[haematologica]
2004;89:1168-1171

Myosin-like sequences in the malaria parasite *Plasmodium falciparum* bind human erythrocyte membrane protein 4.1

ROBERTO LANZILLOTTI
THERESA L. COETZER

A B S T R A C T

Background and Objectives. *Plasmodium falciparum* malaria is one of the most lethal infectious diseases afflicting humanity. During development within the erythrocyte, *P. falciparum* induces significant modifications to the structure and function of the human erythrocyte membrane. This study focused on the identification of new protein-protein interactions between host and parasite.

Design and Methods. A novel application of *in vitro* display technology was used: *P. falciparum* phage display expression libraries were screened against purified human erythrocyte protein 4.1. DNA sequencing and bioinformatic analyses were used to identify parasite proteins that bind protein 4.1.

Results. *P. falciparum* proteins displaying strong binding specificity toward protein 4.1 included five hypothetical proteins, erythrocyte binding antigen-175, erythrocyte binding ligand-1 like protein and a putative serine/threonine kinase. A common binding motif displaying homology to muscle myosin and neurofilament sequences was also identified in four of the eight proteins.

Interpretation and Conclusions. These proteins are potentially involved in the invasion and/or release, as well as the growth and survival of malaria parasites during development with the red blood cell. The characterization of novel protein interactions between *P. falciparum* and erythrocyte membrane protein 4.1 will lead to a better understanding of malaria pathogenesis and parasite biology.

Key words: protein 4.1, *Plasmodium falciparum*, myosin.

From the Department of Molecular Medicine and Haematology, National Health Laboratory Service, School of Pathology, University of the Witwatersrand, Parktown, Johannesburg 2193, South Africa.

Correspondence:
Theresa L. Coetzer, MD, PhD, Dept. of Molecular Medicine and Hematology, University of the Witwatersrand Medical School 7Q11, 7 York Rd, Parktown, Johannesburg, Gauteng, South Africa 2193. E-mail: theresa.coetzer@nhls.ac.za

The completion of the *Plasmodium falciparum* (clone 3D7) genome sequence has provided a comprehensive blueprint for analyzing gene expression and protein function in the malaria parasite.[1] Biological profiling of the 5,268 predicted *P. falciparum* proteins has resulted in the functional classification of about 30% of the parasite's proteome.[2] Assigning function to parasite proteins with no known protein homology (i.e. hypothetical proteins) and refining the mode of action of putative proteins have been aided by investigating protein-protein interactions involved in malaria cell biology.[3] The association of *P. falciparum* proteins with the erythrocyte membrane has initiated widespread interest in defining new proteins that interact at the host-parasite interface.[4] Despite our current understanding, many questions remain unanswered concerning this dynamic interplay. Protein 4.1 (4.1R) is a peripheral protein involved in maintaining the structural integrity of the erythrocyte membrane through its binding with spectrin and actin.[5] *P. falciparum* requires 4.1R for optimal parasite growth and viability.[6-8] Specific 4.1R-parasite protein interactions that play a role include the mature parasite-infected erythrocyte surface antigen (MESA), which binds 4.1R at a 51 residue region within the 30-KDa domain.[9] In addition, Hanspal *et al.*[10] have shown that falcipain-2, a *P. falciparum* cysteine protease, cleaves native and recombinant 4.1R within the spectrin-actin binding domain. Proteolysis of 4.1R results in destabilization of the membrane skeleton, which would assist in the release of merozoites from erythrocytes into the circulation. The phosphorylation status of 4.1R is altered during erythrocyte parasitism by unidentified parasite kinases, which may

affect the biophysical properties of the host membrane and in so doing, support parasite growth.[11]

In this study we identify novel interactions between *P. falciparum* proteins and 4.1R by using *P. falciparum* phage display libraries. This indicates new mechanism(s) by which the parasite associates with the erythrocyte skeleton.

