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Primary structure and subcellular localization of the knob-associated histidine-rich protein of *Plasmodium falciparum*

(malaria/cDNA/tandem repeat/*Escherichia coli* overexpression/cytoadherence)

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ABSTRACT **_Plasmodium falciparum_-infected erythrocytes bind to venular endothelial cells by means of electron-dense deformations (knobs) on the parasitized erythrocyte surface. The primary structure of a parasite-derived histidine-rich protein associated with the knob structure was deduced from cDNA sequence analysis. The 634 amino acid sequence is rich in lysine and histidine and contains three distinct, tandemly repeated domains. Indirect immunofluorescence, using affinity-purified monospecific antibodies directed against recombinant protein synthesized in *Escherichia coli*, localized the knob-associated histidine-rich protein to the membrane of knobby infected erythrocytes. Immunoelectron microscopy established that the protein is clustered on the cytoplasmic side of the erythrocyte membrane and is associated with the electron-dense knobs. A role for this histidine-rich protein in knob structure and cytoadherence is suggested based upon these data.**

Plasmodium falciparum is the most virulent of the four species causing human malaria. Erythrocytes infected with *P. falciparum* develop electron-dense protrusions (knobs) on their plasma membranes during the asexual intraerythrocytic cycle of the parasite (1). The knobs are associated with binding of the parasitized erythrocytes to the venular endothelium (2). This interaction, termed sequestration, maintains the parasites in a low-Po_2 environment, which may favor their development (3) and may also protect the parasites from the filtering action of the spleen (4). The knobby phenotype (knob$^+$) may therefore be partially responsible for the high parasitemias and the hypoxia associated with *P. falciparum* malaria and may also contribute to the pathophysiology of cerebral malaria.

The knob structure is necessary but not sufficient for *P. falciparum*-infected erythrocytes to bind to endothelial cells. Under *in vitro* culture conditions parasites often maintain the capacity to cause knob formation on the infected host cell while losing the ability to mediate cytoadherence (5). Several parasite- and host-derived molecules have been implicated in the cytoadherence reaction, including a high molecular weight strain variant parasite protein localized to the erythrocyte surface (6, 7), a M_r 88,000 endothelial cell surface molecule defined by the OKM5 antibody (8), and human thrombospondin (9). However, the parasite and host molecules responsible for the ultrastructural morphology of the knobs on the erythrocyte membrane are not known.

Expression of a M_r 80,000–90,000 parasite-derived histidine-rich protein has been correlated with the presence of knobs and sequestration (10, 11). This protein has been found to be enriched in membrane preparations of infected erythrocytes that contain knobs (12). It is synthesized in the ring stage of intraerythrocytic development of the parasite and accumulates in infected erythrocyte membranes later, paralleling the appearance of knobs in the trophozoite and schizont developmental stages (13). Spontaneous mutations of knob$^+$ parasites have been described that do not cause knob formation (knob$^-$), do not mediate cytoadherence, and do not express the knob-associated histidine-rich protein (KAHRP) (14). Reintroduction of these knob$^-$ variants *in vivo* resulted in rapid clearance by the spleen and nonproductive infections (15), consistent with a role for the knob in evading splenic defenses. The genetic basis for the loss of knob expression in these knob$^-$ variants was shown to include a chromosome rearrangement and a deletion in the coding sequence of the KAHRP gene resulting in a telomeric location for the truncated gene (16).

To investigate the role of the KAHRP in knob formation and sequestration, we have focused on defining the structure of the KAHRP and determining its function in the knob$^+$ phenotype. We isolated and characterized *P. falciparum* cDNA clones from a knob$^+$ strain; the cDNA sequence‡ encodes a 634 amino acid sequence consisting of a hydrophobic signal peptide, a potential transient propeptide, and a mature protein that contains a domain of polyhistidine tracts of from six to nine contiguous residues. Two additional repeated-sequence domains are present in the lysine-rich, carboxyl-terminal half of the predicted protein. Immunization of rabbits with a recombinant protein expressed in *Escherichia coli* resulted in high-titer polyclonal, monospecific antiserum, which was used to localize the protein to the infected erythrocyte membrane by immunofluorescence. Immunoelectron microscopy revealed the KAHRP to be associated with the knobs on the cytoplasmic side of the erythrocyte membrane.

