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Plasmodium falciparum histidine-rich protein 1 associates with the band 3 binding domain of ankyrin in the infected red cell membrane

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Abstract

Infection of erythrocytes by the malaria parasite *Plasmodium falciparum* results in the export of several parasite proteins into the erythrocyte cytoplasm. Changes occur in the infected erythrocyte due to altered phosphorylation of proteins and to novel interactions between host and parasite proteins, particularly at the membrane skeleton. In erythrocytes, the spectrin based red cell membrane skeleton is linked to the erythrocyte plasma membrane through interactions of ankyrin with spectrin and band 3. Here we report an association between the *P. falciparum* histidine-rich protein (PfHRP1) and phosphorylated proteolytic fragments of red cell ankyrin. Immunochemical, biochemical and biophysical studies indicate that the 89 kDa band 3 binding domain and the 62 kDa spectrin-binding domain of ankyrin are co-precipitated by mAb 89 against PfHRP1, and that native and recombinant ankyrin fragments bind to the 5′ repeat region of PfHRP1. PfHRP1 is responsible for anchoring the parasite cytoadherence ligand to the erythrocyte membrane skeleton, and this additional interaction with ankyrin would strengthen the ability of PfEMP1 to resist shear stress. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Infection with *Plasmodium falciparum* is one of the leading causes of death worldwide. Over 500 million people are infected each year resulting in 2–3 million deaths annually. The asexual form of the parasite living within the red blood cell is responsible for the pathology and resulting morbidity and mortality associated with infection. Changes in membrane properties of infected red cells are secondary to the insertion of parasite proteins into the red cell mem-

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brane and through interaction of parasite proteins with red cell membrane proteins. Parasite proteins participate in a number of protein–protein interactions, both with proteins of the host red cell and with other parasite proteins. Interactions that have been mapped include those of the parasite protein MESA with protein 4.1 [1], of RESA with spectrin [2], of PfHRP1 with PfEMP1 [3], and of PfEMP1 with actin and spectrin [4].

One of the key processes involved in parasite virulence is sequestration, in which red cells containing mature parasites bind to receptors on endothelial cells of the deep vasculature. Receptors include CD36, ICAM-1, and thrombospondin [5]. The parasite ligand involved is PfEMP1 (*P. falciparum* erythrocyte membrane protein 1), a transmembrane protein with a 45 kDa cytoplasmic domain that mediates its interaction with the infected red cell membrane skeleton [5,6]. PfEMP1 has been localized to specific sites on the infected red cell surface, overlying a number of parasite-induced structures called knobs [7]. Knobs are electron-dense membrane protrusions with a diameter of ~100 nm that are present in about 5000 copies per infected cell [8,9]. One of the major components of the knob is the knob associated histidine rich protein (KAHRP or PfHRP1), an 80–108 kDa parasite protein with extensive repeat and histidine-rich regions. Several host proteins can be found in purified knobs [10,11], and among these, PfHRP1 has been shown to bind to spectrin and actin [12], as has PfEMP1 [4]. Thus the cytoplasmic domain of PfEMP1 binds to the spectrin-actin junction [4] and to PfHRP1 [3], which in turn binds to host red cell membrane skeletal proteins to anchor PfEMP1 to the membrane skeleton of the infected red cell. This anchoring is crucial to parasite virulence, and in parasites from which PfHRP1 is missing, PfEMP1 mediated binding is either completely absent [13] or is decreased such that it is unable to occur under flow conditions equivalent to those found in vivo [14].

Another important alteration in the infected red cell is in the levels of phosphorylation of host red cell proteins, as well as the appearance of parasite phosphoproteins at the red cell membrane skeleton [1,15–17]. These induced changes in protein phosphorylation may modulate the membrane mechanical properties of the infected red cell [18]. MESA (the mature-parasite-infected red cell surface antigen, also called PfEMP2) is a phosphoprotein of parasite origin that is exported to the red cell membrane skeleton where it binds to protein 4.1 [1,16,19]. We have recently shown that the presence of knobs and thus the expression of PfHRP1 can influence phosphorylation of protein 4.1 and MESA, and/or expression of the kinase(s) for which these proteins are substrates in infected red cells [20].

