Plasmodium falciparum Erythrocyte Membrane Protein 3 (PfEMP3) Destabilizes Erythrocyte Membrane Skeleton*

Xinhong Pei†, Xinhua Guo†, Ross Coppell‡, Narla Mohandas§, and Xiuli An†1

From the †Red Cell Physiology Laboratory, New York Blood Center, New York, New York 10065 and ‡Department of Microbiology, Monash University, Monash, Victoria 3800, Australia

Plasmodium falciparum erythrocyte membrane protein 3 (PfEMP3) is a parasite-derived protein that appears on the cytoplasmic surface of the host cell membrane in the later stages of the parasite's development where it associates with membrane skeleton. We have recently demonstrated that a 60-residue fragment (FIa1, residues 38–97) of PfEMP3 bound to spectrin. Here we show that this polypeptide binds specifically to a site near the C terminus of α-spectrin at the point that spectrin attaches to actin and protein 4.1R in forming the junctions of the membrane skeletal network. We further show that this polypeptide disrupts formation of the ternary spectrin-actin-4.1R complex in solution. Importantly, when incorporated into the cell, the PfEMP3 fragment causes extensive reduction in shear resistance of the cell. We conjecture that the loss of mechanical cohesion of the membrane may facilitate the exit of the mature merozoites from the cell.

The intraerythrocytic form of Plasmodium falciparum, the agent of the most severe type of human malaria, exports many (according to a proteomic estimate, up to 400) proteins into the host cell in the course of its growth and development (1–3). Certain of these proteins are known to associate with the host cell membrane skeleton and thereby modify its structure and properties. Among the changes that have been reported are an altered morphology, an increased membrane rigidity, and an enhanced propensity to adhere to the vascular endothelium (4). Several such proteins that bind to the red cell membrane skeleton have so far been characterized. When the parasite first invades red cells, it deposits RESA (the ring parasite-infected erythrocyte surface antigen) at the membrane skeleton of the newly invaded cell where it binds to spectrin and protects against thermal damage (5–7). As the intracellular parasite further matures, it traffics further proteins to the skeleton. P. falciparum erythrocyte membrane protein 1, or PfEMP1,2 is inserted into the red cell membrane and its extracellular domain adheres to receptors on endothelial cells (8). PfEMP1 clusters on the red cell surface at dimpled areas called knobs by binding of its intracellular domain to the parasite-encoded protein KAHRP (knob-associated histidine-rich protein) from which knobs are formed (9) and to spectrin and actin (10). KAHRP itself is stabilized at the membrane by interactions with the fourth repeat of spectrin α-chain (11). MESA (the mature parasite-infected erythrocyte surface antigen) binds to the 30-kDa domain of protein 4.1R and displaces the host protein p55 (12, 13). Its role in parasite biology is still uncertain, but interference with MESA binding is lethal to the parasite (14). The P. falciparum erythrocyte membrane protein 3 (PfEMP3), with which we are concerned here, is a large protein of 315 kDa that is expressed from the late ring stage onward and is exported via the Maurer’s clefts, vesicular structures of parasite origin, into the cytoplasm of the red cell, where it associates with membrane skeleton. However, the function of PfEMP3-skeleton interaction has not been explored.

Spectrin, the major component of erythrocyte membrane skeleton, is composed of an α-chain and a β-chain that associate side to side in an antiparallel orientation to form αβ-heterodimers (15). Spectrin heterodimers then self-associate head to head to form spectrin tetramers (16). The tetramers are connected at their ends to junctional complexes that are primarily comprised of spectrin, actin, and 4.1R (17). The importance of the spectrin-actin-4.1R ternary complex in maintaining erythrocyte membrane integrity is demonstrated by the findings that mutations in β-spectrin (18) or 4.1R (19) result in decreased membrane mechanical stability and cell fragmentation in vivo. The ternary complex formation of spectrin with actin and 4.1R involves the β-spectrin N terminus, which harbors two actin and two 4.1R binding sites (20). In contrast to the β-spectrin N terminus, very little is known regarding the α-spectrin C terminus that is in apposition to the β-spectrin N terminus. Studies from sph1 mice (where a nonsense mutation in exon 52 of the erythroid α-spectrin gene eliminates the C-terminal 13 amino acids) strongly suggested the α-spectrin C terminus is also critical to the stability of the junctional complex (21).

