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cDNA sequence encoding a *Plasmodium falciparum* protein associated with knobs and localization of the protein to electron-dense regions in membranes of infected erythrocytes

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***Plasmodium falciparum* modifies the host erythrocyte's plasma membrane by the formation of electron-dense structures called knobs. We have produced monoclonal antibodies (McAbs) which specifically bind to the knobs in immunoelectron microscopic experiments with thin sections of parasitized erythrocytes. However, the McAbs fail to bind to the surface of live parasitized erythrocytes. Immunoblotting experiments with these McAbs show the antigen is localized to the erythrocyte plasma membrane. The antigen with which the McAbs react varies in mol. wt from 80 to 95 kd in different knob-producing isolates of *P. falciparum* and is absent in knobless variants. The McAbs react with the expressed product of a *P. falciparum* cDNA clone, thus demonstrating that the clone encodes part of this knob-associated protein. The sequence of the cDNA fragment partially overlaps a published cDNA sequence reported to encode the amino-terminal portion of the knob protein, and extends the predicted open reading frame by 190 amino acids. The carboxyl-terminal portion of the predicted amino acid sequence contains a highly charged stretch of ~100 amino acid residues. We suggest that this unusual, highly charged region participates in intermolecular salt bridging leading to dense packing of these molecules. This would create the electron-dense regions observed by electron microscopy and might also explain the insolubility of the knob-associated protein in the absence of strong ionic detergents or chaotropic agents.**

Key words: cDNA sequence/erythrocyte membrane/immunoelectron microscopic localization/monoclonal antibodies/*Plasmodium falciparum* knob protein

Introduction

Plasmodium falciparum is an intracellular parasite of human erythrocytes. During the development of the organism from the ring stage to the trophozoite stage the parasite alters the surface of the host cell plasma membrane. An obvious structural modification is the appearance of electron-dense deposits, detectable by transmission electron microscopy, under the intact lipid bilayer of the membrane in the areas where protuberances occur. Trager *et al.* (1966) referred to these regions as knobs. The membrane over these structures (knobs) is antigenically different from the adjoining membrane since antibodies from immune monkeys bind preferentially at these sites (Langreth and Reese, 1979). In addition, it has been hypothesized that the presence of the knobs makes this membrane into an activator of the alternative complement pathway (Stanley *et al.*, 1984). The presence of knobs may be linked with the virulence of the parasites (Langreth and Peterson, 1985) since these structures are proposed to be necessary, although not sufficient, for the binding of parasitized cells to the capillary endothelium (Raventos-Suarez *et al.*, 1985). The appearance of knobs is correlated with an increase in the rigidity of the parasitized erythrocyte. One manifestation of this altered rigidity is that cells which are parasitized with knob-producing strains of *P. falciparum* are unable to form biconcave discs and cannot fit within Rouleaux, while cells infected with knobless variants are still able to participate in Rouleaux formation (Langreth *et al.*, 1979).

Biochemical data suggest that a protein of ~90 kd, which varies in size among isolates, is associated with the presence of knobs (Kilejian, 1979; Hadley *et al.*, 1983; Leech *et al.*, 1984; Vernot-Hernandez and Heidrich, 1984). A number of these studies, including one in which a knobless (K^-) clone was derived from a knob producing (K^+) parental clone (Gritzmacher and Reese, 1984), demonstrate that the ~90-kd protein is present in K^+ organisms while lacking in K^-. The solubility properties of this molecule suggest that it is involved in strong protein-protein interactions and may be linked to the erythrocyte membrane skeleton (Kilejian, 1980; Leech *et al.*, 1984). Metabolic studies using radiolabeled amino acids demonstrate that this knob-associated protein (KP) can be labeled with histidine, lysine and a number of other amino acids, but that it does not label efficiently with [^{35}S]methionine (Hadley *et al.*, 1983; Kilejian, 1984). Based on the presumed high histidine content of the KP, and its proposed homology with the *P. lophurae* histidine-rich protein (HRP) two groups (Kilejian *et al.*, 1986; Pologe and Ravetch, 1986) have used DNA probes, either based on the gene encoding HRP or simple oligonucleotides encoding polyhistidine, to isolate *P. falciparum* cDNA clones reported to encode the KP. All of these studies provide evidence for a histidine-rich region in the KP of *P. falciparum*.

Although the biochemical and genetic data strongly suggest that the knob-associated electron-dense material is the 90-kd antigen, definitive proof is lacking. In this paper we provide that proof. Monoclonal antibodies (McAbs) were produced that react in immunoblots with a 90-kd protein which is present only in K^+ parasitized cells and is associated with their membranes. These McAbs were shown by immunoelectron microscopy to bind to the electron-dense regions under the lipid bilayer. The KP-specific McAbs were also shown to react with a recombinant protein produced by the *Escherichia coli* clone A12 isolated from a *P. falciparum* cDNA expression library, thus providing evidence that clone encodes part of the 90-kd KP. The nucleotide sequence of the cDNA fragment from clone A12 partially overlaps with the recently published sequence of a cDNA clone which is believed to encode the amino-terminal portion of the KP (Kilejian *et al.*, 1986) and extends the open reading frame by 190 amino acids. The predicted protein sequence contains a large, highly charged region which may explain the known physical and chemical properties of the KP.

Results

Using standard techniques (Stanley and Reese, 1985), six independent mouse McAbs were produced which in indirect immunofluorescence assays produced a fluorescence pattern consistent with binding to the erythrocyte membrane of acetone/methanol-fixed trophozoites of *P. falciparum*. Ascites fluids containing these McAbs had fluorescence titers of ~1: 10 000 using fixed trophozoites as antigen. When the same ascites were tested on live trophozoites to determine if the McAbs could bind to the surface of these cells, no reactivity could be demonstrated even at a 1:20 dilution. Thus, if the antigen being recognized is truly membrane-associated, those portions of it with which the antibodies react are not detectable on the extracellular surface of infected erythrocytes.

