Structural and Functional Studies of Interaction between *Plasmodium falciparum* Knob-associated Histidine-rich Protein (KAHRP) and Erythrocyte Spectrin*

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*Malaria caused by* *Plasmodium falciparum* is the most seri-
ous parasitic disease of humans. Clinical symptoms occur du-
cing the asexual stage of the life cycle of the parasites, at
which time it multiplies within the human erythrocyte. During
intraerythrocytic growth, the parasite extensively modifies the
host erythrocyte resulting in alterations of morphology, me-
chanical properties, and adhesive properties. It is generally
believed that almost all the altered properties of parasitized
erythrocytes are because of the action of a group of parasite
proteins that become associated with erythrocyte membrane
proteins (see Refs. 1–3 for recent reviews). Well studied mem-
proteins that become associated with erythrocyte membrane
believed that almost all the altered properties of parasitized
erythrocytes. As the presence of KAHRP at the erythrocyte
membrane localization of KAHRP in parasitized erythro-
cytes. As the presence of KAHRP at the erythrocyte
membrane necessary for cytoadherence in *vivo*, our
findings have implications for the development of new
therapies for mitigating the severity of malaria infection.

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The glutatione-Sephrose 4B affinity column were from Amersham Biosciences. pET28b(−) was obtained from MR4 (Manassas, VA). pGEX vector and glutathione-Sepharose 4B were purchased from Interstate Blood Bank (Memphis, TN). Parasite clone 3D7 was transformed into BL21 bacteria strain. The expression of recombinant KAHRP fragments were analyzed by 10% SDS-PAGE and stained with GelCode Blue. N and C indicate the N terminus or C terminus of spectrin chains, respectively.

In the present study, we identified the binding site for spectrin in KAHRP and the reciprocal KAHRP-binding site in spectrin. We showed that spectrin bound to a specific region of KAHRP (residues 370–441) and that this region of KAHRP specifically bound to a single repeat of a spectrin (repeat 4, αR4). Furthermore, resealing of the αR4 polypeptide into erythrocytes prior to infection by malarial parasites prevented the membrane association of KAHRP. These findings enabled us to identify an important functional role for the interaction between KAHRP and spectrin in localizing KAHRP to the membrane of the infected erythrocytes.

**Experimental Procedures**

Materials—Type O- fresh blood was taken from healthy volunteers with informed consent. Serum from type A blood donor was obtained from Interstate Blood Bank (Memphis, TN). Parasite clone 3D7 was obtained from MR4 (Manassas, VA). pGEX vector and glutathione-Sepharose 4B were purchased from Amersham Biosciences. pET28b(−)-D-galactopyranoside were purchased from Fisher Scientific (Piscataway, NJ). All other chemicals were reagent grade products from standard sources.

Preparation of Spectrin, Recombinant Spectrin Fragments, and KAHRP Fragments—Spectrin from erythrocytes was prepared according to the method described by Tyler et al. (19). Spectrin fragments were cloned into pGEX-4T-2 (20) and KAHRP fragments were cloned into pGEX-KG vector (9). The cDNA encoding the desired polypeptide was inserted into BL21 bacteria strain. The expression of recombinant proteins was induced by 0.1 mM isopropyl-D-thiogalactopyranoside at 16°C for 3–4 h. The GST-tagged spectrin and KAHRP polypeptides were purified using a glutathione-Sepharose 4B affinity column. Two

![Figure 1](image1.png)  
**Fig. 1. Schematic representation (A) and SDS-PAGE of recombinant spectrin fragments (B) as well as spectrin single repeats (C).** The boundaries of all spectrin fragments and single repeats were defined by SMART annotations. 1 μg of total purified GST fusion proteins was analyzed by 10% SDS-PAGE and stained with GelCode Blue. N and C indicate the N terminus or C terminus of spectrin chains, respectively.

![Figure 2](image2.png)  
**Fig. 2. Schematic representation (A) and SDS-PAGE of recombinant KAHRP fragments (B).** The P. falciparum KAHRP gene contains two exons separated by an intron. Amino acid residue numbers and the lengths of fragments are indicated. His-tagged KAHRP fragments were run in 10% SDS-PAGE and His-tagged KAHRP fragments were run in 15% SDS-PAGE. Lane 1, GST-K1; lane 2, GST-K2; lane 3, GST-K2a; lane 4, GST-K2b; lane 5, GST-K3; lane 6, His-K2; lane 7, His-K2a + b.