In the present study, we detected a red cell phosphoprotein of ~89 kDa in infected cells. This protein is clearly distinct from protein 4.1. Our studies indicate that this protein is the 89 kDa Band 3 binding domain of ankyrin which is phosphorylated in infected red cells, and that it is involved in non-covalent interaction with the *P. falciparum* knob protein PfHRP1. This is the first report of an association between erythrocyte ankyrin and PfHRP1.

2. Materials and methods

2.1. Parasites

K+ Malayan Camp parasites [10], as well as three K+ and K− paired parasite lines were used in these experiments. K+ D10 and K− E12 were derived from the FC-27 isolate [21]. K+ D63 and K− D65 were derived from the D6 clone [22–24]. K+ P37 [24] and K− B8B6 [25] were derived from the ItG2F6 clone. Infected red cells were cultured at 2% hematocrit in RPMI 1640 supplemented with 10% human serum or 0.5% Albumax II as previously described [26]. Knob phenotype was confirmed by indirect immunofluorescence as previously described [19] and, in some cases, by electron microscopy (B.M. Cooke, Monash University, Australia, personal communication).

2.2. Protein 4.1-deficient erythrocytes

Protein 4.1-deficient red cells, obtained with informed consent from two different donors, have been described previously [16,19,27]. The red cells were washed, cryopreserved and thawed when needed as previously described [19].

2.3. Preparation of ankyrin

Human erythrocyte ankyrin and its fragments, the 89 kDa band 3 binding domain, its 43 kDa subfragment, and the 62 kDa spectrin binding domain, were prepared as previously described [28,29].

2.4. Antibodies

Mouse monoclonal antibodies (mAbs) to MESA (mAb Pf8b4.7) and to PfHRP1 (mAb 89) were kindly provided by Drs Jeffrey Lyon (Walter Reed Army Institute of Research, Washington, DC) and Diane Taylor (Georgetown University, Washington, DC) and have been previously described [30,31]. Antibody to protein 4.1 has been previously described [20].

Antibodies to human heat shock proteins (hsp) 84 and 86 were kindly provided by Dr Shyamala Harris (Lawrence Berkeley National Laboratory, Berkeley, CA). Antibody to *P. falciparum* hsp 70 was generated against a recombinant protein expressing the C-terminal half of the protein [32]. Antibodies to spectrin and protein kinase C were purchased from Sigma Chemical Co. (St. Louis, MO). Affinity purified rabbit antibody to the band 3 binding domain of human ankyrin (termed 'anti-ANK' in this paper) was prepared at Purdue University. For use in some experiments, the anti-ANK antibody was absorbed three times on ice and once overnight with inside-out-vesicles that were prepared from normal human red blood cells as previously described [33]. The resulting antibody was reacted with infected and uninfected red blood cells in indirect immunofluorescence, as previously described [19], and immunoprecipitation analyses to determine whether the antibody's activity was against erythrocyte ankyrin or a parasite protein.

2.5. Preparations of recombinant proteins

All recombinant proteins were expressed in *Escherichia coli* [34,35]. The full-length PfHRP1 (second exon) was amplified by PCR from genomic *P. falciparum* D10 DNA, cloned into the pMAL-c2 vector (New England BioLabs, Beverly, MA), and expressed as a fusion of maltose binding protein. Recombinant PfHRP1 fragments [3] were produced as glutathione *S*-transferase (GST) fusion proteins. The minigenes were constructed in pGEX-KG vector. The expression and purification of GST fusion proteins has been described previously [34,35]. The recombinant 43 kDa fragment of ankyrin was cloned into pGEMEX vector (a kind gift from Vann Bennett, Duke University) that has been previously described [28]. The 43 kDa fragment of ankyrin was purified from a bacteria lysate as previously reported [36,37]. The purity of the recombinant proteins was assessed by analysis with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE, 15% gel). The protein concentration was determined as previously reported [34,35].