To establish unequivocally the membrane location for the antibody binding, the McAbs were used in immunoelectron microscopic studies. Three different McAbs produced similar immunoelectron microscopic patterns. The data from two such McAbs are presented (Figure 1). From those studies, it is clear that the molecule to which the McAbs bound is not only membrane-associated, but is localized in the electron-dense region under the membrane protrusions. In addition to the knob-specific staining shown in Figure 1, the McAbs also bound to small electron-dense regions within the parasite (not shown). We hypothesize that these regions probably represent the antigen as it is being transported to the erythrocyte plasma membrane.

To investigate whether the molecule being detected by these McAbs was associated with the presence of knobs, we examined a series of K^+ and K^- isolates for (i) the presence of knobs by transmission electron microscopy, (ii) their reactivity with these McAbs in immunofluorescence assays and (iii) their sedimentation properties in the Rouleaux-inducing agent, Physiogel. Erythrocytes infected with late-stage parasites from knobby isolates are separable from uninfected cells by sedimentation in Physiogel (Reese *et al.*, 1979), and this characteristic is dependent on the presence of knobs (Langreth *et al.*, 1979). The data in Table I demonstrate an absolute correlation between the presence of knobs by electron microscopy, the ability to concentrate the infected cells by Physiogel treatment and the ability of the McAbs to react with these infected cells. Thus the McAbs appear to recognize a knob-specific antigen

When these different parasite isolates were examined by immunoblotting with two different monoclonal antibodies, the McAbs bound antigens that were only found in K^+ organisms and did not react with any antigen in K^- organisms. The results using one McAb are shown in Figure 2. The major protein with which the McAbs reacted varied in size from ~80 kd in some isolates (Figure 2A, lane l) up to 95 kd in others (Figure 2A, lanes 3 and 6). In addition, more than one major protein of similar mol. wt was detectable in some of the K^+ isolates (Figure 2A, lanes 4 and 5). This was true even when several K^+ clones were examined (Figure 2B, lanes 3–9). Thus, the presence of more than one immunoreactive protein was unlikely to be due to allelic variation created by having several different parasite variants within each isolate population. The second band might represent a differentially processed form of the same molecule, or perhaps a structurally related cross-reactive molecule, which is also absent in knobless variants.

To obtain biochemical evidence in support of the membrane localization of the molecule recognized by these McAbs, membrane fragments were prepared from parasitized erythrocytes containing trophozoite-stage parasites by first attaching the cells to polylysine-coated flasks, and then shearing them off, leaving fragments of the plasma membrane attached to the plate (Stanley and Reese, 1986). To control for binding of intracellular antigens

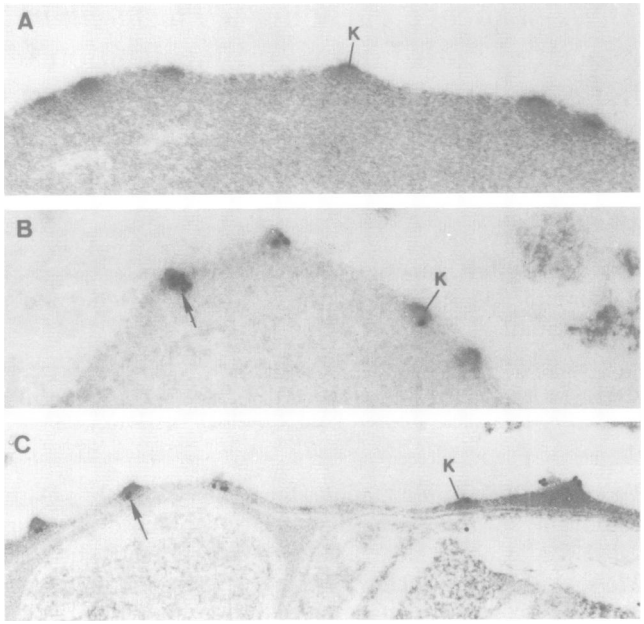

Fig. 1. Immunoelectron micrograph of *P. falciparum*-infected erythrocytes previously incubated with various monoclonal antibodies. (**A**) Surface of an erythrocyte infected with *P. falciparum* which has been stained with non-specific ascites as a control. Note that no gold particles are associated with the knobs (K), ×44 000. (**B**) Surface of an erythrocyte infected with *P. falciparum* which has been stained with McAb P3-9B. Gold particles (arrow) are associated with knobs (K), ×54 000. (**C**) Surface of an erythrocyte infected with *P. falciparum* which has been stained with McAb P6-10F. Gold particles (arrow) are specifically associated with knobs (K), ×34 000.

Table I. Properties correlated with the presence of knobs on isolates and clones of *P. falciparum*

Isolate	K^+ by electron microscopy	IFAT reactivity with McAb	Separable by Physiogel	Hybridization of genomic DNA to A12 cDNA
Honduras I/CDC	+	+	+	+
FVO	+	+	+	+
FVOc (clone)	–	–	–	ND
Tanzania	ND	+	+	+
Sierra Leone	+	+	+	+
Kenya	–	–	–	–
Geneva	+	+	+	+
Philippines	–	–	–	ND
Indochina I/CDC	–	–	–	–
FCR-3	+	+	+	ND
NF-54	+	+	+	ND
K-1	+	+	+	ND
FVO clone H12	+	+	+	+
FVO clone F11	+	+	+	+
FVO clone E6	–	–	–	–
Camp K^- clone[a]	ND	ND	ND	–

[a]The DNA from this clone was a gift from Dr Jim Weber (Weber and Hockmeyer, 1985). The clone has been shown to lack the knob protein (Hadley *et al.*, 1983).

ND, not determined.