![Figure 3](image3.png)  
**Fig. 3. Binding of spectrin dimer to KAHRP fragments.** Spectrin dimer was incubated with various GST-tagged KAHRP fragments at room temperature for 30 min. The interaction was assessed by GST pull-down assay, and the binding was detected by anti-spectrin antibody.

His-tagged KAHRP fragments (K2 and K2a+b) were subcloned into pET-28b (+) vector using Ncol and Xhol cloning sites upstream and downstream, respectively. His-tagged KAHRP fragments were expressed as above and purified with a nickel column. Proteins were dialyzed against PBS (10 mM phosphate, pH 7.4, 150 mM NaCl). Protein concentrations were determined spectrophotometrically using extinction coefficients calculated from the tryptophan and tyrosine contents, taking the molar extinction coefficients of these amino acids at 280 nm as 5500 and 1340, respectively (21). All the proteins were clarified by ultracentrifugation at 230,000 × g for 30 min at 4°C before use.

Pull-down Assay—For GST pull-down assay, GST-tagged recombinant polypeptides were coupled to glutathione-Sepharose 4B beads at room temperature for 30 min. Beads were pelleted and washed. Binding partner was added to the coupled beads in a final volume of 80 μl. The final concentration of the coupled protein was 1 μM. The mixture was incubated for 1 h at room temperature, pelleted, washed, and eluted with 10% SDS. The pellet was analyzed by SDS-PAGE. The binding of full-length spectrin to KAHRP fragments was detected by Western blot using anti-spectrin antibody, and similarly the binding of His-K2a + b was detected by Western blot using anti-His antibody. GST was used as negative control in all experiments. For His pull down, the procedure was basically the same except that the His-tagged protein was coupled to nickel beads.

Surface Plasmon Resonance Assay—Surface plasmon resonance assay was performed using a BIAcore 3000 instrument. His-tagged KAHRP polypeptide (K2 or K2a+b) was covalently coupled to a CM-5
biosensor chip using an amino coupling kit. Binding reactions were done in HBS-EP buffer, containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.05% (v/v) surfactant P20. The surface was regenerated before each new injection using 50 mM NaOH. The BIAcore instrument was programmed to perform a series of binding assays with increasing concentrations of αN-5 or αR4 polypeptide over the same regenerated surface. Sensorsgrams (plots of changes in response unit on the surface as a function of time) derived were analyzed using the software BIAeval 3.0. Affinity constants were estimated by curve fitting using a 1.1 binding model.

**Recombinant Protein Expression and Purification**—A schematic representation of the various spectrin and KAHRP fragments used in the present study are illustrated in Figs. 1A and 2A, respectively. All spectrin fragments and spectrin single repeats were cloned into pGEX-4T-2 protein expression vector (20). To detect by Western blotting the binding of KAHRP fragments to GST-tagged spectrin fragments using anti-His antibody, we constructed His-tagged K2 and K2α+b fragments from previously described GST-tagged KAHRP fragments (9). At 16 °C, all recombinant proteins are well expressed and soluble. The purity of various GST-spectrin fusion proteins and GST-tagged KAHRP, as well as His-tagged KAHRP fragments, used in the present study are shown in Figs. 1, B and C and 2B, respectively.

Mapping the Spectrin-binding Site in KAHRP—To define the spectrin-binding site in KAHRP, three recombinant GST-tagged KAHRP polypeptides (K1, K2, and K3), which encompass the full-length of KAHRP, were purified and examined for their ability to bind the spectrin dimer using the pull-down assay. As shown in Fig. 3A, under the binding conditions used in these experiments only the GST-tagged K2 fragment was able to pull down the spectrin dimer. Spectrin dimer did not bind to GST, GST-tagged K1, or K3 fragments. To further define the spectrin binding region in K2 fragment, the binding
of spectrin dimer to subfragments of K2, K2A, and K2B, was examined. Interestingly, neither of them had the ability to bind spectrin dimer (Fig. 3B). Based on this finding, we reasoned that the spectrin-binding site in K2 fragment probably requires the C-terminal part of K2A and N-terminal part of K2B. To test this hypothesis, we constructed a fragment designated K2a+b, which is composed of 36 amino acids from the C-terminal of K2A and 36 amino acids from the N-terminal of K2B. As shown in Fig. 3B, in contrast to K2A and K2B, K2a+b did bind spectrin dimer. Thus, we localized a distinct spectrin-binding site in KAHRP to a 72-amino-acid region. This finding is in agreement with a previous report, which localized the spectrin-binding site in KAHRP to a 72-amino-acid region. This finding is in agreement with a previous report, which localized the spectrin-binding site in KAHRP to a 72-amino-acid region.