2.6. Binding assay by resonant mirror detection

Protein–protein interactions were studied using the resonant mirror detection method [38,39] of the IAsys system (Affinity Sensors, Cambridge, UK). The immobilization of proteins to the aminosilane cuvette has been previously described [34,35]. All the binding assays were carried out in phosphate-buffered saline (PBS/T: 10 mM Na_2HPO_4/NaH_2PO_4 (pH 7.4), containing 0.15 M NaCl and 0.05% Tween-20) at 25°C under constant stirring.

The dissociation constant (termed $K_{(D)kin}$) was then calculated as $K_{(D)kin} = k_d/k_a$, where k_a is the association rate constant, and k_d is the dissociation rate constant [34,35]. The cuvettes were reused after cleaning with HCl. Original binding curves could be replicated after HCl washes, implying that the washing procedure did not denature the bound ligands.

2.7. Phosphorylation of infected and uninfected erythrocytes

Infected and uninfected red cells were labeled as previously described [20]. Briefly, infected red cells were incubated for 4–8 h with 250 μCi ml^{-1} $^{32}PO_4$ in glutamine- and phosphate-free MEM supplemented with 10% human serum dialyzed against 0.15 M NaCl. Because membrane proteins in uninfected red cells are not heavily phosphorylated [1,16], uninfected red cells were incubated with 4-β-phorbol 12-myristate (PMA, Sigma) at 40% hematocrit in the presence of 1 mCi $^{32}PO_4$ to maximize phosphoryla-

tion of various red cell membrane proteins as previously described [20].

2.8. Biosynthetic labeling

[^{3}H]Histidine and [^{3}H]amino acid mixture were obtained from Amersham (Arlington Heights, IL). [^{35}S]Methionine (Express protein labeling mix) was obtained from NEN Research Products (Boston, MA). Isotopes were added at 250 μCi ml^{-1} to RPMI 1640 Select-Amine media deficient in the appropriate amino acid(s) (Gibco BRL, Gaithersburg, MD). Parasites were cultured from ring stage to trophozoites and immediately extracted for immunoprecipitation at a ratio of 2.5×10^8 infected red cells ml^{-1} of extraction buffer.

2.9. Immunoprecipitation, SDS–PAGE and Western blot analysis

These analyses were carried out as previously described [20]. The Triton X-100-insoluble fraction of protein 4.1-deficient human red cell lysates, or infected red cell lysates, was extracted in 1% SDS to extract non-ionic proteins. For immunoprecipitation, excess Triton X-100 was added before addition of antibody to avoid denaturation by SDS of the antibody [10,23,40,41]. Cellular extracts were precleared with Protein A sepharose beads before the addition of antibody, and 0.35 M NaCl was included in one of the washes of immunoprecipitated Protein A beads to reduce non-specific binding. For Western blots, extracts were directly loaded on 15% SDS–polyacrylamide gels (PAGE). Equal amounts of infected red cell extract were loaded in each experiment. Proteins were separated by SDS–PAGE and either autoradiographed or transferred to nitrocellulose for Western blots. Blots were probed with antibody and reactions were visualized by chemiluminescence using the Renaissance system (Dupont NEN, Boston, MA).

3. Results

3.1. Two ~89 kDa phosphoproteins are detected in infected erythrocytes

P. falciparum-infected red cells were cultured in the presence of [^{32}P]orthophosphate which results in labeling of phosphoproteins of both host and parasite origin. A ^{32}P-labeled band of ~89 kDa was detected in Triton X-100-insoluble extracts of normal red blood cells infected with various cloned parasite lines. As in previous studies, both polyclonal and monoclonal antibody to MESA ($M_r > 250$ kDa) coprecipitated this phosphoprotein from K+ and K− infected red blood cells [1,20]. That this phosphoprotein is protein 4.1 has previously been shown by one- and two-dimensional peptide mapping and by immunoprecipitation with an antibody specific to protein 4.1 [1,16,20,42].