MATERIALS AND METHODS

Parasites. FCR-3 is a nonclonal Gambian line of *P. falciparum* established by Jensen and Trager (17). FVO parasites were kindly provided by R. Reese. FVO$^+$ is a knob$^+$ line cloned from a Vietnam isolate and FVO$^-$ is a knob$^-$ clone derived from it (11). All parasites were maintained in synchronous culture as described (4, 18, 19).

cDNA Clones and DNA Sequencing. cDNA clones for the KAHRP of *P. falciparum* strain FCR-3 were isolated as described (16). DNA sequencing was performed by the chemical degradation method of Maxam and Gilbert (20) and by the dideoxy chain-terminating method (21).

Overexpression of KAHRP Peptides in *E. coli* and Antipeptide Antibody Preparations. A DNA fragment beginning at the

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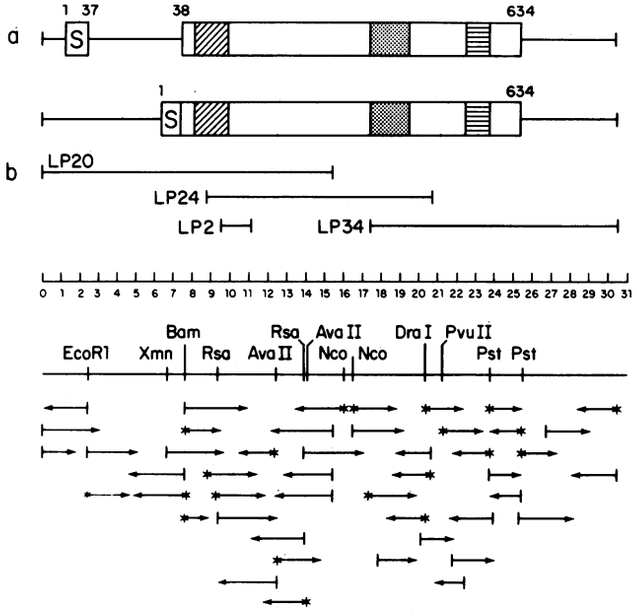
Abbreviations: HRP, histidine-rich protein; KAHRP, knob-associated HRP; knob$^+$, knob-inducing phenotype; knob$^-$, knobless phenotype.

‡This sequence is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J02972).



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*Bam*HI site within the coding sequence of the KAHRP gene (nucleotide 764, Fig. 1 *a* and *c*) and extending 3′ to nucleotide 1548 (see Fig. 1*c*) was fused in-frame to the coding sequence of the amino-terminal 81 amino acids of the influenza virus NS1 nonstructural protein (KAHRP–NS1 fusion) in the plasmid vector pB4$^+$ and also to the amino-terminal 3 amino acids of the NS1 protein (KAHRP nonfusion) in the related plasmid vector pT17 (22, 23). Overexpression and isolation of the KAHRP–NS1 fusion and KAHRP nonfusion peptides from temperature-induced AR58 (*c*Its857) lysates and immunization of rabbits were as described (24). Specific antibodies were affinity-purified from immune serum by chromatography on KAHRP–NS1 coupled to CNBr-activated Sepharose 4B according to the manufacturer's specifications (Pharmacia).

Immunoblot Analysis. The native KAHRP protein was identified in NaDodSO$_4$-soluble extracts of FVO$^+$ and FVO$^-$ schizont-infected erythrocytes by immunoblot analysis using a 1:200 dilution of the affinity-purified rabbit antiserum to the KAHRP–NS1 fusion peptide followed by a 1:1000 dilution of ^{125}I-labeled protein A (specific activity 9 μCi/μg; 1 Ci = 37 GBq) as described (24).