Mapping the KAHRP-binding Site in Spectrin—The same experimental approach was used to identify the KAHRP-binding site in the spectrin chains. Nine recombinant GST-tagged spectrin fragments encompassing the entire α and β spectrin chains were purified, and the binding of His-tagged K2 and His-tagged K2a+b to these fragments was examined. As shown in Fig. 4A, the His-tagged K2 fragment bound specifically to only one α spectrin fragment, αN-5 but not to any of the other eight spectrin fragments or to GST. Among the various structural elements that constitute the αN-5 fragment, K2 specifically bound to one single repeat, α4 (Fig. 4B). The K2a+b fragment exhibited the same behavior as the K2 fragment in that it specifically bound only the αN-5 fragment and the single repeat, α4 (data not shown). Thus among the 37 repeats of α and β spectrin, we have localized the KAHRP-binding site on spectrin to one single repeat, α4.

Interactions between KAHRP Fragments and Spectrin Fragments as Assessed by Surface Plasmon Resonance Assay—To further confirm and characterize the interactions between KAHRP fragments and spectrin polypeptides, real-time plasmon resonance assays were performed. In these experiments, His-tagged KAHRP fragment (K2 or K2a+b) was immobilized onto the surface of a sensor chip, and the binding of GST-tagged spectrin polypeptide (αN-5 or α4) was assessed. In agreement with the pull-down assay data, GST-αN-5 and GST-α4 bound to the immobilized K2 fragment, whereas GST itself did not show any detectable binding (Fig. 5A). The dose-dependent binding of αN-5 to K2 is shown in Fig. 5B. Table I summarizes the characteristics of interactions between spectrin polypeptides (αN-5, α4) and KAHRP fragments (K2, K2a+b). The binding affinities of all of these interactions are in the micromolar range, values comparable with those reported previously for various other protein-protein interactions in normal and infected erythrocytes (2).

Inhibition of Full-length Spectrin Binding to K2 Fragment by the Single Spectrin Repeat, α4—To confirm the specificity of the interaction between the single spectrin repeat α4 and KAHRP, we performed a competitive inhibition assay. In this experiment, the K2 fragment was preincubated with various concentrations of α4 prior to the addition of the full-length spectrin dimer. As shown in Fig. 6, the binding of spectrin dimer to the K2 fragment was decreased with progressively increasing concentrations of α4. At a concentration of 1 μM α4, spectrin binding to KAHRP was inhibited by 50%.

Effect of α4 on KAHRP Distribution and Localization in Infected Erythrocytes—To examine the functional implication of KAHRP-spectrin interaction, we examined the localization of KAHRP in infected erythrocytes that were resealed with excess amount of α4 polypeptide prior to infection with P. falciparum parasites. It was anticipated that the exogenously added α4 polypeptide would compete with native membrane-associated spectrin for interaction with KAHRP, which is expressed in the later stages of malaria infection. The location and distribution of KAHRP in the infected erythrocytes was monitored following 48 h of culture. As shown in Fig. 7A, KAHRP localized at the membrane in erythrocytes resealed with no added polypeptide as described previously (28, 29). A very similar localization of KAHRP was seen in erythrocytes resealed with GST prior to infection (Fig. 7B). Strikingly, in erythrocytes resealed with GST-α4, KAHRP was diffusely distributed in the cytoplasm of infected erythrocytes (Fig. 7C). Uninfected erythrocytes did not show any staining with anti-KAHRP antibody (data not shown).

**DISCUSSION**

In the present study, we performed a detailed molecular characterization of the interaction between the P. falciparum-encoded protein, KAHRP, and erythrocyte membrane skeletal protein, spectrin. A surprising feature of our finding is that only one unique repeat of a spectrin binding KAHRP. Importantly, when this spectrin repeat was resealed into erythrocytes it blocked the membrane assembly of KAHRP in infected erythrocytes. This finding implies that spectrin plays a key role in localizing KAHRP at the membrane of infected erythrocytes.