A ^{32}P-labeled band with similar apparent mobility was detected in Triton X-100-insoluble extracts of protein 4.1-deficient infected red cells, indicating that in addition to protein 4.1 there was a second

Table 1
PfHRP1 binding to ankyrin

Ankyrin	PfHRP1[a]	k_a (M^{-1} s^{-1})	k_d (s^{-1})	$K_{(D)kin}$ (μM)
Band 3 binding domain				
89 kDa	Full length	$7.0\pm0.1\times10^3$	$1.3\pm0.1\times10^{-2}$	1.8
	K2A	$6.8\pm0.3\times10^3$	$1.6\pm0.1\times10^{-2}$	2.4
43 kDa	Full length	$1.5\pm0.1\times10^4$	$2.0\pm0.3\times10^{-2}$	1.3
	K2A	$2.2\pm0.2\times10^4$	$6.7\pm0.3\times10^{-2}$	3.0
Spectrin binding domain				
62 kDa	Full length	$2.9\pm0.1\times10^3$	$1.4\pm0.2\times10^{-2}$	4.8
	K2A	$3.5\pm0.1\times10^3$	$2.9\pm0.2\times10^{-2}$	8.3

The binding assay was carried out in PBS containing 0.05% Tween-20. From the binding curves obtained by the resonant mirror detection method, k_a, k_d, and $K_{(D)kin}$ were determined using the software package FAST-fit.
[a]Full-length PfHRP1, and the K2A region of this parasite protein, bind to fragments of human erythrocyte ankyrin.

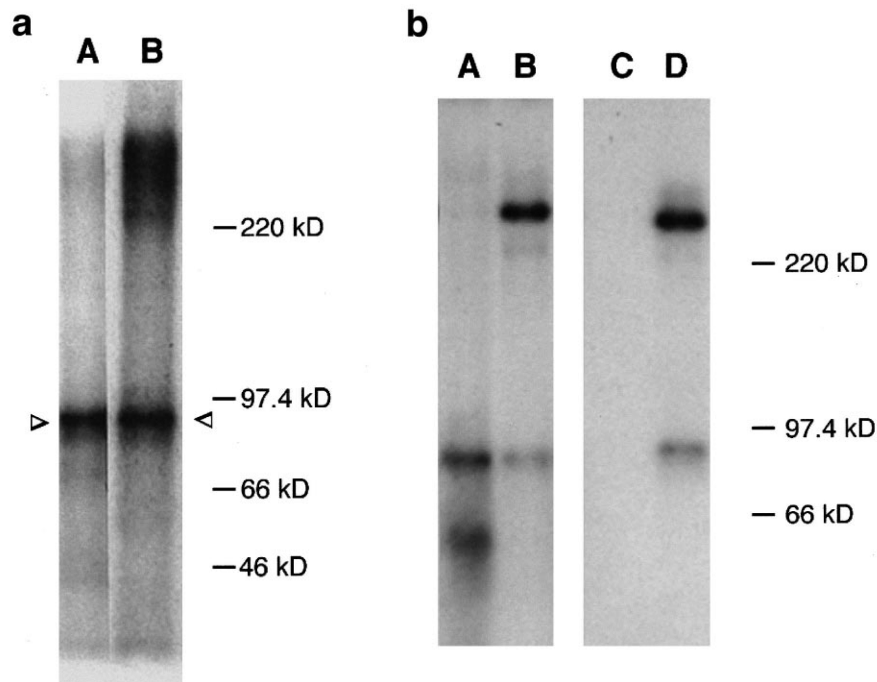

Fig. 1. (a) Autoradiographs of SDS–PAGE gels of Triton X-100-insoluble extracts of ^{32}P-labeled infected normal red blood cells (lane A) and infected protein 4.1-deficient red blood cells (lane B). Extracts from equal amounts of red cells were loaded in each lane. A phosphoprotein that migrates at a M_r similar to protein 4.1 (∼89 kDa) is present in protein 4.1-deficient red cells (arrows). (b) Autoradiograph of SDS–PAGE gels of Triton X-100-insoluble extracts of ^{32}P-labeled red cells infected with K+ parasite line FC27/D10 immunoprecipitated with anti-PfHRP1 mAb 89 (lane A) or anti-MESA antibody (lane B). Triton X-100 extracts of ^{32}P-labeled red cells infected with K− parasite line FC27/E12 immunoprecipitated with anti-PfHRP1 mAb 89 (lane C) or anti-MESA antibody (lane D). Extracts from equal amounts of red cells were loaded in each lane. Note that mAb 89 did not coprecipitate the second phosphoprotein in K− infected red cells (lanes A compared with C), whereas anti-MESA antibody coprecipitated protein 4.1 in both K+ and K− infected cells (lanes B compared with D).