Immunolocalization of the KAHRP. Indirect immunofluorescence was performed on acetone-fixed parasites, using a 1:100 dilution of affinity-purified anti-KAHRP antibodies followed by a 1:100 dilution of affinity-purified fluorescein-conjugated goat anti-rabbit antibodies (Boehringer Mannheim) (25). For immunoelectron microscopy, FCR-3 schizonts were fixed in 0.25% glutaraldehyde, dehydrated in graded ethanol solutions, and embedded in LR white resin (Fullam, Schenectady, NY). The same antibodies, at a 1:1 dilution in phosphate-buffered saline containing 0.1% bovine serum albumin, were used to stain the samples, followed by protein A bound to 5-nm gold particles. Some sections were counterstained with uranyl acetate.

RESULTS

The complete amino acid sequence for the KAHRP of *P. falciparum* was deduced from cDNA sequence analysis. Fig.

FIG. 1. Primary structure analysis of the KAHRP. (*a*) Genomic and cDNA sequence organization. Residue numbers above the genomic map indicate initiating methionine, residues preceding and following the intron, and the carboxyl-terminal residue. The protein is divided into a signal sequence (S), a hydrophilic sequence, a histidine-rich sequence (hatching), and a charged hydrophilic sequence. Two types of repeat domains within the charged region are indicated by the stippled region and the horizontal lines. The 5′ and 3′ untranslated sequences are indicated in the schematic by a single line. (*b*) Sequencing strategy for the KAHRP cDNA. Overlapping cDNA clones 20, 24, 2, and 34 were sequenced. Scale shows length in hundreds of base pairs. DNA fragments indicated by arrows originating at vertical lines were sequenced by the dideoxy chain-termination method, and DNA fragments indicated by arrows originating at stars were sequenced by the chemical degradation method. The entire coding sequence, except for the 48-base-pair *Nco* I fragment, was sequenced by the chemical degradation method in order to avoid the possible introduction of deletions and insertions upon subcloning into M13 vectors. The sequences obtained with the dideoxy method generally used the original cDNA plasmid templates and synthetic oligonucleotide primers. (*c*) Nucleotide sequence of the cDNA and predicted amino acid sequence of the KAHRP. Three thousand forty-seven nucleotides have been determined. The predicted amino acid sequence (standard one-letter symbols) is given above the nucleotide sequence, beginning with the initiating methionine at position 638 in the cDNA sequence. The signal sequence is overlined. The arrow represents the proposed signal-peptidase cleavage site. A potential recognition sequence for attachment of Asn-linked carbohydrate is boxed. An open triangle indicates a second possible processing site. The polyhistidine sequence and the two carboxyl-terminal repeat domains are boxed.

1 *a* and *b* show a composite map derived from four overlapping cDNA clones containing representative restriction sites that were used for sequencing and also shows the sequencing strategy. Electrophoretic comigration of multiple genomic restriction fragments with corresponding fragments in the cDNA clones indicated that from the *Eco*RI site to the *Xmn* I site and from the *Bam*HI site to a *Dra* I site (428 base pairs from the 3′ end of the cDNA), there are no discernible insertions or deletions of sequence relative to genomic DNA (data not shown). However, restriction fragments from cDNA clones containing the *Xmn* I–*Bam*HI sites do not comigrate with their corresponding genomic DNA fragment. The genomic *Xmn* I–*Bam*HI fragment migrates as a 600-base-pair fragment, compared to an 88-base-pair sequence in the cDNA. Nucleotide sequence analysis of genomic clones (data not shown) demonstrates the presence of an intron occurring between nucleotides 748 and 749 in the cDNA (Fig. 1*a*). The genomic sequence contains splice donor and acceptor sequences similar to those seen in the gene encoding the histidine-rich protein of *Plasmodium lophurae* (26). The discontinuity in the cDNA occurs between the coding sequence for amino acids 37 and 38 (Fig. 1*a*), 3 amino acids to the carboxyl side of the predicted signal-peptidase cleavage site as discussed below.