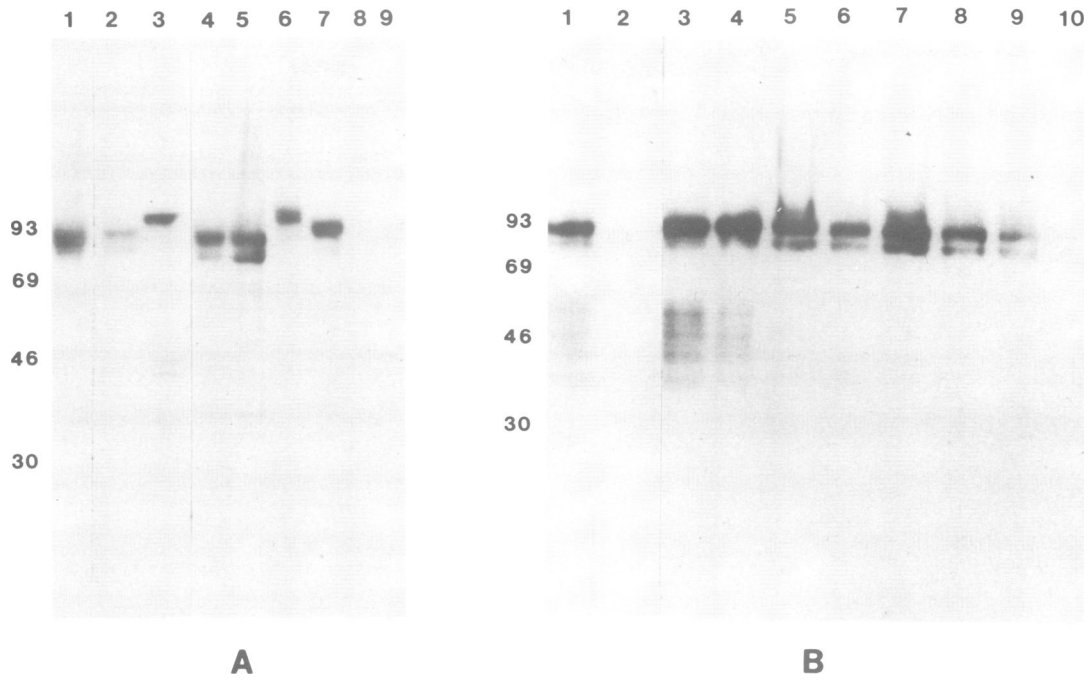

Fig. 2. Immunoblots of the proteins of *P. falciparum* isolates and clones, probed with McAb P3-9B. Cultures were harvested as a mixture of trophozoites and schizonts. **A** and **B** represent independent experiments. Lysates of the following were examined: **(A) lane 1**, FVO; **lane 2**, FVO clone, K^+; **lane 3** Honduras I; **lane 4**, Geneva; **lane 5**, Sierra Leone; **lane 6**, NF54; **lane 7**, Kl; **lane 8**, Indochina I; **lane 9**, Philippines. **(B) lane 1**, FVO isolate; **lane 2**, FVO clone c, K^-; **lanes 3–9**, K^+ clones derived from FVO, in the following order: H12, F11, A10, B3, B4, H6, C12; **lanes 10**, K^- clone E6, derived from FVO.

to the plates during the shearing process, a separate plate was processed using a lysate prepared by freezing and thawing parasitized cells, thus allowing binding of any antigens from parasitized erythrocytes which have an affinity for the polylysine coated plates. The bound membrane fragments and control cell lysate, along with a total protein extract from parasitized erythrocytes were each solubilized in SDS, electrophoresed in 6–14% gradient SDS–polyacrylamide gels, and examined by immunoblotting for their reactivity with two of the six McAbs. Similar results were obtained with both McAbs. The McAbs-bound to a major antigen of 95 kd and two minor antigens of ~90 and >205 kd present in unfractionated Honduras I CDC infected cells (Figure 3, lane 1). However, only the 95-kd antigen was present in the plasma membrane fragments of these parasitized erythrocytes while the minor antigens appeared to be absent from this fraction (lanes 3 and 4). The McAbs did not bind to any of the antigens from the freeze–thaw lysates which had an affinity for the polylysine coated plates (lane 2). Thus, of the three structures recognized by these McAbs, the 95-kd antigen can be defined as the knob protein (KP), as it is biochemically linked with the parasitized erythrocyte membrane and it can be localized to the electron-dense region under the lipid bilayer by immunoelectron microscopy.

A *P. falciparum* cDNA library constructed in the expression vector pUC8 contains clones reactive with antibodies from owl monkeys immune to *P. falciparum* (Ardeshir *et al.*, 1985). Lysates of some of the reactive clones were injected into mice and rats to produce antisera which were tested for their ability to bind parasite antigens. Clones E9 and A12 stimulated antibodies which by immunofluorescence of fixed parasitized erythrocytes appeared to react with antigens associated with the membrane of *P. falciparum*-infected erythrocytes. Both E9 and A12 produce recombinant proteins of the same size which can be detected by immune owl monkey serum on immunoblots of bacterial proteins, but whose concentrations are too low to be visualized by Coomassie blue staining of bacterial proteins fractionated on SDS–polyacrylamide gels. The clones E9 and A12 contained cDNA fragments of identical size which cross-hybridized. They are likely to be identical siblings since the cDNA pool from which they were isolated had been amplified.

Several monoclonal antibodies which recognize antigens of the infected erythrocyte membrane were tested by immunoblotting for their ability to bind the recombinant protein produced by the clone A12, because of the indication that A12 might be encoding a plasmodial protein localized in the membranes of infected erythrocytes. Three of the six monoclonal antibodies reactive with the 80–95-kd KP were found to bind the expressed recombinant protein of clone A12. Thus, there are at least two different epitope specificities among the six KP-specific McAbs. Two of the three monoclonal antibodies which bound the recombinant protein were those which by immunoelectron microscopy had been shown to react with the electron-dense regions under the infected erythrocyte membranes. These results suggested that the polypeptide encoded by the cDNA insert of the clone A12 is a fragment of the 95-kd KP of the Honduras isolate.