phosphoprotein of ∼89 kDa present in infected red cells (Fig. 1a). This second phosphoprotein was detected in infected protein 4.1-deficient red blood cells from two different donors [16,19,27]. As expected, antibodies directed to protein 4.1 failed to recognize an antigen from these mutant red cells in the 89 kDa region by Western blot or by immunoprecipitation. Moreover, anti-MESA antibodies did not coprecipitate this band from protein 4.1-deficient cells. Thus, this second 89 kDa phosphoprotein does not possess the immunochemical properties of protein 4.1, and is not associated with MESA.

3.2. *Novel phosphoprotein is recognized by mAb to PfHRP1*

Based on the size and detergent solubility properties of this phosphoprotein, we suspected it might be PfHRP1, although PfHRP1 has not previously been reported to be a phosphoprotein. mAb 89 against parasite PfHRP1 immunoprecipitated an ∼89 kDa phosphorylated protein from extracts of several K+ parasites but not from extracts of K− parasites (Fig. 1b). On the other hand, anti-MESA antibody, which has been shown to coprecipitate protein 4.1, immunoprecipitated an 89 kDa phosphoprotein from Triton X-100-insoluble extracts of both K+ and K− parasites (Fig. 1b).

PfHRP1 is a protein that varies in size in different isolates due to differences in the number of repeats within the protein [43]. When immunoprecipitation studies using mAb 89 were performed with [^{3}H]histidine biosynthetically labeled parasite extracts and ^{32}P-labeled infected red cell extracts of the same lines, the precipitated phosphoprotein did not show the same pattern of variation in size seen with the biosynthetically labeled PfHRP1. Instead it was found to be constant in size (∼89 kDa) in all

isolates examined (Fig. 2). Thus, the 89 kDa protein is a phosphoprotein that is co-precipitated with PfHRP1 by mAb 89 and thus presumably binds to PfHRP1 in both normal and protein 4.1-deficient infected red blood cells.

3.3. *Antibody to 43 kDa ankyrin fragment immunoprecipitates the ∼89 kDa phosphoprotein*

Antibodies against several proteins in this molecular mass range of both malarial and host origin were used for immunoprecipitation and Western blot analysis. Antibodies directed against protein kinase C, *P. falciparum* hsp 70, human hsp 84 and 86, and spectrin failed to recognize a phosphoprotein of ∼89 kDa in Triton X-100-insoluble extracts. However, anti-ANK antibody against the ankyrin repeats 13–24 (subdomains 3 and 4) of the 89 kDa band 3 binding domain immunoprecipitated a phosphoprotein which comigrated with both protein 4.1 and the protein immunoprecipitated by mAb 89 (Fig. 3). The intensity of phosphorylation of the polypeptide recognized by anti-ANK is not as strong as the intensity of phosphorylation of the polypeptide coprecipitated by anti-PfHRP1. This might result from increased phosphorylation of the ankyrin fragment in association with PfHRP1, or an increased association of PfHRP1 with a subpopulation of more heavily phosphorylated ankyrin fragments.