We show here that the receptor site of PFEMP3 on the membrane is a motif close to the C terminus of the spectrin α-chain. This is the region of the spectrin αβ-heterodimer that forms the junctions of the membrane skeletal network by ternary interaction with actin and protein 4.1R. We further show that the peptide acts to destabilize the junction and thereby the membrane as a whole. These findings enabled us to identify a role of
the interaction between PfEMP3 and spectrin in the infected erythrocytes.

EXPERIMENTAL PROCEDURES

Materials—Fresh blood was taken from healthy volunteers with informed consent. pGEX-4T-2 vector and glutathione-Sepharose 4B were purchased from Amersham Biosciences Inc., restriction enzymes, amylose resin affinity column, and monoclonal anti-MBP antibody from New England BioLabs (Beverly, MA), and reduced form glutathione and isopropyl-1-thio-β-d-galactopyranoside from Sigma. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG was from Jackson ImmunoResearch Laboratories Inc. (Gaithersburg, MD). The CM-5 sensor chip, and other reagents for surface plasmon resonance assay were purchased from BIAcore (Piscataway, NJ). Dextran T40 was from Amersham Biosciences, and electrophoresis reagents from Bio-Rad (Hercules, CA).

Preparation of Spectrin, Recombinant Spectrin Fragments, and PfEMP3 Fragment—Spectrin from erythrocytes was prepared according to Tyler et al. (22). Spectrin fragments and single repeats were cloned, expressed, and purified as described previously (23). The F1a1 fragment of PfEMP3 was cloned into pMAL-p2X vector using BglII and EcoRI upstream and downstream, respectively. The MBP-tagged F1a1 polypeptide was purified using an amylose resin affinity column. Protein concentrations were determined spectrophotometrically, using extinction coefficients calculated from the tryptophan and tyrosine contents. Proteins were dialyzed against phosphate-buffered saline (10 mM phosphate, pH 7.4, 150 mM NaCl) for pulldown assay and against HBS-EP buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.05% (v/v) surfactant P20) for surface plasma resonance assay. For rescaling experiments, polypeptides were dialyzed against hypotonic buffer (5 mM Tris, 5 mM KCl, pH 7.4). All proteins were clearly ultracentrifuged at 230,000 × g for 30 min at 4 °C before use.

GST Pulldown Assay—Glutathione S-transferase (GST)-tagged recombinant spectrin polypeptides were coupled to glutathione-Sepharose 4B beads at room temperature for 30 min. Beads were pelleted and washed. MBP-tagged F1a1 fragment (at a final concentration of 1 μM) was added to the coupled beads to a final volume of 100 μl and a coupled protein concentration of 1 μM, incubated for 1 h at room temperature, and pelleted, washed, and eluted with 10% SDS. The pellet was analyzed by SDS-PAGE, followed by transfer to nitrocellulose membrane and exposure to anti-MBP antibody to detect F1a1 binding to spectrin polypeptides. GST was used as negative control in all experiments. To study the effect of PfEMP3 F1a1 peptide on its ability to interfere with spectrin heterodimer assembly, 1 μM GST-tagged α17-C was coupled to beads. The binding of His-tagged β 1–5 fragment to the immobilized α fragment in the absence or presence of PfEMP3 peptide at various concentrations (ranging from 1 to 24 μM) was assessed using the same approach described above.