Kilejian *et al.* (1986) recently published a cDNA sequence believed to encode the amino-terminal portion (Figure 4, bases 1–810) of the KP from the *P. falciparum* isolate FCR-3. We used the chain termination method of DNA sequencing to determine the entire sequence of the cDNA fragment from the clone A12, and discovered that 254 bases at the 5′ end of our cDNA sequence overlap with the 3′ end of the published sequence (Figure 4, bases 556–810, boxed). This amalgamated sequence contains a single open reading frame that encodes a predicted protein of 461 amino acids. The fact that the overlapping segments of the two sequences are almost identical supports our

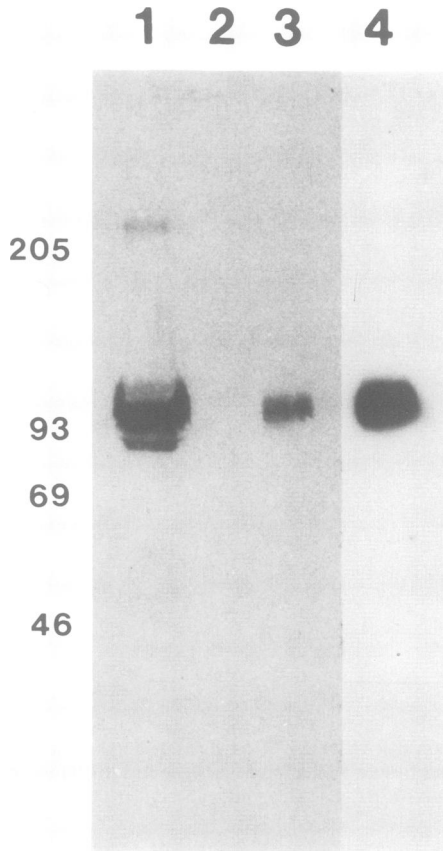

Fig. 3. Immunoblot of *P. falciparum* antigens associated with the erythrocyte plasma membrane. The blot was first incubated with McAb followed by [^{125}I]-labeled rabbit anti-mouse Ig. **Lane 1**, protein extract from intact Honduras isolate trophozoites; **lane 2**, proteins which bind to polylysine coated flasks from a freeze−thaw lysate of trophozoites (control for non-specific binding); **lane 3**, proteins from the membranes of parasitized erythrocytes prepared by binding parasitized cells to polylysine coated flasks, shearing the cells off, and solubilizing attached membrane fragments in sample buffer; **lane 4**, longer exposure of lane 3.

conclusion that A12 encodes part of the KP. The only difference in the two DNA sequences is at residue 778 (underlined) which is a G in our sequence, and an A in the sequence of Kilejian *et al.*(1986). This results in a conservative amino acid change, from threonine in the sequence predicted for KP from the FCR-3 isolate, to alanine in the KP sequence for the Honduras isolate. There is no stop codon within the A12 cDNA sequence; presumably the oligo(dT) primer used during the cDNA synthesis bound to an internal stretch of adenosine residues within the coding region for this protein.

Because of the many stretches of polyadenosine residues at the 3′ end of the A12 cDNA fragment, the nucleotide sequence from bases 1183 to 1380 was confirmed by the chemical degradation method on both strands of the DNA. In addition, the sequence of the cDNA insert from the sibling clone E9 was also determined by the dideoxy method (on one strand only) from bases 556−806 and 1149−1380. We obtained the same sequence in all cases.

Rearrangements in plasmodial DNA sequences during cloning and sequencing procedures have been known to create artifactual results (Irving *et al.*, 1986). Presumably this occurs because of the repetitive nature of *P. falciparum* DNA and its high A + T content. To demonstrate that the A12 cDNA sequence did not contain any major rearrangements, we show in Figure

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ATGAAAAGTTTTAAGAACAAAAATACTTTGAGGAGAAAGAAGGCTTTCCCTGTTTTTACT   60
MetLysSerPheLysAsnLysAsnThrLeuArgArgLysLysAlaProValPheThr

AAAATTCTTTTAGTCTCTTTTTTAGTATGGGTTTTGAAGTGCTCTAATAACTGCAATAAT  120
LysIleLeuLeuValSerPheLeuValTrpValLeuLysCysSerAsnAsnCysAsnAsn

GGAAACGGATCCGGTGACTCCTTCGATTTCAGAAATAAGAGAACTTTAGCACAAAAGCAA  180
GlyAsnGlySerGlyAspSerPheAspPheArgAsnLysArgThrLeuAlaGlnLysGln

CATGAACACCATCACCACCATCACCATCAACATCAACACCAACACCAAGCTCCACACCAA  240
HisGluHisHisHisHisHisHisHisGlnHisGlnHisGlnHisGlnAlaProHisGln

GCACACCACCATCATCATCATGGAGAAGTAAATCACCAAGCACCACAGGTTCACCAACAA  300
AlaHisHisHisHisHisGlyGluValAsnHisGlnAlaProGlnValHisGlnGln

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GTACATGGTCAAGACCAAGCACACCATCACCATCATCACCACCATCATCAATTACAACCT  360
ValHisGlyGlnAspGlnAlaHisHisHisHisHisHisHisHisGlnLeuGlnPro

CAACAACTCCAGGGAACAGTTGCTAATCCTCCTAGTAATGAACCAGTTGTAAAAACCCAA  420
GlnGlnLeuGlnGlyThrValAlaAsnProProSerAsnGluProValValLysThrGln

GTATTCAGGGAAGCAAGACCAGGTGGAGGTTTCAAAGCATATGAAGAAAAATACGAATCA  480
ValPheArgGluAlaArgProGlyGlyGlyPheLysAlaTyrGluGluLysTyrGluSer