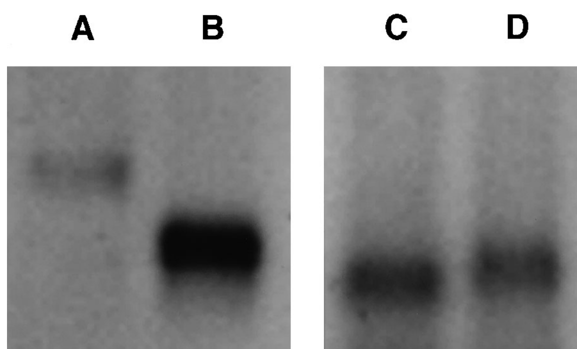

Fig. 2. Autoradiograph of SDS–PAGE gels of Triton X-100-insoluble extracts immunoprecipitated with anti-PfHRP1 mAb 89. Compare the migration of [^{3}H]histidine biosynthetically labeled parasite lines D63 (lane A) and Malayan Camp (lane B) with ^{32}P-labeled phosphoprotein in D63 (lane C) and Malayan Camp (lane D). Note the size polymorphism of PfHRP1 in lanes A and B, and the lack thereof for the second phosphoprotein in lanes C and D. Extracts from equal amounts of red cells were loaded in each lane.

3.4. *Novel phosphoprotein is of red cell origin*

To determine whether the anti-ANK antibody was reacting with red cell ankyrin or a malarial ankyrin-like protein [44], efforts were made to biosynthetically label the protein. [^{3}H]histidine, [^{3}H]amino acid mixture and [^{35}S]methionine failed to label a protein in the 89 kDa region that was immunoprecipitated by anti-ANK antibody in either K+ or K− infected red cells. Further evidence that the phosphoprotein is of red cell origin was provided by absorbing anti-ANK antibody with inside-out vesicles generated from uninfected red cells and showing that this depleted antibody failed to recognize the phosphoprotein in infected red cell extracts by immunoprecipitation, and did not react by indirect immunofluorescence with the membranes of either infected or uninfected red blood cells. Taken together, these data offer evidence that the phosphoprotein we identified is of red cell origin.

3.5. *Biophysical assays demonstrate binding of ankyrin and PfHRP1*

We went on to examine the PfHRP1-ankyrin fragment binding in detail. To do this, we generated full-length PfHRP1, and a number of purified native and recombinant fragments of PfHRP1 and ankyrin. The IAsys system was used to determine the affinity of binding between these proteins. Binding studies of full-length and various regions of PfHRP1, and the 89 kDa fragment of ankyrin, indicated that the 89 kDa ankyrin fragment bound specifically with the 5′ repeat region of PfHRP1, region K2A. Deletion of the 5′ repeat region K2 [3] resulted in loss of binding (data not shown). Similar results were obtained using the smaller recombinant 43 kDa subfragment of ankyrin instead of the 89 kDa ankyrin fragment. Table 1 shows the $K_{(D)kin}$ values of full-length PfHRP1 and the K2A region, and the fragments of ankyrin. The $K_{(D)kin}$ of binding between full-length PfHRP1 and the 89 kDa and the 43 kDa ankyrin fragments were 1.8 and 1.3 μM, respectively. The $K_{(D)kin}$ of binding between the PfHRP1 region K2A and the 89 kDa and 43 kDa fragments were 2.4 and 3.0 μM, respectively. Both the association rate constant (k_a) and the dissociation rate constant (k_d) of K2A binding with the 43 kDa ankyrin fragment were about three

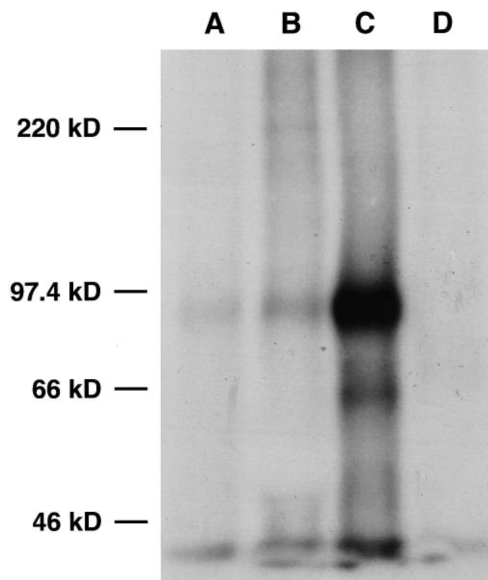

Fig. 3. Autoradiograph of SDS–PAGE gels of Triton X-100-insoluble extracts of ^{32}P-labeled red cells infected with K+ parasite line D10 immunoprecipitated with anti-protein 4.1 (lane A), anti-ANK (lane B), anti-PfHRP1 (mAb 89) (lane C), and anti-heat shock protein (lane D). Note that the phosphoproteins in lanes A–C migrate very similarly. Extracts from equal amounts of red cells were loaded in each lane.

times higher than K2A binding with the 89 kDa fragment.