The nucleotide sequence of the cDNA for the KAHRP and the predicted amino acid sequence of the primary translation product are shown in Fig. 1*c*. An open reading frame coding for 634 amino acids extends from nucleotide 638 to nucleotide 2539. Clone 24 (Fig. 1*b*) is notable in lacking base 1745, creating a frameshift in the sequence of that clone and a premature termination site at nucleotides 1848–1850. This base is found in both clone 34 (Fig. 1*b*) and a genomic clone obtained from FCR-3 DNA. Whether this single-base deletion results from infidelity of reverse transcriptase during cDNA cloning or from the parasite RNA polymerase is not known. The assignment of residue 1 as the initiating methionine was made on the basis that the corresponding AUG is the only methionine codon in-frame with and preceding the polyhistidine-encoding sequence. The peptide in an unprocessed form has a calculated molecular weight of 69,160, a value lower than the 80,000–90,000 observed for the native protein by $NaDodSO_4$/PAGE (11). However, anomalous mobilities in $NaDodSO_4$/PAGE have been observed for other malaria proteins (27), including the *P. lophurae* HRP (26) and the HRPs II and III of *P. falciparum* (28).

The predicted amino acid sequence (Fig. 1*c*) begins with a positively charged amino-terminal peptide ending with a lysine at position 21, which is followed by an 11 amino acid hydrophobic sequence highly characteristic of a signal peptide (29). A potential signal-peptidase cleavage site occurs between cysteine-34 and serine-35, based upon empirical data derived from the sequences of other processed proteins (29).

This signal peptide is followed by a hydrophilic peptide containing a consensus N-glycosylation site (Asn-Xaa-Ser) at residue 42. Since no carbohydrate has been detected in the mature protein (30), this peptide may represent a propeptide that is glycosylated and subsequently cleaved during maturation of the KAHRP, in a manner analogous to an avian malaria HRP counterpart that has been shown to be synthesized as a prepromature protein (31).

The predicted KAHRP is a highly charged basic peptide: 29% of the amino acids in the primary translation product are charged, including 91 lysines. An extraordinary feature of this sequence is a histidine-rich region from residue 61 through residue 116. Thirty histidines are encoded in this 56 amino acid peptide. Two types of histidine-containing repeats are found in this region: tandemly repeating arrays of 6–9 contiguous histidine residues (amino acids 63–69, 82–87, and 108–116) and an alternating Gln-His sequence (amino acids 70–76). The remainder of the protein from residue 117 to the carboxyl terminus contains predominantly charged or neutral hydrophilic residues. Two more repeat sequences are found in this region. A 13 amino acid sequence, Asp-Gly-Glu-Gly-Glu-Lys-Lys-Lys-Ser-Lys-Lys-His-Lys, is imprecisely repeated five times from residue 370 to residue 438. The 40 amino acid sequence beginning at residue 540 contains four copies of the consensus sequence (Thr/Ser)-Lys-Gly-Ala-Thr-Lys-Glu-Ala-Ser-Thr with only three nonconservative substitutions.

To establish the identity of the corresponding *P. falciparum* protein, antiserum was raised against a recombinant peptide that contains residues 43–303 and includes the histidine-rich region. Immunoblot analysis with this antiserum detected a protein of M_r 89,000 from extracts of FVO^+ ($knob^+$) parasites, but no protein was detected by immunoblotting in extracts of $knob^-$ parasites (Fig. 2). Preimmune serum did not detect protein from extracts of either $knob^+$ or $knob^-$ parasites (data not shown). That the molecular weight calculated from the predicted amino acid sequence (M_r 69,160) is lower than the relative molecular weight observed on $NaDodSO_4$/PAGE may be due to the peculiar tandem repeat sequences common to malaria proteins (32). In fact, the recombinant peptide migrated with a relative M_r of 47,000, compared to a predicted M_r of 38,400.