AAACACTATAAATTAAAGGAAAATGTTGTCGATGGTAAAAAAGATTGTGATGAAAAATAC  540
LysHisTyrLysLeuLysGluAsnValValAspGlyLysLysAspCysAspGluLysTyr

|
GAAGCTGCCAATTATGCTTTCTCCGAAGAGTGCCCATACACCGTAAACGATTATAGCCAA  600
GluAlaAlaAsnTyrAlaPheSerGluGluCysProTyrThrValAsnAspTyrSerGln

GAAAATGGTCCAAATATATTTGCCTTAAGAAAAAGATTCCCTCTTGGAATGAATGATGAA  660
GluAsnGlyProAsnIlePheAlaLeuArgLysArgPheProLeuGlyMetAsnAspGlu

GATGAAGAAGGTAAAGAAGCATTAGCAATAAAAGATAAATTACCAGGTGGTTTAGATGAA  720
AspGluGluGlyLysGluAlaLeuAlaIleLysAspLysLeuProGlyGlyLeuAspGlu

                    *                    |
TACCAAAACCAATTATATGGAATATGTAATGAGACATGTACCACATGTGGACCTGCCGCT  780
TyrGlnAsnGlnLeuTyrGlyIleCysAsnGluThrCysThrThrCysGlyProAlaAla

ATAGATTATGTTCCAGCAGATGCACCAAATGGCTATGCTTATGGAGGAAGTGCACACGAT  840
IleAspTyrValProAlaAspAlaProAsnGlyTyrAlaTyrGlyGlySerAlaHisAsp

GGTTCTCACGGTAATTTAAGAGGACACGATAATAAAGGTTCAGAAGGTTATGGATATGAA  900
GlySerHisGlyAsnLeuArgGlyHisAspAsnLysGlySerGluGlyTyrGlyTyrGlu

GCTCCATATAACCCAGGATTTAATGGTGCTCCTGGAAGTAATGGTATGCAAAATTATGTC  960
AlaProTyrAsnProGlyPheAsnGlyAlaProGlySerAsnGlyMetGlnAsnTyrVal

                                    |
CCACCCCATGGTGCAGGCTATTCAGCTCCATACGGAGTTCCACATGGTGCAGCCCATGGT 1020
ProProHisGlyAlaGlyTyrSerAlaProTyrGlyValProHisGlyAlaAlaHisGly

TCAAGATATAGTTCATTCAGTTCCGTAAATAAATATGGAAAACACGGTGATGAAAAACAC 1080
SerArgTyrSerSerPheSerSerValAsnLysTyrGlyLysHisGlyAspGluLysHis

CATTCCTCTAAAAAGCATGAAGGAAATGACGGTGAAGGAGAAAAAAAGAAAAAATCAAAA 1140
HisSerSerLysLysHisGluGlyAsnAspGlyGluGlyGluLysLysLysLysSerLys

AAACACAAAGACCACGATGGAGAAAAGAAAAAATCAAAAAAACACAAAGACAATGAAGAT 1200
LysHisLysAspHisAspGlyGluLysLysLysSerLysLysHisLysAspAsnGluAsp

GCAGAAAGCGTAAAATCAAAAAAACACAAAAGCCACGATTGTGAAAAGAAAAAATCAAAA 1260
AlaGluSerValLysSerLysLysHisLysSerHisAspCysGluLysLysLysSerLys

AAACACAAAGACAATGAAGATGCAGAAAGCGTAAAATCAAAAAAAAAGTGTTAAAGAAAAG 1320
LysHisLysAspAsnGluAspAlaGluSerValLysSerLysLysSerValLysGluLys

                                    |
GGAGAAAAGCATAATGGAAAAAAACCATGCAGCAAAAAAACTAACGAAGAAAAAAAAAAA 1380
GlyGluLysHisAsnGlyLysLysProCysSerLysLysThrAsnGluGluLysLysLys
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Fig. 4. Nucleotide sequence of the cDNA encoding part of the KP, obtained by combining the published cDNA sequence of the coding region from the FCR-3 isolate (bases 1−810, corresponding to bases 92−901 of Kilejian *et al.*, 1986) with the sequence of the cDNA clone A12, derived from the Honduras isolate (bases 556−1380). The region of overlap between the two sequences is boxed and the only non-identical residue (position 778) is underlined. Sites for the restriction enzymes *Rsa*I and *Fnu*4H are marked above the sequence with stars and vertical lines respectively. The deduced amino acid sequence is shown below the DNA sequence.

5A that the internal restriction fragments produced by digesting the cDNA fragment with the enzyme *Fnu*4H have the sizes predicted from the cDNA sequence, and that they co-migrate with