A second phosphoprotein that shared the biochemical characteristics of the 89 kDa fragment was also coprecipitated by anti-PfHRP1 mAb 89 (Fig. 1b, lane A and Fig. 3, lane C). We suspected this might be the 62 kDa spectrin binding domain of ankyrin. Therefore, we also examined binding of this domain, derived from native ankyrin, to PfHRP1. The 62 kDa fragment also specifically bound to PfHRP1 region K2A with a $K_{(D)kin}$ of 8.3 μM, and to full-length PfHRP1 with a $K_{(D)kin}$ of 4.8 μM (Table 1). These experiments used native or recombinant fragments of ankyrin that were not phosphorylated. The repeat region of PfHRP1 K2A contains several basic amino acid clusters (e.g., KKKKSKKHK), and it may be that phosphorylated ankyrin fragments would bind to the K2A region with higher affinity.

4. Discussion

In the present study, we have shown that two phosphorylated proteins of ~89 kDa are present in *P. falciparum*-infected red cells. As previously documented, one of these proteins is red cell protein 4.1, which is more heavily phosphorylated in infected red cells compared to uninfected red cells and is bound by the parasite protein MESA. The second phosphorylated protein, identified in the present study in both normal and 4.1-deficient red cells, is the 89 kDa band 3 binding domain of ankyrin. It was coprecipitated by anti-PfHRP1 antibody but not by anti-MESA antibodies. It was detected by anti-ANK antibody in both K+ and K− infected red cells but was coprecipitated by antibody against PfHRP1 only from K+ infected red blood cells, suggesting a non-covalent association with PfHRP1.

Although red cells contain about 200 000 copies of ankyrin, as few as 5000 knobs are induced on the infected red cell membrane. The number of copies of PfHRP1 per knob is unknown, as is the fraction of ankyrin that is associated with PfHRP1. However, a phosphorylated fragment of ankyrin, even in low abundance, might significantly alter properties of the infected red cell membrane, particularly locally at the knob. Possible effects include changes in the adhesive properties of the erythrocyte through activation of cryptic red cell adhesion proteins that are themselves present in only a few thousand copies per cell. Modified band 3 has been suggested to be a cytoadherence ligand in infected cells [40]. Whether phosphorylation and binding of the band 3 binding domain of ankyrin to PfHRP1 leads to changes in the distribution and/or mobility of band 3 that would affect cytoadherence remains to be elucidated.

A 62 kDa phosphoprotein that shared the biochemical and immunochemical characteristics of the 89 kDa ankyrin fragment was also coprecipitated by anti-PfHRP1 antibodies. Data from our in vitro binding studies confirm that both the 89 kDa band 3 binding domain and the 62 kDa spectrin binding domain of ankyrin bind specifically to the K2A region of PfHRP1. Thus the 62 kDa spectrin binding domain of ankyrin appears to be an additional binding site on the ankyrin molecule for PfHRP1, which has also been shown to bind to spectrin and actin [12].

Ankyrins constitute a family of proteins that appear to serve a general role in coupling diverse integral proteins to the spectrin–actin network [45]. Erythrocyte ankyrin is a structural protein that links

the spectrin based red cell membrane skeleton to the red cell plasma membrane through interactions with band 3 and spectrin [46]. It is comprised of an 89 kDa domain that binds to the cytoplasmic domain of band 3, a 62 kDa spectrin-binding domain, and a 30–55 kDa regulatory domain [36]. These red cell protein associations may be regulated by phosphorylation which generally reduces a red cell protein's affinity for other membrane proteins [18,47–52]. Ankyrin and band 3 are substrates for membrane-associated and cytosolic casein kinases [50,53,54]. Casein kinase phosphorylation affects the interaction of ankyrin with the cytoplasmic domain of band 3 by decreasing the stoichiometry but not the affinity of ankyrin binding [48,52], whereas casein kinase phosphorylation of ankyrin decreases its binding affinity for spectrin [50,52].