Design and Methods

The *P. falciparum* strain FCR-3 was cultured according to the method of Trager and Jensen.[12] Parasite-infected erythrocytes were lysed with 0.5% saponin and total RNA isolated using guanidinium isothiocyanate.[13] Messenger RNA was extracted using the Dynal® (Oslo, Norway) mRNA direct kit. cDNA was synthesized and packaged into T7 bacteriophage extracts as previously described.[14] 4.1R was extracted and purified from human erythrocyte membranes (15) and the purity verified using 12% SDS polyacrylamide gels and silver staining (detection limit: 2-5 ng protein) (Figure 1). Purified 4.1R was subsequently biotinylated with D-biotin-N-hydroxysuccinimide ester (Roche, Germany) and immobilized on streptavidin-coated magnetic beads (Roche, Germany). *P. falciparum* phage display libraries were biopanned against 4.1R and insert sequences analyzed as described by Lauterbach *et al.*[14] Sequences were matched to annotated *P. falciparum* genes by performing bioinformatic analyses in the PlasmoDB database version 4.1 (*http://www.plasmodb.org*).

Results

P. falciparum phage display libraries provide a powerful tool and novel approach for identifying new protein interactions between the parasite and the human erythrocyte membrane. A phage display library was used to identify eight proteins that demonstrated binding specificity towards 4.1R. These included five hypothetical proteins, a putative serine/ threonine protein kinase, erythrocyte binding antigen-175 (EBA-175) and erythrocyte binding ligand-1 (Ebl-1)-like protein (Table 1). A single library is not representative of the entire parasite's genome and therefore these proteins do not necessarily represent all *P. falciparum* proteins that interact with 4.1R.

The amino acid composition of the 4.1R binding sequences consists predominantly of charged residues at physiologic pH, implying that the interaction of these proteins with 4.1R is most likely to be ionic in nature. Sequences encoded by PF11_0191 and MAL6P1.48 consist of 30% and 18% lysine, respectively, and have net positive charges. Ebl-1-like protein and sequences encoded by PF14_0201, PFB0150 and PFA0420w have a predominance of acidic amino acids (glutamic acid and aspartic acid) and have net negative charges. The 4.1R binding sequence encoded by PFE0570w has no net charge, whereas EBA-175 has a net negative charge due to the high proportion (15%) of glutamic acid.

Table 1. *Plasmodium falciparum* proteins containing binding sequences specific for human erythrocyte membrane protein 4.1.

PlasmoDB V4.1Gene Identification	*Annotated protein*	*Amino acid binding sequence*	*Predicted motif*
PF07_0128	EBA-175	1349-1378	Duffy antigen
PFA0125c	putative Ebl-1 like protein	971-1001	myosin-like motif
PFB0150	putative protein kinase (serine/threonine)	26-55	unknown
PFA0420w	hypothetical	150-139	neurofilament-like motif
PFE0570w	hypothetical	8611-8710	unknown
PF11_0191	hypothetical	459-488	myosin-like motif
PF14_0201	hypothetical	801-860	myosin-like motif
MAL6P1.48	hypothetical	2675-2704	unknown

Discussion

Erythrocyte binding proteins

EBA-175 and Ebl-1-like protein belong to a family of homologous Duffy-binding-like erythrocyte-binding proteins (DBL-EBP). Upon erythrocyte invasion by *P. falciparum* merozoites, the release of DBL-EBP from the micronemes has been linked to erythrocyte entry and junction formation.[16] However, the molecular mechanisms governing parasite invasion remain unclear. We propose that binding of EBA-175 and Ebl-1-like protein to 4.1R helps to destabilize spectrin-actin interactions and thus compromise erythrocyte membrane integrity. This would facilitate the movement of merozoites into the erythrocytes through the action of the parasite's actinomyosin motor.[17] During merozoite invasion, a 12-amino acid sequence in the N-terminal cysteine-rich region of EBA-175 binds the sialic residues of glycophorin A.[18,19] We have localized