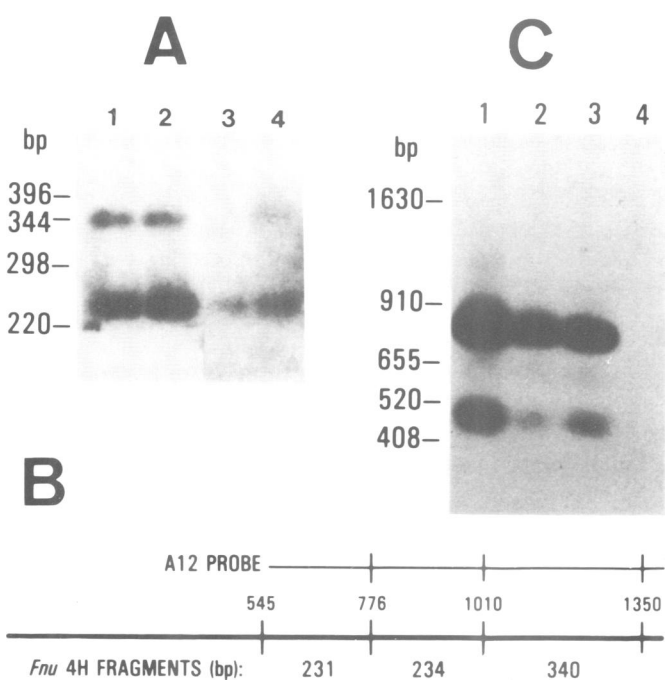

Fig. 5. (**A**) Southern blot of *Fnu*4H digests of: **lane 1**, 3 μg of genomic DNA from the FVO K^+ clone F11; **lane 2**, 3 μg of genomic DNA from the Honduras isolate; **lane 3**, 0.2 ng of the cDNA fragment from clone A12; **lane 4**, 0.4 ng of the A12 cDNA fragment. Sizes of marker fragments are indicated on the left. The fragments were resolved on a 4% NuSieve agarose gel, and the blot was probed with the cDNA fragment from clone A12. (**B**) Map of the sites for the restriction enzyme *Fnu*4H within the cDNA sequence encoding part of KP. The thick line represents the entire sequence shown in Figure 4. The positions of the sites are shown above this line, and the sizes of the predicted fragments are indicated below it. The thin line represents the A12 cDNA fragement which was used to probe the blot in Figure 5A, with the *Fnu*4H sites marked by vertical lines. (**C**) Southern blots of *Rsa*I digests of genomic DNA from:**lane 1**, FVO isolate (K^+); **lane 2**, FVO K^+ clone H12; **lane 3**, FVO K^+ clone F11; **lane 4**, FVO K^- clone E6. DNA fragments were resolved on a 1% agarose gel and probed with the cDNA fragment from clone A12. Sizes of marker fragments are indicated on the left.

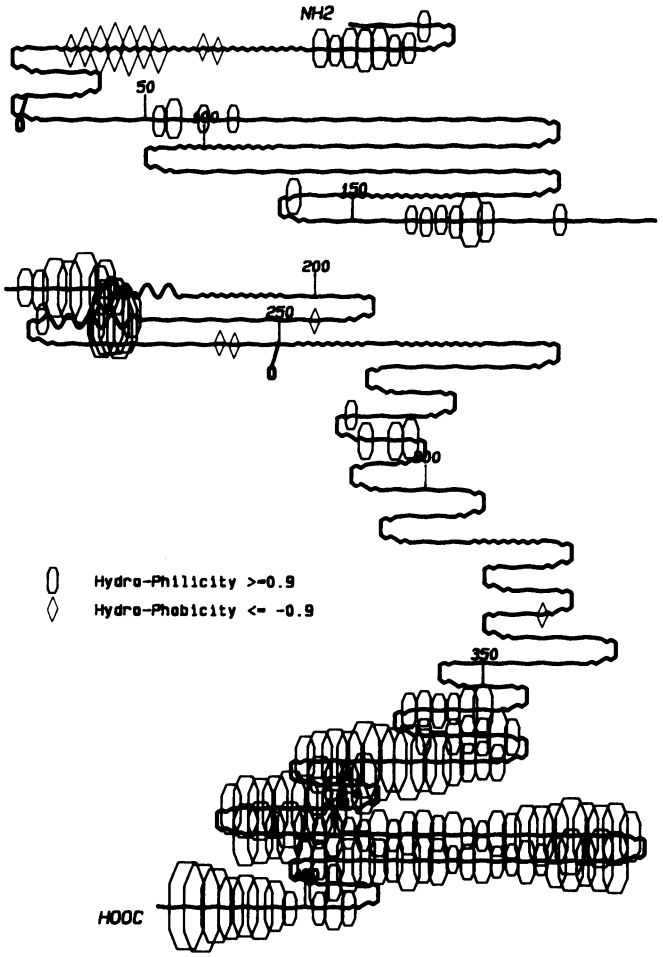

Fig. 6. Diagrammatic representation of the predicted secondary structure for part of the KP. The 460 amino acid sequence derived from the cDNA sequence was analyzed by the PLOT CHOUFASMAN program from UWGCG (Devereux *et al.*, 1984, Jameson and Wolf, in preparation) as modified by Reese to reflect the fact that at least four amino acid residues are usually involved in any turn. The compressed zig-zag lines represent regions of β-pleated sheet, the expanded zig-zag lines indicate predicted α-helical regions, while the gently undulating line represents stretches of random coil. The line representing the peptide backbone changes direction when turns are predicted. Hexagons and diamonds represent regions of hydrophilicity and hydrophobicity respectively, with the size of the symbol being proportional to the value of the attribute. Potential glycosylation sites are marked.

the homologous *Fnu*4H fragments in the genomic DNA of both the Honduras isolate and a clone derived from FVO, a Vietnamese isolate. A map of the *Fnu*4H sites predicted from the cDNA sequence is shown in Figure 5B (lower line), aligned with the cDNA insert from clone A12 (upper line). Note that the A12 cDNA fragment is predicted to hybridize with three *Fnu*4H fragments in the genomic DNA, one of 340 bp, and a co-migrating pair at 231 and 234 bp. This co-migration results in the stronger hybridization signal for the lower band which is observed in Figure 5A.

There have been two reports (Pologe and Ravetch, 1986; Kilejian *et al.*, 1986) that the gene for KP is rearranged or deleted in some knobless isolates and clones of *P. falciparum*. The cDNA insert from A12 was used to probe *Eco*RI digests of genomic DNA from several K^+ and K^- isolates and clones. The results are summarized in Table I. The probe hybridized with a 14-kb *Eco*RI fragment in all the K^+ isolates and clones, but no hybridization was observed to the DNA of any of the K^- strains tested under the conditions used, suggesting that at least the DNA encoding the central third of the 95-kd protein is deleted from the genome in these K^- strains. The A12 cDNA fragment was also used to probe *Hin*dIII and *Rsa*I digests of genomic DNA from two K^+ clones (F11, H12) and a K^- clone (E6) derived from the FVO isolate. The probe hydridized with a 10.5-kb *Hin*dIII fragment (data not shown) and two *Rsa*I fragments (850 bp and 460 bp) in DNA from the K^+ clones (Figure 5C), while again, no hybridization was detectable to the DNA of the K^- clone. A *Rsa*I fragment of 457 bp is predicted from the combined cDNA sequence shown in Figure 4. The occurrence of a homologous *Rsa*I fragment of this size in the genome suggests co-linearity between the cDNA and genomic sequences in this region. Also, the sizes of the *Hin*dIII fragment and the smaller *Rsa*I fragment agree with those calculated from a published restriction map for the gene encoding the KP from strain FCR-3 (Pologe and Ravetch, 1986).