Knob-like, electron-dense protrusions located at the membrane surface of infected erythrocytes are localized at the points of adhesive interaction between infected erythrocytes and vascular endothelial cells (30). KAHRP is associated with these knob-like structures. Although PfEMP1, the adhesive receptor expressed on the surface of infected erythrocytes is the mediator of adhesive interactions, KAHRP plays an important
role in modulating the avidity of these interactions, presumably by clustering the receptors. Strong support for this thesis comes from the finding that in the absence of KAHRP, infected erythrocytes adhere very weakly to endothelial cells under physiological flow conditions (6). As adherence of infected erythrocytes to vascular endothelium is a major determinant of the pathogenicity of *P. falciparum* (11), reducing or eliminating adhesive interactions by interfering with the interaction of KAHRP with α spectrin is a potential therapeutic strategy to prevent serious complications of *P. falciparum* malaria.

Previous studies have identified interactions between a number of parasite proteins including RESA (31–33), MSP-1 (34), MESA (35), and PEMP1 (7) and erythrocyte membrane proteins. Two recent studies have suggested that over 400 parasite-encoded proteins are exported into erythrocyte cytoplasm (36, 37), and a number of these are likely to interact with membrane proteins. Most studies, to date, on the interactions of parasite proteins with erythrocyte membrane proteins have focused on defining the domains in malaria proteins that participate in these interactions. For example, the 4.1R-binding site in MESA has been localized to 19 residues located in the N-terminal region of MESA (35). The spectrin-binding domain in RESA has been mapped to a 48-residue region (33), whereas the spectrin-binding domain in MSP-1 has been localized to a 30-residue region (34).

A unique feature of many malaria proteins is the presence of extensive regions of tandemly repeated sequences (38). The functional role of these repeats is not clear. It is interesting to note that the binding regions of MESA, RESA and MSP-1 mentioned above are all found in non-repetitive domains (33–35). Previous work that mapped the spectrin-binding domain of KAHRP to a 271-residue region (27), and this region contained the 5′-repeat. However, in the present study we found that K2A, which is composed of the complete 5′-repeat and 21 amino acids downstream of this 5′-repeat sequence did not bind spectrin. This suggests that the repeat sequence in KAHRP is not involved in KAHRP binding to spectrin. The binding site of some sporozoite proteins for hepatocytes has also been mapped to non-repetitive regions (39, 40). Thus it appears that the repeat sequences of parasite proteins are probably not involved in association of malaria proteins with host proteins. Another common feature of binding motifs in malaria proteins for erythrocyte skeleton proteins is the relatively short length of these sequences. This reliance on short linear sequences may arise from the necessity of the exported malaria protein to interact with a preexisting network of proteins. However, the above considerations may not be relevant for malarial protein-protein interactions, as multiple binding regions in KAHRP have been identified to interact with PEMP1, and the binding regions do contain repetitive sequence (41).

In contrast to our detailed understanding of the binding motifs on malaria proteins much less is known regarding the binding motifs on host cell proteins. To date, only the MESA-binding site on 4.1R has been mapped to a 51-residue region encoded by exon 10 of the 4.1R gene (39). Spectrin is a long, rod-like molecule with a contour length of 200 nm. It has been traditionally thought that the major function of spectrin is to provide the structural basis for the flexibility of erythrocyte membranes because of its unique repetitive triple α-helical structure. The successful localization of the KAHRP binding region to a single repeat (of 37 repeats) of spectrin highlights the specificity of KAHRP-spectrin interaction. In fact, this is the first demonstration that a specific spectrin repeat could serve as a docking site for malaria proteins in infected erythrocytes. This finding further implies that spectrin may serve as a scaffold for assembly of malarial proteins in infected erythrocytes.

The most striking outcome of this study is the observation that resealing of α4 polypeptide into erythrocytes alters the localization of KAHRP in infected cells. This is probably because of the failure of interaction of KAHRP with membrane-associated spectrin in the presence of excess amount of the competitive polypeptide α4. Thus, binding of KAHRP to spectrin is required for its assembly onto the erythrocyte membrane skeleton. These findings suggest that molecules that are able to block the binding of KAHRP (or other malarial proteins) to spectrin (or other red cell membrane skeleton proteins) could provide a novel therapeutic approach to mitigate the severity of malaria.

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