrocytes, but the distribution of knob-associated histidine-rich protein (KAHRP) was altered (35). This suggests that the normal function of PF10_0381 promotes knob formation. The deletion of PF14_0018 resulted in parasite-infected cells that were less rigid than the wild-type parasite-infected controls, indicating that PF14_0018 functions to increase infected RBC rigidity (35). No obvious phenotypes were observed when the other MEC motif genes were deleted.

Given the large number of proteins with the MEC motif, it seems unlikely that all of them target functions of 4.1R. Rather, we favor the hypothesis that the MEC motif is a protein module that is used to dock exported P. falciparum proteins at erythrocyte membranes. A prediction of this hypothesis is that peptides or small molecules that inhibit the binding of the MEC motif to the RBC cytoskeleton would disrupt the functions of multiple proteins. Although six of the MEC motif proteins have been demonstrated to be dispensable for the growth of P. falciparum in cell culture, simultaneously interfering with the localizations of all of them may cause a more severe phenotype. Indeed, the disruption of MESA localization has been suggested to interfere with P. falciparum replication based on the observation that parasites expressing MESA invaded and replicated in 4.1R-deficient RBCs less efficiently than their MESA-negative counterparts (32). Since MESA was found distributed throughout the erythrocyte cytosol in 4.1R-deficient cells, it was suggested that improperly localized MESA was toxic for the parasite (32). Thus, small molecules that cause the mislocalization of MESA and other MEC motif proteins would be predicted to have the same deleterious effect on parasite growth. However, the results from Magowan et al. (32) must be interpreted with caution, since the study was performed prior to the advent of genome-wide microarrays that could reveal the extent of genetic changes (16) and the development of technologies to complement the deficiency in MESA (19). It is formally possible that the spontaneous mutation that deleted MESA is deleted from adjacent genes, many of which are predicted to be exported, and that a different protein is responsible for the poor growth in 4.1R-deficient cells.

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**REFERENCES**


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**TABLE 1. Features of P. falciparum MEC motif proteins**

<table>
<thead>
<tr>
<th>Designation</th>
<th>Name</th>
<th>Gene disruption</th>
<th>Knockout phenotype</th>
<th>Domain(s)</th>
<th>Binding partner(s)</th>
<th>Reference(s)</th>
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<tr>
<td>PF10_0378</td>
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<td>Defective knob formation; reduced adhesion</td>
<td>DNAJ, PHIST-b</td>
<td>IOVs, 4.1R</td>
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<tr>
<td>PF10_0381</td>
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<td>ND</td>
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*NA, gene disruption not attempted; ND, not determined.*