Discussion

We have produced six independent monoclonal antibodies that react with a molecule of ~90 kd which has the properties of the KP of *P. falciparum*: it is present in K^+ and absent from K^- parasites; it is associated with the membranes of infected cells; and it varies in size among isolates. Immunoelectron

microscopic experiments with these monoclonal antibodies localized this protein to the electron-dense region below the lipid bilayer in the area where knobs occur. The KP has previously been shown to be poorly soluble in non-ionic detergents but is extractable in the ionic detergent SDS (Kilejian, 1979; Leech *et al.*, 1984). These solubility properties, together with its location, suggest that the molecule is linked to the erythrocyte membrane skeleton. There is currently no evidence that the KP is a transmembrane protein, as none of the six McAbs detect epitopes on the external surface of parasitized cells.

The proposed association of the KP with the erythrocyte skeleton might be expected to change the relationship of at least some of the normal erythrocyte membrane skeletal proteins. We now know there are at least seven parasite proteins which become associated with the erythrocyte membrane (Stanley and Reese, 1986), and that two of these appear to be linked with the membrane skeleton [a >240-kd protein (H.Stanley, unpublished data) and KP]. Since the KP is the only one of these proteins which is absent in K^- variants we propose that its insertion into the membrane is the essential event which creates the knobs and causes a parasitized erythrocyte to have a dramatically altered surface similar to that of a crenated red blood cell.

The KP-specific McAbs were used to identify a cDNA clone that appears to express an internal segment of the KP which is recognized by these McAbs. The DNA sequence at the 5′ end of the cDNA fragment is almost identical with, and therefore corroborates, the sequence at the 3′ end of a published cDNA sequence (Kilejian *et al.*, 1986) which is thought to encode the amino-terminal end of the KP.

The open reading frame predicted from the combined cDNA sequences contains no stop codons and would encode a protein with a mol. wt of 52 kd, approximately two-thirds of the size of the KP, as determined by the mobility of KP in SDS−polyacrylamide gels. The protein is predicted to have an unusually high content of lysine residues preferentially distributed towards its C-terminal end. This prediction is consistent with two know properties of the KP: (i) it incorporates substantial amounts of exogenous lysine (Kilejian, 1984); and (ii) there is a differential distribution of lysine and histidine in protease-cleavage products of KP (Kilejian *et al.*, 1986).

The amino acid sequence derived for the KP from the combined cDNA sequences was analyzed using computer programs for secondary structure prediction (Devereux *et al.*, 1984) (Figure 6). Although these predictions are theoretical, they are useful for elucidation of the biochemical properties of the protein. The amino-terminal portion of the molecule contains the only major hydrophobic center. This was suggested to be a signal peptide sequence (Kilejian *et al.*, 1986). It is also possible that this protein has no cleavable signal peptide as is the case with a number of membrane proteins including the erythrocyte anion transport protein Band III (Braell and Lodish, 1982). In such a case, we might expect this hydrophobic region to interact with the lipid bilayer of the erythrocyte.

There appear to be two major hydrophilic centers, one in the amino-terminal portion (Figure 6, residues 172−180 and 219−226, which are probably brought together by a disulfide bridge and a turn) and a second, very large, highly hydrophilic region which runs from residue 357 to the end of the encoded structure.

Of the residues composing the total sequence, 30% are charged, and the net charge is clearly basic. However, the charged residues are clustered and appear to be distributed in a non-random fashion. Thus, closely linked groups of three to six amino acids of like charge are found interspersed with stretches containing groups of residues of the opposite charge. For example, the amino acid sequence corresponding to base pairs 1136−1200 (Figure 4) contains two clusters of positively charged residues alternating with two clusters of negatively charged residues. This pattern of interspersed patches of opposite charge appears to promote turns in the peptide backbone in these regions.

That ionic detergents such as SDS are necessary to solubilize the authentic parasite knob protein is consistent with the hypothesis that the predicted charged regions create strong intermolecular salt bridges (like an ionic *velcro*) which lead to insoluble polymer formation. If such bridges are *not* formed, it is difficult to understand why a molecule predicted to be so highly charged would not be readily soluble in an aqueous buffer. Based on the proposed highly charged nature of this molecule, it might play some role in modifying the normal flow of ions through the membranes of infected erythrocytes, since it is known that new permeation pathways with a positively charged character appear in the membranes of parasitized erythrocytes at the trophozoite stage (Ginsburg *et al.*, 1985).

The KP of *P. falciparum* has also been referred to as the knob-associated histidine-rich protein for two reasons: it is readily labeled with radioactive histidine, and it is proposed to have homology with the well-characterized histidine-rich protein from *P. lophurae*, which is 73% histidine in composition. Although the 460 amino acid sequence predicted for the amino-terminal two-thirds of the KP contains 48 histidine residues (10%) it also contains 64 lysines (14%). Most of the histidines are concentrated in three polyhistidine stretches localized between nucleotides 180 and 360 (Figure 4), as previously reported. However, several single histidines are also found in this region, often adjacent to glutamines which comprise 25% of the sequence of this portion of the molecule. Such a grouping of these amino acids should provide ample opportunity for hydrogen bonding. In addition, the second large hydrophilic region contains several histidine residues adjacent to lysine residues, creating an environment which is likely to suppress the weakly basic properties of histidine.

To summarize, we can derive, based on DNA sequence information, a partial amino acid sequence for the KP which suggests it is a highly charged molecule with an unusual composition and distribution of amino acids, resulting in a predicted secondary structure consistent with the known properties of the molecule.

