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Histidine-rich domain of the knob protein of the human malaria parasite *Plasmodium falciparum*

(*Plasmodium lophurae*/histidine-rich protein)

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ABSTRACT **Membranes of erythrocytes infected with the human malaria parasite *Plasmodium falciparum* develop protrusions called knobs. These structures are essential for the survival of the parasite in the host, and their induction requires the synthesis of the knob protein by the parasite. We describe the isolation of a cDNA clone encoding the amino-terminal half of the knob protein. A cDNA library was constructed from RNA prepared from ring stages of a *P. falciparum* isolate that has retained its ability to induce knobs (knob$^+$ phenotype). A synthetic oligonucleotide probe encoding polyhistidine was used to isolate the cDNA clone, which encodes the amino-terminal half of a polypeptide with all the known attributes of the knob protein. The gene is not transcribed in variants that do not synthesize the knob protein and thereby cannot induce knobs (knob$^-$ phenotype). The apparent lack of transcription in knob$^-$ variants is due to different mechanisms: although the gene is present in one knob$^-$ isolate, it has been deleted in a cloned knob$^-$ variant. The primary structure of the polypeptide deduced from a partial sequence of the cDNA is distinctly different from other malarial histidine-rich polypeptides. The amino-terminal sequence shows the characteristic features of a signal peptide. This is followed by a histidine-rich domain and a subsequent region which contains one histidine. Peptide map analysis of the knob protein is consistent with the structural features deduced from the sequence analysis of the cDNA.**

Plasmodium falciparum causes the most severe form of human malaria. During the course of the erythrocytic development of the parasite, the membranes of the host erythrocyte develop protrusions called knobs (1). These structures mediate the adhesion of infected erythrocytes to vascular endothelium and have been implicated in conferring virulence to the parasites (2, 3). Under laboratory culture conditions some parasites lose their ability to induce knobs (knob$^-$ phenotype) (4). Knob$^-$ variants are not pathogenic to experimental hosts (5, 6).

The only identified biochemical deficiency of knob$^-$ variants is their inability to synthesize the knob protein (KP) (7–9). Several lines of evidence have suggested a relationship of KP with the histidine-rich protein (HRP) of *Plasmodium lophurae*, a polypeptide that contains over 70% histidine (10). KP is one of a few identified polypeptides of *P. falciparum* that incorporate relatively large amounts of exogenous histidine (8, 9, 11, 12), and it is serologically crossreactive with the HRP (25). Moreover, RNA isolated from mixed developmental stages of knob-inducing (knob$^+$) *P. falciparum* isolates contains a HRP-related transcript which is absent in knob$^-$ variants; it has been suggested that this transcript represents the mRNA of KP (13).

The HRP of *P. lophurae* contains a series of tandemly repeated polyhistidine sequences separated by two or three intervening amino acids (14–16). In this study we used an oligonucleotide probe encoding polyhistidine to isolate a cDNA clone that encodes a polypeptide with all the known characteristics of KP.

MATERIALS AND METHODS

Parasites. *P. falciparum* cultures were derived from the Gambian isolate FCR-3 and the Honduran isolate I/CDC. The preparation of knob$^+$ and knob$^-$ uncloned (referred to as wild-type) FCR-3 (7), FCR-3 knob$^+$ clone A-2 and knob$^-$ clone D-4 (17), and FCR-3 knob$^-$ clone C-6 and knob$^+$ clone C-3 (6) has been described. The knob$^+$ clone HB-3 was prepared from a Honduran isolate (18). *In vitro* cultures were maintained in Petri dishes in a candle jar (19). The preparation of synchronized knob$^+$ and knob$^-$ cultures has been described (20).

Preparation of RNA and DNA. RNA was prepared from synchronously growing rings or multinucleate parasites as described previously (21). DNA was isolated from multinucleate parasites which were concentrated by lysis with saponin (20). The parasite pellets were suspended in 100 mM EDTA, pH 8.5/50 mM Tris·HCl, pH 8.5/2% $NaDodSO_4$ with ribonuclease A at 10 μg/ml and incubated 30 min at 37°C. Proteinase K was added (0.1 mg/ml), and after overnight digestion, the samples were deproteinized with phenol. High molecular weight DNA was "spooled" (collected on a glass rod) after the addition of two volumes of cold ethanol.

Construction and Screening of the cDNA Library. RNA prepared from knob$^+$ rings of wild-type FCR-3 was used for construction of a cDNA library in the bacterial plasmid pBR322 by published procedures (15). The library was screened with a synthetic tetradecanucleotide probe encoding polyhistidine (15).

Hybridization Analysis. DNA was digested with restriction endonucleases, size-fractionated by electrophoresis in agarose gels, and transferred to nitrocellulose filters by the method of Southern (22). The methods for denaturation of RNA, electrophoresis in formaldehyde/agarose gels, and transfer to nitrocellulose were as described (21). DNA and RNA blots were hybridized at 42°C in the presence of 30% (vol/vol) formamide (15). The synthetic oligonucleotide probe was end-labeled using T4 polynucleotide kinase, whereas cDNA probes were labeled by nick-translation (23).

DNA Sequence Analysis. Restriction fragments were subcloned in M13 mp18 and mp19 phage and sequenced by the dideoxy chain-termination method (24).

Peptide Mapping. Synchronously growing knob$^+$ FCR-3 rings were labeled for 3 hr with L-[U-^{14}C]histidine or L-[U-^{14}C]lysine at 10 μCi/ml (1 Ci = 37 GBq), and the label was "chased" by incubation in medium without radioactive amino acid for 10 hr (12). The KP of labeled parasites was

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Abbreviations: knob$^+$, knob-inducing; knob$^-$, knobless; KP, knob protein; HRP, histidine-rich protein of *Plasmodium lophurae*; kb, kilobase(s).