RESULTS

Mapping the PfEMP3 Binding Site in Spectrin—The positions in the α- and β-spectrin sequences of the recombinant fragments used in this study are shown in Fig. 1A. All recombinant proteins were expressed at 16 °C, at which the yield was optimal, and allowed the isolation of soluble products in a state of assembly, 1 μM GST-F1a1 polypeptide at a series of concentrations was added to hypotonically lysed cells. After restoration of isotonicity and warming, as described previously, the stability of the ghosts was assayed in the ektacytometer and expressed in terms of the rate of decline of the deformability index, as the membranes vesiculated under constant shear (25).

Surface Plasma Resonance Assay—Binding was measured in a BIAcore 3000 instrument. The instrument was programmed to perform a series of binding measurements with increasing concentrations of analyte over the same surface. Conditions were as described previously (11). Spectrin or the GST-tagged fragment containing EF1/2 of the C terminus of α-spectrin was covalently coupled to a CM-5 biosensor chip using an amino coupling kit. MBP-F1a1 at various concentrations was passed through the cell. The surface was regenerated between injections with 50 mM NaOH. Binding and dissociation kinetics were analyzed with the aid of the software BIAeval 3.0, and binding constants were derived, with the assumption of a 1:1 interaction.

ELISA Assay—Competitive inhibition was measured by an enzyme-linked immunosorbent assay (ELISA). Spectrin dimer was coated on a 96-well plate overnight at 4 °C. The plate was washed and then blocked with 1% bovine serum albumin and 0.05% Tween 20 in phosphate-buffered saline for 1 h at room temperature. F1a1 fragment (preincubated without or with increasing concentrations of EF1/2) was added to the spectrin-coated plate and incubated for 1 h at room temperature. After three washes, the binding of MBP-tagged F1a1 to spectrin was detected by anti-MBP antibody, followed by HRP-conjugated anti-mouse IgG. The color was developed by adding 3’3’5’5’-tetra-methylbenzidine microwell peroxidase substrate and read by an ELISA plate reader at 450 nm.

Introduction of F1a1 Polypeptide into Erythrocyte Ghosts and Measurement of Membrane Stability—MBP or MBP-tagged F1a1 polypeptide at a series of concentrations was added to hypotonically lysed cells. After restoration of isotonicity and warming, as described previously, the stability of the ghosts was assayed in the ektacytometer and expressed in terms of the rate of decline of the deformability index, as the membranes vesiculated under constant shear (25).
fragment was immobilized onto the CM-5 sensor chip. The experiment was performed as described in the Methods section. A binding assay was performed with each of the GST-tagged spectrin fragments, and the bound fragment was detected by blotting with anti-MBP antibody after SDS-PAGE.

FIGURE 2. Interaction between F1a1 and spectrin or the EF1/2 fragment of spectrin as assessed by surface plasmon resonance assay. A, schematic representation of the spectrin α- and β-chains, showing the domain structure and locations in the sequence of expressed recombinant fragments. The boundaries of all spectrin fragments and single repeats were defined by SMART annotations. B, schematic representation of PFEMP3. The P. falciparum PFEMP3 gene contains two exons separated by an intron. The first exon encodes a putative signal sequence, and the second exon contains three repeat regions as indicated. Amino acid residue numbers and boundaries of the F1a1 fragment are indicated. C, binding of the PFEMP3 F1a1 fragment to spectrin fragments. MBP-tagged F1a1 was incubated for 30 min at room temperature with each of the GST-tagged spectrin fragments, and binding was assayed by GST pulldown assay. The bound fragment was detected by blotting with anti-MBP antibody after SDS-PAGE. D, binding of F1a1 to spectrin repeats. Binding assays were performed as above. Note binding to EF 1/2 of α-spectrin only.