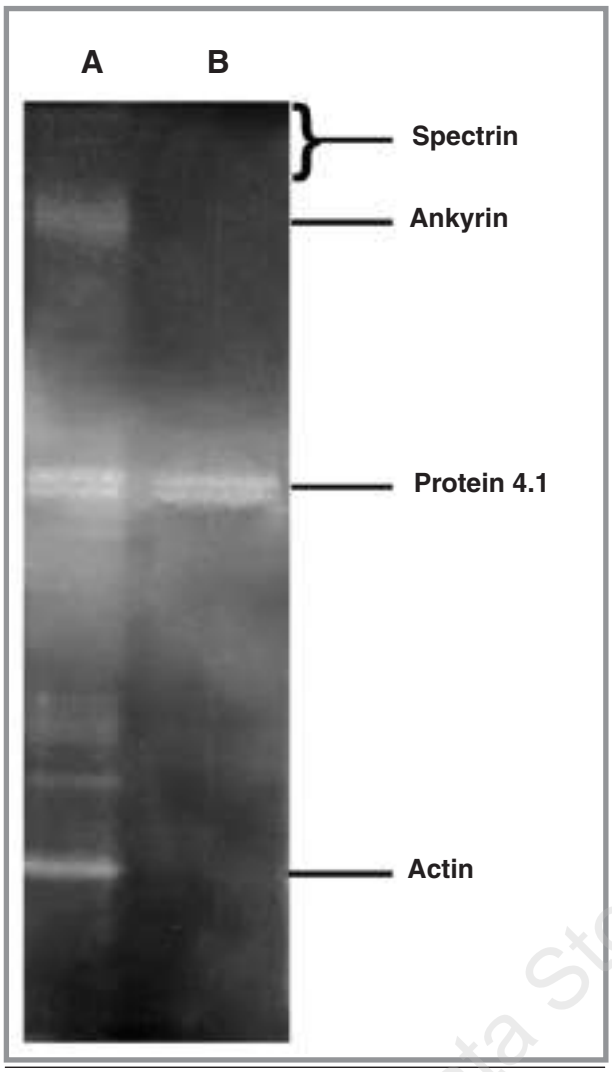

Figure 1. Purification of 4.1R from human erythrocyte membranes. Crude protein 4.1 was extracted from erythrocyte membranes with 1M KCl (A). Anion exchange chromatography was used to purify 4.1R from the crude extract (B). Proteins were separated on a 12% SDS polyacrylamide gel (Laemmli) and visualized with silver staining.

the protein 4.1 binding site in EBA-175 to the C-terminal cysteine region, found adjacent to a transmembrane domain. We therefore infer that following the initial attachment of EBA-175 to the erythrocyte surface, the transmembrane domain of EBA-175 inserts into the erythrocyte membrane. In so doing, the 4.1R binding site is brought in close proximity to 4.1R, thus initiating interaction between the two proteins.

P. falciparum protein kinase

The identification of a putative *P. falciparum* kinase provides a potential mechanism of 4.1R phosphorylation. Together with a host of other erythrocyte skeletal proteins (including spectrin, ankyrin and band 3), the phosphorylation of 4.1R influences protein-protein interactions which may lead to changes in membrane structure and function.[20] The discovery of novel kinases may aid in understanding merozoite invasion and release, as well as the development of *P. falciparum* with the erythrocyte. Furthermore, kinases have become attractive targets for the development of anti-malaria agents.[2]

Hypothetical P. falciparum proteins

As expected, approximately 62% of the proteins that were identified by biopanning were hypothetical (Table 1), confirming that the proteome of *P. falciparum* is largely uncharacterized. The interaction of these proteins with 4.1R infers that they localize and/ or function at the erythrocyte membrane. Proteins encoded by PF14_0201 and MAL6P.48 have predicted transmembrane regions and presumably associate with 4.1R by inserting into the host membrane. These hypothetical proteins may modulate the interaction between 4.1R and other erythrocyte membrane proteins, since 4.1R enhances the binding affinities between spectrin and actin, as well as between glycophorin and P55.[22] *P. falciparum* proteins that interact with 4.1R may therefore influence normal 4.1R functioning, resulting in reduced protein interactions and structural alterations to the membrane skeleton.