The affinity-purified anti-KAHRP antibodies were used to establish the subcellular localization of the KAHRP. Immunofluorescence demonstrated a pattern consistent with a membrane or cytoskeletal association for the KAHRP (Fig. 3 *a* and *b*) only on parasitized erythrocytes. Three independent isolates of $knob^+$ parasites from different geographical locations showed a similar pattern of KAHRP immunofluorescence (data not shown). Immunofluorescence on live parasites was negative, indicating that the KAHRP epitopes recognized by this rabbit polyclonal serum are not exposed on the outer surface of the infected cells (data not shown). Detailed subcellular localization was performed by indirect immunoelectron microscopy using protein A–gold as a second reagent. Gold labeling was specific to the membranes of FCR-3-infected erythrocytes (Fig. 3*c*). No gold particles were seen associated with the erythrocyte cytoplasm or with internal structures of the schizont. In uninfected erythrocytes, no staining was observed (data not shown). A section grazing an erythrocyte membrane through several knobs (Fig. 3*c*, *Upper Inset*) demonstrates that gold staining is specific to these structures. With higher magnification of a section perpendicular to the plane of the schizont-infected erythrocyte membrane (*Lower Inset*), gold labeling occurs on the cytoplasmic side of the membrane and only in

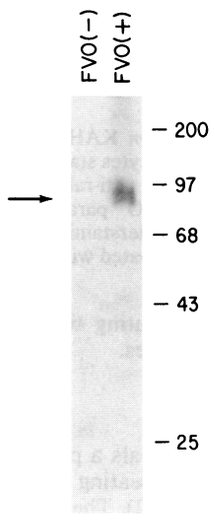

FIG. 2. Identification of the native KAHRP by immunoblot analysis. Rabbit antiserum raised to the recombinant NS1–KAHRP fusion protein was used to identify the mature parasite protein following electrophoresis of detergent-solubilized erythrocytes infected with FVO^- ($knob^-$) or FVO^+ ($knob^+$) parasites. Molecular weight markers ($M_r \times 10^{-3}$) are indicated at right. Parallel experiments using antiserum to the glycophorin-binding protein (27) showed that comparable amounts of parasite extract were loaded in FVO^- and FVO^+ lanes.