FIGURE 2. Interaction between F1a1 and spectrin or the EF1/2 fragment of α-spectrin as assessed by surface plasmon resonance assay. A, spectrin was immobilized onto a CM-5 sensor chip. MBP-F1a1 at different concentrations as indicated was injected at 20 μl/min over the surface in a BIAcore 3000 instrument. The figure shows dose-response curves of F1a1 binding. B, the experiment was performed as described in panel A except that the EF1/2 fragment was immobilized onto the CM-5 chip.

occurred to only one of the α-spectrin fragments, α17-C. There was no binding to GST or to other spectrin fragments. The α17-C construct comprises repeats 17, 18, 19, 20, 21, and the C-terminal element with four EF-hands. We next examined the binding of F1a1 to all these parts of the large fragment individually and obtained a positive result for only one of them. This comprised 65 amino acids, embracing EF-hands 1 and 2 (Fig. 1D). Thus we localized the PFEMP3 binding site to the C terminus of the α-spectrin chain. The binding of F1a1 to spectrin and to the C-terminal fragment of the α-chain, which we designate EF1/2, was further characterized by surface plasmon resonance assay. The binding profiles are shown in Fig. 2, and the derived dissociation constants of binding of F1a1 to spectrin dimer and to EF1/2 are 2.16 × 10⁻⁸ M and 7.48 × 10⁻⁸ M, respectively.

Inhibition of Spectrin Binding to F1a1 by the EF1/2—To confirm the specificity of the interaction between EF1/2 of the α-chain C-terminal region and PFEMP3, we performed a competitive inhibition assay. The result of the competitive ELISA assay is shown in Fig. 3, from which it can be seen that the binding of F1a1 to full-length spectrin is significantly reduced in the presence of increasing concentrations of EF1/2 fragment.

Effect of F1a1 on Formation of the Spectrin-Actin-4.1R Ternary Complex—The N-terminal domain of the spectrin β-chain engages with F-actin and 4.1R, for each of which it has two binding sites (20), to form a ternary complex. In the cell this complex is at the seat of the nodes, or junctions, of the membrane skeletal network, and its disruption leads to breakdown of the membrane. Because the N-terminal region of the spectrin β-chain is in close apposition to the C-terminal part of the α-chain, we examined the possibility that attachment of PFEMP3, as represented by its active fragment, F1a1, might hinder formation of the ternary complex. This was tested in the falling ball viscosity assay. Addition of F1a1 to the mixture of F-actin, 4.1R, and spectrin was indeed found to inhibit the interaction (Fig. 4).

No Effect of F1a1 on Spectrin Heterodimer Assembly—As the PFEMP3 binding is in close proximity to the nucleation site involved in spectrin heterodimer assembly, we examined the ability of PFEMP3 peptide to interfere spectrin heterodimer formation. The interaction between dimerizing spectrin fragments α17-C and β 1–5 in the absence and presence of the PFEMP3 peptide was examined by GST pulldown assay. Peptide concentrations up to 24 μM had no effect on spectrin heterodimer assembly (data not shown).

Effect of F1a1 on Erythrocyte Membrane Stability—From the above result it might be expected that PFEMP3, or its active fragment, would act in a similar way inside the erythrocyte, reducing the cohesion of the junctions and thus the stability of the membrane. We accordingly introduced F1a1 into resealed...
ghosts at increasing concentrations and measured their stability under shear in the ektacytometer. The mixture was added to ELISA wells coated with spectrin dimer. After incubation and washing, the fraction of Fla1 remaining was estimated by antibody staining.

The site of attachment to the membrane lies at the C-terminal extremity of the spectrin α-chain, which is also in, or in intimate proximity to, the site of interaction of spectrin with actin and 4.1R that forms the junctions of the membrane skeletal network. The N-terminal domain of the spectrin β-chain, to which the C-terminal part of the α-chain is linked, is sufficient to generate the ternary complex in vitro (20), and what we have observed here hints at a possible participation of the α-chain in the junction complex. This has indeed been suggested on the basis of the properties of erythrocytes of sphJ mice, in which the C-terminal 13 amino acids of the α-chain were deleted (27). We cannot, at the same time, exclude that the effect of PfEMP3 or its active fragment in weakening the junction by binding to α-spectrin may be due to steric crowding.