Common myosin-like binding motif

Bioinformatic analyses in the PlasmoDB database revealed a common, negatively charged binding motif in four of the eight identified *P. falciparum* proteins. The 4.1R binding sequences in PF14_0201, PF11_0191 and Ebl-1-like protein were mapped to a region within the proteins that showed homology to the coiled-coil domain of eukaryotic myosin (E values: $1.5e^{-28}$, $1.4e^{-14}$ and $1.0e^{-23}$ respectively), whereas the binding sequence in PFA0420w revealed homology to the coiled repeat pattern in eukaryotic neurofilament sequences (E value: 4.9^{e-10}). The coiled-coil structure of eukaryotic myosin is a distinctive structural feature of the tail domain in class II myosins, typically skeletal and smooth muscle types.[23] The coiled-coil forms an important structural element in fibrous proteins, however, coiled-coil motifs in globular proteins perform a variety of cellular functions, the most common of which are protein-protein interactions.[24] This study demonstrates that eukaryotic myosin and neurofilament-like motifs in specific *P. falciparum* proteins have the ability to bind directly to 4.1R. In support of our findings, the 10-KDa domain of 4.1R has been demonstrated to bind and regulate myosin.[25]

Other binding proteins

MESA, a protein containing a large number of repeat regions and charged residues, facilitates the survival of

P. falciparum by associating with 4.1R.[8,26] Bennett *et al.* have mapped the 4.1R binding site in MESA to a 19-amino acid sequence, a region suggested to be of insufficient length to support this interaction.[27] Interestingly, our bioinformatic analyses revealed that the binding sequence is located at an N-terminal position near a myosin-like motif. We speculate that the myosin motif in MESA enhances the affinity of the 19-amino acid binding sequence for 4.1R.

On further examination of the 4.1R binding sequence in MESA, namely SIRNY--C---APYI, we identified a similar SMKNY--C---APYI motif in the RESA-like protein. The motif in the RESA-like protein is also located at the N-terminal end of a myosin-like sequence.

These findings, as well as the structural similarity between RESA-like protein and MESA, allow us to infer that RESA-like protein may bind 4.1R. Further bioinformatic analyses revealed homology between the myosin sequence and six other *P. falciparum* proteins, MAEBL (PF11_0486), PFD0207c, PFB0115w, PF11_0035, PFE0230w and PFE0440w, indicating that these proteins are potentially involved in 4.1R binding.

Conclusions

In this study *P. falciparum* phage display expression libraries have facilitated the identification of novel interactions between the parasite and 4.1R. Addressing the detailed binding kinetics between 4.1R and myosin-like motifs will aid in unravelling the functional importance of these interactions to *P. falciparum* growth and survival. Furthermore, the interaction between 4.1R and parasite proteins containing myosin and neurofilament-like sequences may emerge as an important finding for the development of novel drugs against malaria.

TLC was responsible for designing and co-ordinating the study, interpretating the data, as well as critical revision and final approval of the manuscript; RL was responsible for the experimental work, interpretation of data, drafting the article, tabulating the data and final approval of the manuscript.

We thank the Department of Pharmacy and Pharmacology, University of the Witwatersrand, for supplying stock cultures of P. falciparum (strain FCR-3). This material is based upon work supported by the National Research Foundation under grant number 2053200, as well as research grants from the Medical Research Council of South Africa, National Health Laboratory Service and University of the Witwatersrand.

Manuscript received May 5, 2004. Accepted July 19, 2004.