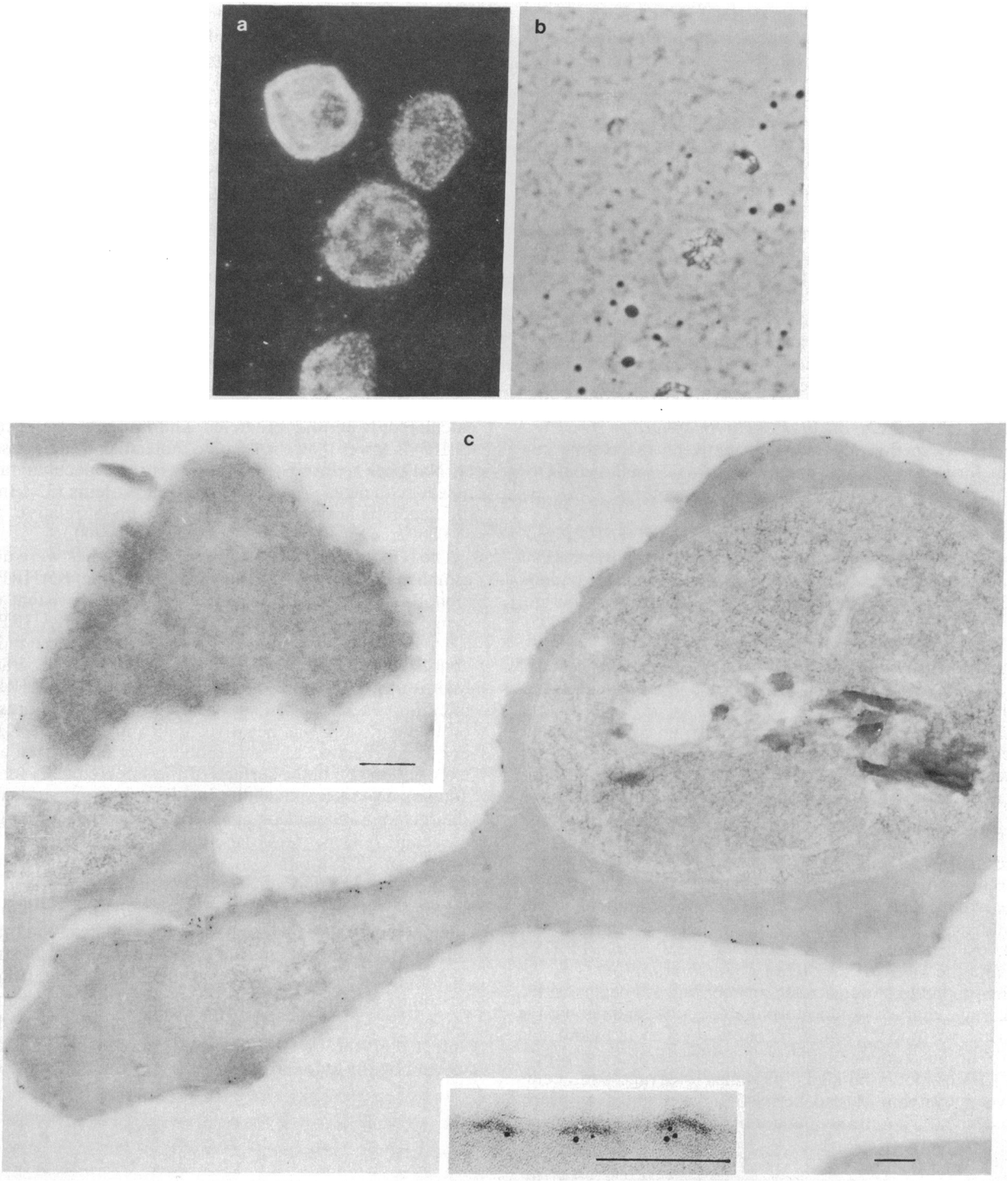

FIG. 3. Immunolocalization of the *P. falciparum* KAHRP by indirect immunofluorescence (*a* and *b*) and electron microscopy (*c*). (*a*) Fluorescence micrograph of FVO^+-infected erythrocytes stained with affinity-purified rabbit antibodies against the KAHRP nonfusion peptide. The second reagent was fluorescein-conjugated goat anti-rabbit IgG. (× 1440.) (*b*) Phase micrograph of the same field. In control experiments using FVO^+ parasites and preimmune serum or FVO^- parasites and anti-KAHRP antibodies, no fluorescence was observed and parasitized erythrocytes could only be distinguished by counterstaining the parasite nucleus with ethidium bromide (data not shown). (*c*) Electron micrographs of an FCR-3-infected erythrocyte incubated with the same anti-KAHRP antiserum followed by protein A–gold. (Bars = 200 nm.)

association with electron-dense knobs, indicating that the KAHRP is restricted to these unique structures.

DISCUSSION

cDNA sequence analysis of the KAHRP reveals a primary translation product containing tandemly repeating amino acids, a feature common to malaria antigens (32). The repeat domains of some malaria proteins are highly conserved, whereas others are strain-variant (32). The KAHRP is synthesized by all $knob^+$ parasites that have been examined to date (10–12, 33) and is antigenically conserved in multiple geographical isolates (ref. 33 and unpublished observations). This conservation may reflect that the KAHRP has evolved to perform a function critical to parasite survival. However, the KAHRP also displays strain-dependent variation in

molecular weight, as seen by $NaDodSO_4$/PAGE (12, 33), that may result from variation in repeat number or sequence as has been seen for other plasmodial antigens (32). In this regard, the complete sequence of a Ghanian isolate, NF7 (34), reveals two additional copies of the 10 amino acid repeat sequence in the carboxyl-terminal repeat domain, which may partially account for the observed strain-dependent size variation in this protein. The NF7 KAHRP sequence also displays multiple single amino acid substitutions, which may reflect a different mechanism for strain-dependent variation or trivial sequencing discrepancies. The nucleotide sequence reported here is also quite similar to two other recently reported, partial cDNA sequences (35, 36).