The site for attachment of PfEMP3 on spectrin is remote from that for KAHRP, which is in repeat 4 of the α-chain. It is also clear that the two proteins serve quite different functions, KAHRP being involved in knob formation (28) and thus probably adhesive interactions (29), with no detectable influence on shear resistance of the membrane, whereas PfEMP3 affects this membrane property profoundly. RESA, which has a single binding site on the spectrin β-chain, also perturbs shear response but in the opposite direction (32), while MESA binds to a site on 4.1R, probably interfering with its attachment to the membrane (14).

Merozoites have to exit the host cells before infecting new erythrocytes, but the mechanism of merozoite release remains largely unknown. It is generally believed that proteases are basically involved. One proposed model is that these proteases will proteolyze the erythrocyte skeleton proteins and the proteolytic breakdown of these proteins in turn will lead to disruption of membrane skeleton. Indeed, a number of plasmodial proteases have been identified that have activities against erythrocyte proteins (30). More specifically, a P. falciparum-derived cysteine protease, falcipain-2 (FP-2), has been shown to cleave...
both ankyrin and 4.1R and this cleavage is accompanied by diminished membrane stability (31). Interestingly, in the present study we found that resealing of fragment PfEMP3 also led to membrane instability. Our finding implies that disruption of the skeleton network by malaria proteins that are expressed at late stages of malaria development could potentially provide a mechanism for merozoite release from erythrocytes.

In summary, our results shed some light on the function of the major exported proteins. While PfEMP1 is unquestionably, and KAHRP probably, involved in promoting adhesion of the parasitized erythrocyte to endothelial surfaces, RESA, which is expressed early in development, reinforces the membrane against a second invasion by a merozoite (32). As RESA disappears from the cell, late in development, PfEMP3 is secreted and accrues on the erythrocyte membrane, which it greatly weakens. A plausible function of PfEMP3 may then be to facilitate the escape of the new generation of merozoites into the bloodstream. The identification, however tentative, of these various functions may suggest a new range of targets for therapeutic intervention. It should at the same time be recalled that, in summary, our results shed some light on the function of PfEMP3 and Erythrocyte Spectrin.