Our evidence that part of the gene for KP is deleted in all the K^- variants of *P. falciparum* we tested is consistent with Pologe and Ravetch's (1986) report of genomic rearrangements resulting in deletions of the 3′ coding sequence for KP in K^- mutants. In contrast, Kilejian *et al.* (1986) found one uncloned K^- variant with no obvious deletion of the KP gene. Although K^- mutants arise readily *in vitro*, it is presently not clear whether they persist during the natural cycle of *P. falciparum* and what their role might be in the biology of the infection.

Materials and methods

Parasites

Isolates and clones of *P. falciparum* were grown using the method of Trager and Jensen (1976). The isolates used were: Honduras I CDC, FVO, Geneva, Sierra Leone, Kenya, Indochina I, Philippines, FCR-3, NF54, K1 and Tanzania. K^+ and K^- clones had been derived previously from the FVO isolate by limiting dilution (Gritzmacher and Reese, 1984).

McAbs

Erythrocytes infected with Honduras I CDC trophozoites were concentrated by sedimentation in Physiogel (Reese *et al.*, 1979). Fragments of the infected erythrocyte membranes were prepared using polylysine-coated tissue culture flasks (Stanley and Reese, 1986). The fragments were then solubilized in 0.1% SDS and lyophilized. The lyophilized fragments were resuspended in phosphate-buffered saline (PBS), mixed with complete Freund's adjuvant and used to immunize a mouse. McAbs-producing hybridomas were prepared from the mouse as previously described (Stanley and Reese, 1985) and used to prepare ascites fluid.

Immunological assays

The McAbs were used in indirect fluorescent antibody tests (IFAT), Western blots and immunoelectron microscopy. The IFAT were initially done using slides of trophozoite-infected erythrocytes fixed at −20°C using 20% methanol in acetone. In subsequent experiments suspensions of unfixed trophozoite-infected cells were used as antigen. For the IFAT, the antigen was incubated with various dilutions of the McAbs for 20 min. After washing with PBS, the bound antibody was detected using an appropriately diluted, affinity-purified, fluorescein−iso-thiocyanate-conjugated goat anti-mouse immunoglobulin (Tago).

The Western blots were done as previously described (Ardeshir *et al.*, 1985). The antigens employed in the Western blots were either entire intact infected erythrocytes solubilized in SDS sample buffer, preparations of the infected erythrocyte surface membranes or lysates of parasitized cells (as a control for the membrane preparations), prepared as previously described (Stanley and Reese, 1986).

Immunoelectron microscopy

Parasitized erythrocytes were fixed with 1% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB) pH 7.3 for 10 min at room temperature. Cells were then washed three times with 0.1 M PB, dehydrated in a graded series of ethanols at −20°C, and embedded in LR White resin (Poly sciences Inc) according to the method described by Atkinson *et al.* (in preparation). The resin was polymerized at 37°C for five days. Thin sections were cut with a diamond knife and mounted on nickel grids.

The sections were pretreated with a saturated aqueous solution of sodium metaperiodate for 1 h at 37°C, then washed several times in distilled water. They were then incubated with 0.1 M PBS containing 5% non-fat dry milk and 0.01% Tween 20, transferred to a drop of purified McAb (diluted at 1:250 to 1:500 with PBS containing 1% BSA and 0.01% Tween 20) and incubated for 2 h at room temperature. Control sections were incubated with the same concentration of ascites prepared using a non-secreting parent cell line. The grids were rinsed thoroughly in PBS−BSA−Tween and then incubated for 1 h at 37°C with a drop of rabbit anti-mouse IgG (Miles Scientific), diluted at 1:100 with PBS−BSA−Tween. Following several rinses in PBS−BSA−Tween, the grids were incubated for 1 h at room temperature in a drop of anti-rabbit IgG bound to gold particles (50 nm), in PBS−non-fat dry milk−Tween. The grids were again rinsed thoroughly in PBS−BSA−Tween followed by distilled water. They were dried and stained with 1% uranyl acetate, 0.4% lead citrate and then examined by a JEOL 100CX electron microscope.

DNA sequencing

The 830-bp cDNA insert from clone A12 was purified on NA45 membrane (Schleicher and Schuell), self-ligated and randomly sheared by sonication. The pieces were subcloned into M13mp8 and sequenced by the chain-termination method as modified by Bankier and Barrell (1983) using [^{35}S]thio-dATP. The chemical degradation method (Maxam and Gilbert, 1977) was also used to confirm parts of the sequence. Both strands were entirely sequenced. The computer programs of Staden (1984) and the University of Wisconsin Genetics Computer Group (UWGCG) (Devereux *et al.*, 1984) were used to analyze the sequence data.

DNA blots

Genomic DNA was prepared from late-stage parasites according to the method of Williamson *et al.* (1985), except that the DNA was banded only once in gradients containing 50% w/v CsCl and 250 mg/ml ethidium bromide. Restriction enzymes were used according to the manufacturer's directions. *Eco*RI, *Hin*dIII and *Rsa*I were purchased from Boehringer Mannheim Biochemicals, *Fnu*4H was from New England BioLabs. Approximately 3 μg of genomic DNA was digested per sample, with a 5-fold excess of enzyme to ensure completion of the digest. Fragments were resolved by agarose gel electrophoresis and transferred (Southern, 1975) to nylon membranes (Biotrans, ICN). The cDNA insert from clone A12 was labeled by nick-translation and hybridized to the blots at 42°C in a mix containing 0.75 M NaCl, 75 mM sodium citrate, 50 mM $NaPO_4$, pH 6.8, 100 μg/ml calf thymus DNA, 0.1% SDS, 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinyl pyrrolidone. Blots were washed at 42°C in 0.1% SDS, 30 mM NaCl and 3 mM sodium citrate (0.2×SSC).

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