The kinase responsible for phosphorylation of ankyrin that is associated with PfHRP1 is unknown. We note, however, that in addition to red cell kinases, a casein kinase of apparent parasite origin has been reported [16], as has a serine–threonine kinase the parasite exports to the red cell membrane skeleton [55], although the role either of these kinases plays in this process is yet to be determined. The results of the present study show that the phosphorylated 89 kDa band 3 binding domain of ankyrin is associated with PfHRP1. In a previous study, we have shown in infected red cells that expression of PfHRP1 influences phosphorylation, by a casein kinase, of two other membrane-associated proteins, the parasite protein MESA, as well as the red cell membrane protein 4.1 [20]. We suggest that PfHRP1, in addition to the role it plays in knob formation and cytoadherence [14], may have a role in pathways that affect phosphorylation of several membrane proteins in the infected red cell.

Proteolysis also may be utilized by malarial parasites in the extensive restructuring of the infected red cell membrane that occurs during the association of PfHRP1 and other malarial proteins with red cell skeletal proteins. There are several reports of proteolytic digestion of spectrin in infected red cells [56–58]. Cleavage of ankyrin and association of the band 3 and spectrin binding domains with PfHRP1 may allow reorganization of cytoskeletal structures to suit the parasite's needs. Recently Hanspal et al. detected a truncated 155 kDa fragment of ankyrin in soluble cytosolic extracts of trophozoite/schizont-infected erythrocytes cleaved within the regulatory domain by a cysteine protease [59]. Additional cleavage steps would be required to generate the smaller ankyrin fragments we recognized in association with PfHRP1, although the cleavage mechanism is as yet not known.

The specific binding region of PfHRP1 for the 89 kDa and the 62 kDa ankyrin fragments has been localized to the K2A fragment that encompasses the 5′ repeat region of PfHRP1. Removal of the 5′ repeats resulted in loss of ankyrin binding, suggesting that the repeats were the binding site. These repeats consist of five copies of a 13–16 residue region rich in lysine and glutamic acid [43]. This is the second binding interaction localized to this region, as it has already been demonstrated that the cytoplasmic tail of PfEMP1 is strongly bound by this region [3]. The binding constants involved are very similar with the $K_{(D)kin}$ of K2A binding to ankyrin being 2.4 μM and for PfEMP1 being 3.3 μM [3]. It is not clear whether these two molecules compete against each other for binding to K2A, and what the effect of this might be. This would not necessarily destabilize the PfHRPI–PfEMPI interaction, as there are three distinct binding sites on PfHRP1 for this protein, one with a $K_{(D)kin}$ of 0.1 μM. However, these interactions could lead to formation of an aggregated protein complex through the operation of multiple intermolecular associations. K2A partially overlaps the region of PfHRPI that has been reported to bind to spectrin [12], and recently published work shows that the cytoplasmic tail of PfEMP1 also binds to the spectrin–actin junction in vitro [4], thus further emphasizing the complex network of protein–protein interactions formed at the knobs of the infected red blood cell.

This study presents new information about an interaction between the parasite protein PfHRP1 and erythrocyte ankyrin that would appear to play an indirect role in anchoring PfEMP1 to the membrane skeleton of the infected cell. It seems that PfHRP1 participates in a number of interactions that anchor it to the membrane skeleton including those involving spectrin and actin that have been previously reported [12]. The combination of these interactions is likely to be extremely strong, thus providing a secure base to which PfEMP1 can attach. Further, this would allow transmission to the red cell skeleton of

stresses experienced by PfEMP1 as it mediates adherence in vivo, under flow, in the microvasculature. Approaches that interfere with the anchoring of PfEMP1 to PfHRP1, or PfHRP1 to the membrane skeleton, would weaken the ability of infected red cells to adhere. This may have the effect of ameliorating the severity of malaria infection, and may provide a novel chemotherapeutic approach to controlling this disease.

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