REFERENCES

1. Hiller, N. L., Bhattacharjee, S., van Ooij, C., Liolios, K., Harrison, T., Lopez-Estrano, C., and Haldar, K. (2004) Science 306, 1934–1937
2. Cowman, A. F., and Kappe, S. H. (2006) Science 313, 1934–1937
3. Sargeant, T. J., Marti, M., Caler, E., Carlton, J. M., Simpson, K., Speed, T. P., and Cowman, A. F. (2006) Mol. Biochem. Parasitol. 146, 1–86
4. Cooke, B. M., Mohandas, N., and Coppel, R. L. (2001) Adv. Parasitol. 50, 1–86
5. Foley, M., Tilley, L., Sawyer, W. H., and Anders, R. F. (1991) Mol. Biochem. Parasitol. 46, 137–147
6. Da Silva, E., Foley, M., Dluzewski, A. R., Murray, L. J., Anders, R. F., and Tilley, L. (1994) Mol. Biochem. Parasitol. 66, 59–69
7. Silva, M. D., Cooke, B. M., Guillot, M., Buckingham, D. W., Sauzet, J. P., Le Scanf, C., Contamin, H., David, P., Mercereau-Puijalon, O., and Bonnefoy, S. (2005) Mol. Microbiol. 56, 990–1003
8. Baruch, D. I., Gormely, J. A., Ma, C., Howard, R. J., and Pasloske, B. L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 3497–3502
9. Waller, K. L., Nunomura, W., Cooke, B. M., Mohandas, N., and Coppel, R. L. (2002) Mol. Biochem. Parasitol. 119, 125–129
10. Oh, S. S., Voigt, S., Fisher, D., Yi, S. J., LeRoy, P. J., Derick, L. H., Liu, S., and Chishti, A. H. (2000) Mol. Biochem. Parasitol. 108, 237–247
11. Pei, X., An, X., Guo, X., Tarnawski, M., Coppel, R., and Mohandas, N. (2005) J. Biol. Chem. 280, 31166–31171
12. Bennett, B. J., Mohandas, N., and Coppel, R. L. (1997) J. Biol. Chem. 272, 15299–15306
13. Waller, K. L., Nunomura, W., An, X., Cooke, B. M., Mohandas, N., and Coppel, R. L. (2003) Blood 102, 1911–1914
14. Magowan, C., Coppel, R. L., Lau, A. O., Moronne, M. M., Tchernia, G., and Mohandas, N. (1995) Blood 86, 3196–3204
15. Ursitti, J. A., Kotula, L., DeSilva, T. M., Curtis, P. J., and Speicher, D. W. (1996) J. Biol. Chem. 271, 6636–6644
16. Ungewickell, E., and Gratzger, W. (1978) Eur. J. Immunol. 8, 379–385
17. Cohen, C. M., Tyler, J. M., and Branton, D. (1980) Cell 21, 875–883
18. Becker, P. S., Morrow, J. S., and Lux, S. E. (1987) J. Clin. Invest. 80, 557–565
19. Conboy, J. G., Shitamoto, R., Parra, M., Winardi, R., Kabra, A., Smith, J., and Mohandas, N. (1991) Blood 78, 2438–2443
20. An, X., Debnath, G., Guo, X., Liu, S., Lux, S. E., Baines, A., Gratzier, W., and Mohandas, N. (2005) Biochemistry 44, 10681–10688
21. Wandersee, N. J., Birkenmeier, C. S., Bodine, D. M., Mohandas, N., and Barker, J. E. (2003) Blood 101, 325–330
22. Tyler, J. M., Hargreaves, W. R., and Branton, D. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 5192–5196
23. An, X., Guo, X., Sum, H., Morrow, J., Gratzier, W., and Mohandas, N. (2004) Biochemistry 43, 310–315
24. Gim, J. A., An, X., Nunomura, W., and Mohandas, N. (2002) Biochemistry 41, 7275–7282
25. Mohandas, N., Clark, M. R., Health, B. P., Rossi, M., Wolfe, L. C., Lux, S. E., and Shohet, S. B. (1982) Blood 59, 768–774
26. An, X., Guo, X., Zhang, X., Baines, A. J., Debnath, G., Moyo, D., Salomao, M., Bhasin, N., Johnson, C., Discher, D., Gratzier, W. B., and Mohandas, N. (2006) J. Biol. Chem. 281, 10527–10532
27. Wandersee, N. J., Birkenmeier, C. S., Gifford, E. J., Mohandas, N., and Barker, J. E. (2000) Hematol. J. 1, 235–242
28. Crabb, B. S., Cooke, B. M., Reeder, J. C., Waller, R. F., Caruana, S. R., Davern, K. M., Wickham, M. E., Brown, G. V., Coppel, R. L., and Cowman, A. F. (1997) Cell 89, 287–296
29. Cooke, B. M., Glenister, F. K., Mohandas, N., and Coppel, R. L. (2002) Br. J. Haematol. 117, 203–211
30. Raphael, P., Takakuwa, Y., Manno, S., Liu, S. C., Chishti, A. H., and Hanspal, M. (2000) Mol. Biochem. Parasitol. 110, 259–272
31. Hanspal, M., Dua, M., Takakuwa, Y., Chishti, A. H., and Mizuno, A. (2002) Blood 100, 1048–1054
32. Pei, X., Guo, X., Coppel, R., Bhattacharjee, S., Halder, K., Gratzier, W., Mohandas, N., and An, X. (2007) Blood 110, 1036–1042