The sequence organization of the KAHRP contains a polyhistidine repeat structure that is quite different from the short direct repeat sequences found for two other HRPs of unknown function, synthesized by both knob$^+$ and knob$^-$ *P. falciparum* parasites, HRP II and HRP III (28). The unusual polyhistidine arrays characteristic of the KAHRP are similar to the polyhistidine repeat structure of the HRP of *P. lophurae*, an avian malarial parasite that does not cause knob formation but does synthesize a HRP with a 74% histidine content arranged in tandem repeats of the general structure Xaa-Xaa-$(His)_8$ (26). The genes encoding these proteins reveal an intron in approximately the same location, further suggesting an evolutionary relationship between these parasites. The primary amino acid squence of the KAHRP also reveals the existence of a hydrophobic signal sequence and the possibility of a short-lived core glycosylated propeptide, suggesting a role for these transient sequences in the targeting and translocation of this protein from its site of synthesis within the parasitophorous vacuole to the erythrocyte membrane.

The significance of the knob is in its ability to mediate cytoadherence. A possible role for the *P. falciparum* KAHRP in cytoadherence is suggested by its homology to a histidine-rich glycoprotein from human serum. This protein has been shown to possess a domain of 60 amino acids with a 53% histidine content, arranged in tandem repeats of the general pattern Gly-His-His-Pro-His (37), and has been shown to interact specifically with human thrombospondin (38), a molecule that binds both to parasitized erythrocytes (9) and to the OKM5-defined endothelial cell receptor for infected erythrocytes (39). Thus, by mimicking the human protein, the KAHRP may act to anchor host thrombospondin or a parasite analogue in a binding complex with the endothelial cell receptor.

Localization of the KAHRP to the knob structure on the cytoplasmic side of the erythrocyte membrane suggests the possibility of a structural role for the protein in the observed morphologic changes on the surface of *P. falciparum*-infected erythrocytes.