References

1. Gardner M J, Hall N, Fung E, White O, Berriman M, Hyman RW, et al. Genome sequence of the human malaria parasite Plasmodium falciparum. Nature 2002; 419: 498-511.
2. Florens L, Washburn MP, Raine JD, Anthony RM, Grainger M, Haynes JD, et al. A proteomic view of the Plasmodium falciparum lifecycle. Nature 2002;419:520-6.
3. Horrocks P, Bowman S, Kyes S, Waters AP, Craig A. Entering the post-genomic era of malaria research. Bull World Health Organ 2000;78:1424-37.
4. Cooke BM, Mohandas N, Coppel RL. The malaria-infected red blood cell: structural and functional changes. Adv Parasitol 2001; 50:1-86.
5. Conboy JG. Structure, function, and molecular genetics of erythroid membrane skeletal protein 4.1 in normal and abnormal red blood cells. Semin Hematol 1993;30:58-73.
6. Schulman S, Roth EF, Cheng B, Rybicki AC, Sussman II, Wong M, et al. Growth of Plasmodium falciparum in human erythrocytes containing abnormal membrane proteins. Proc Natl Acad Sci USA 1990;87:7339-43.
7. Chishti AH, Palek J, Fisher D, Maalouf GJ, Liu SC. Reduced invasion and growth of Plasmodium falciparum into elliptocytic red blood cells with combined deficiency of protein 4.1, glycophorin C and p55. Blood 1996; 87:3462-9.
8. Magowan C, Coppel RL, Lau AOT, Moronne MM, Tchernia G, Mohandas N. Role of the Plasmodium falciparum mature-parasite erythrocyte surface antigen (MESA/PfEMP2) in malarial infection of erythrocytes. Blood 1995;86:3196-204.
9. Waller KL, Nunomura W, An X, Cooke BM, Mohandas N, Coppel RL. Mature parasite-infected erythrocyte surface antigen (MESA) of Plasmodium falciparum binds to the 30-KDa domain of protein 4.1 in malaria-infected red blood cells. Blood 2003;102: 1911-14.
10. Hanspal M, Dua M, Takakuwa Y, Chishti AH, Mizuno A. Plasmodium falciparum cysteine protease falcipain-2 cleaves erythrocyte membrane skeletal proteins at late stages of parasite development. Blood 2002; 100: 1048-54.
11. Chishti AH, Maalouf GJ, Marfatia S, Palek J, Wang W, Fisher D, et al. Phosphorylation of protein 4.1 in Plasmodium falciparum-infected human red blood cells. Blood 1994; 83:3339-45.
12. Trager W, Jensen JB. Human malaria parasites in continuous culture. Science 1976; 193:673-5.
13. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156-9.
14. Lauterbach SB, Lanzillotti R, Coetzer TL. Construction and use of Plasmodium falciparum phage display libraries to identify host parasite interactions. Malaria J 2003; 2:47.
15. Tyler JM, Reinhardt BN, Branton D. Associations of erythrocyte membrane proteins: binding of purified bands 2.1 and 4.1 to spectrin. J Biol Chem 1980; 255:7034-9.
16. Chitnis CE, Blackman MJ. Host cell invasion by malaria parasites. Parasitol Today 2000; 16:411-5.
17. Kappe SHI, Buscaglia CA, Bergman LW, Coppens I, Nussenzweig V. Apicomplexan gliding motility and host cell invasion: overhauling the motor model. Trends Parasitol 2004;20:13-6.
18. Orlandi PA, Klotz FW, Haynes JD. A malaria invasion receptor, the 175-kilodalton erythrocyte binding antigen of Plasmodium falciparum recognises the terminal NeuAc(α2-3) Gal-sequences of glycophorin A. J Cell Biol 1992;116:901-9.
19. Jakobsen PH, Heegaard PM, Koch C, Wasniowska K, Lemnge MM, Jensen JB, et al. Identification of an erythrocyte binding peptide from the erythrocyte binding antigen, EBA-175, which blocks parasites multiplication and induces peptide-blocking antibodies. Infect Immun 1998;66:4203-7.
20. Magowan C, Liang J, Yeung J, Takakuwa Y, Coppel RL, Mohandas N. Plasmodium falciparum: influence of malaria and host erythrocyte skeletal protein interactions on phosphorylation in infected erythrocytes. Exp Parasitology 1998;89:40-9.
21. Go ML. Novel antiplasmodial agents. Med Res Rev 2003;23:456-87.
22. Marfatia SM, Lue RA, Branton D, Chishti AH. In vitro binding studies suggest a membrane-associated complex between erythroid p55, protein 4.1, and glycophorin C. J Biol Chem 1994;269:8631-4.
23. Sellers JR. Myosins: a diverse superfamily. Biochim Biophys Acta 2000;1496:3-22.
24. Lupas A. Coiled coils: new structures and new functions. Trends in Biochemical Sciences 1996;21:375-82.
25. Pasternack GR, Racusen RH. Erythrocyte protein 4.1 binds and regulates myosin. Proc Natl Acad Sci USA 1989;86:9712-6.
26. Lustigman S, Anders RF, Brown GV, Coppel RL. The mature-parasite-infected erythrocyte surface antigen (MESA) of Plasmodium falciparum associates with the erythrocyte membrane skeletal protein, band 4.1. Mol Biochem Parasitol 1990;38:261-70.
27. Bennett BJ, Mohandas N, Coppel RL. Defining the minimal binding domain of the Plasmodium falciparum protein MESA involved in the interaction with red cell membrane skeletal protein 4.1. J Biol Chem 1997; 272: 15299-306.