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1. Trager, W., Rudzinska, M. A. & Bradbury, P. C. (1966) *Bull. W.H.O.* **35,** 883–885.
2. Luse, S. A. & Miller, L. H. (1971) *Am. J. Trop. Med. Hyg.* **20,** 655–660.
3. Trager, W. & Jensen, J. B. (1976) *Science* **193,** 673–675.
4. Kreier, J. P. & Green, T. J. (1980) in *Malaria*, ed. Kreier, J. P. (Academic, New York), Vol. 2, pp. 1–46.
5. Udeinya, I. J., Graves, P. M., Carter, R., Aikawa, M. & Miller, L. H. (1983) *Exp. Parasitol.* **56,** 207–214.
6. Udeinya, I. J., Miller, L. H., McGregor, I. A. & Jensen, J. B. (1983) *Nature (London)* **303,** 429–431.
7. Leech, J. H., Barnwell, J. W., Miller, L. H. & Howard, R. J. (1984) *J. Exp. Med.* **159,** 1567–1575.
8. Barnwell, J. W., Ockenhouse, C. F. & Knowles, D. M. (1985) *J. Immunol.* **135,** 3494–3497.
9. Roberts, D. D., Sherwood, J. A., Spitalnik, S. L., Panton, L. J., Howard, R. J., Dixit, V. M., Frazier, W. A., Miller, L. H. & Ginsburg, V. (1985) *Nature (London)* **318,** 64–66.
10. Kilejian, A. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4650–4653.
11. Gritzmacher, C. A. & Reese, R. T. (1984) *Science* **226,** 65–67.
12. Leech, J. H., Barnwell, J. W., Masumichi, A., Miller, L. H. & Howard, R. J. (1984) *J. Cell Biol.* **98,** 1256–1264.
13. Vernot-Hernandez, J. P. & Heidrich, H.-G. (1984) *Mol. Biochem. Parasitol.* **12,** 337–350.
14. Langreth, S. G., Reese, R. T., Motyl, M. R. & Trager, W. (1979) *Exp. Parasitol.* **48,** 213–219.
15. Langreth, S. G. & Peterson, E. (1985) *Infect. Immun.* **47,** 760–766.
16. Pologe, L. G. & Ravetch, J. V. (1986) *Nature (London)* **322,** 474–477.
17. Jensen, J. B. & Trager, W. (1978) *Am. J. Trop. Med. Hyg.* **27,** 743–746.
18. Pasvol, G., Wilson, R. J. M., Smalley, M. E. & Brown, J. (1978) *Ann. Trop. Med. Parasitol.* **72,** 87–88.
19. Lambros, C. & Vanderberg, J. P. (1979) *J. Parasitol.* **65,** 418–420.
20. Maxam, A. M. & Gilbert, W. (1980) *Methods Enzymol.* **65,** 499–560.
21. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
22. Young, J. F., Desselberger, V., Palese, P., Ferguson, B., Shatzman, A. R. & Rosenberg, M. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 6105–6109.
23. Shatzman, A., Ho, Y. S. & Rosenberg, M. (1983) in *Experimental Manipulation of Gene Expression*, ed. Inouye, M. (Academic, New York), pp. 1–14.
24. Portnoy, D. A., Erickson, A. H., Kochan, J., Ravetch, J. V. & Unkeless, J. C. (1986) *J. Biol. Chem.* **261,** 14697–14703.
25. Pirson, P. J. & Perkins, M. E. (1985) *J. Immunol.* **134,** 1946–1951.
26. Ravetch, J. V., Feder, R., Pavlovec, A. & Blobel, G. (1984) *Nature (London)* **312,** 616–620.
27. Kochan, J., Perkins, M. & Ravetch, J. V. (1986) *Cell* **44,** 689–696.
28. Wellems, T. E. & Howard, R. J. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 6065–6069.
29. Perlman, D. & Halvorson, H. O. (1983) *J. Mol. Biol.* **167,** 391–409.
30. Vernot-Hernandez, J. P. & Heidrich, H.-G. (1985) *Z. Parasitenkd.* **71,** 41–51.
31. Feder, R. & Blobel, G. (1983) *Mol. Biochem. Parasitol.* **9,** 351–362.
32. Ravetch, J. V., Young, J. & Poste, G. (1985) *Biotechnology* **3,** 729–740.
33. Culvenor, J. G., Langford, C. J., Crewther, P. E., Saint, R. B., Coppel, R. L., Kemp, D. J., Anders, R. F. & Brown, G. V. (1987) *Exp. Parasitol.* **63,** 58–67.
34. Triglia, T., Stahl, H. D., Crewther, P. E., Scanlon, D., Brown, G. V., Anders, R. F. & Kemp, D. J. (1987) *EMBO J.* **6,** 1413–1419.
35. Kilejian, A., Sharma, Y. D., Karoui, H. & Naslund, L. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 7938–7941.
36. Ardeshir, F., Flint, J. E., Matsumoto, Y., Aikawa, M., Reese, R. T. & Stanley, H. (1987) *EMBO J.* **6,** 1421–1427.
37. Koide, T., Foster, D., Yoshitake, S. & Davie, E. W. (1986) *Biochemistry* **25,** 2220–2225.
38. Leung, L. L. K., Nachman, R. L. & Harpel, P. C. (1984) *J. Clin. Invest.* **73,** 5–12.
39. Asch, A. S., Barnwell, J., Silverstein, R. L. & Nachman, R. L. (1987) *J. Clin. Invest.* **79,** 1054–1061.