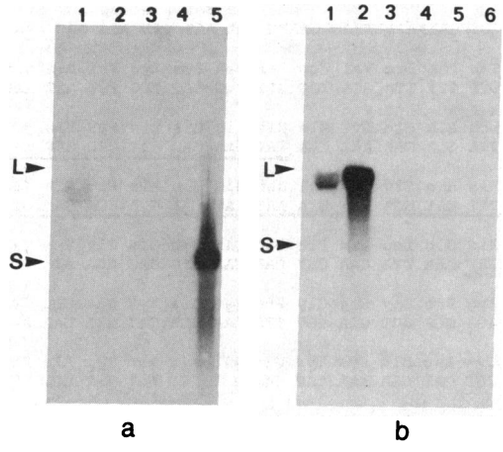

FIG. 1. Transfer blot hybridization of stage-specific RNA from knob$^+$ and knob$^-$ variants of FCR-3. (*a*) Ten micrograms of total RNA from knob$^+$ rings (lane 1), knob$^-$ rings (lane 2), knob$^+$ schizonts (lane 3), knob$^-$ schizonts (lane 4), or *P. lophurae* (lane 5) was hybridized with an oligonucleotide probe encoding polyhistidine. (*b*) Ten micrograms of total RNA from rings of knob$^+$ clone A-2 (lane 1) or 1 μg of oligo(dT)-cellulose-selected RNA from wild-type knob$^+$ rings (lane 2), wild-type knob$^-$ rings (lane 3), wild-type knob$^+$ schizonts (lane 4), knob$^-$ clone D-4 rings (lane 5), or knob$^-$ clone C-6 rings (lane 6) was hybridized with pfc43. Arrowheads mark the positions of large (L) and small (S) ribosomal RNAs of *P. falciparum*.

immunoprecipitated with antibodies produced against the HRP of *P. lophurae* as described (25). Equal amounts of radioactivity from [^{14}C]lysine- and [^{14}C]histidine-labeled immunoprecipitates were electrophoresed in a NaDodSO$_4$/7.5% polyacrylamide gel (26). Gel slices containing radioactive KP were digested with 0.2 μg of *Staphylococcus aureus* V8 protease (Miles) and partial cleavage products were analyzed by electrophoresis in a 10–20% gradient gel by the method of Cleveland *et al.* (27). The radioactive bands were enhanced with Enlightning (DuPont) prior to exposure to x-ray film.

RESULTS

The biosynthesis of KP during the developmental cycle is ring stage-specific and is limited to the 9- to 21-hr period after infection (30). To test for the presence of stage-specific histidine-codon-rich transcripts, RNA prepared from ring and multinucleate stages of knob$^+$ and knob$^-$ variants of wild-type FCR-3 was hybridized with an oligonucleotide probe encoding polyhistidine. (The term wild-type is used to describe parasites obtained from continuous culture of a natural population, in order to distinguish them from parasites derived from a single clone.) The probe hybridized to a knob$^+$ ring-specific transcript (Fig. 1*a*, lane 1) that was not apparent in total RNA from knob$^+$ schizonts (lane 3) or from knob$^-$ rings or schizonts (lanes 2 and 4). RNA from *P. lophurae* (lane 5) was included as a control; as noted previously (13), the HRP mRNA gave a very intense hybridization signal. Long exposure of the autoradiogram did not reveal additional signals. Based on these findings, we used RNA from knob$^+$ rings to construct a cDNA library, which was screened with the synthetic probe. One out of nine positive clones, denoted pfc43, was selected for further analysis.

Similar to the synthetic probe, pfc43 hybridized to knob$^+$ ring RNA of wild-type FCR-3 (Fig. 1*b*, lane 2). The correlation of the knob$^+$ phenotype with the expression of this transcript is further substantiated by the presence of a strong hybridization signal in RNA from a cloned knob$^+$ variant (lane 1) and its absence in RNA of two cloned knob$^-$ variants of FCR-3 (lanes 5 and 6). Only after long exposure of the autoradiogram did a weak hybridization signal become apparent in RNA samples prepared from knob$^+$ multinucleate parasites (lane 4). The lack of a hybridization signal in RNA from rings of knob$^-$ variants cannot be ascribed to qualitative or quantitative differences between knob$^+$ and knob$^-$ samples used in this study. These same RNA samples gave an identical pattern of *in vitro* translation products when tested in a rabbit reticulocyte lysate system (data not shown). Unfortunately, this system proved to be unsuitable for the *in vitro* translation of KP mRNA.

The KP of wild-type FCR-3 is synthesized as a precursor polypeptide of M_r 75,000 (12). The 3- to 3.5-kilobase (kb) RNA transcript that hybridizes with pfc43 is large enough to encode a polypeptide the size of KP. However, the cDNA of pfc43 is only 1.6 kb long, indicating that it is not a complete copy. The cDNA library was rescreened and a second cDNA clone was isolated. The 5′ end of the latter clone overlaps with the 475 base pairs of the 3′ end of pfc43. From a comparison with the restriction map and sequence analysis of a genomic DNA clone (data not shown), we concluded that pfc43 represents the 5′ half of the gene and that reverse transcription of the mRNA could have been primed from a centrally located adenine-rich region. The cDNA of pfc43 was mapped with restriction enzymes and partially sequenced by the strategy shown in Fig. 2.

The primary structure of the polypeptide deduced from the nucleotide sequence has some unusual features (Fig. 3). Unlike most other malarial antigens and histidine-rich proteins that have been analyzed, the polypeptide does not contain tandem repeats composed of a few amino acids. The amino-terminal sequence has the characteristics of a signal peptide: two lysine residues are followed by a stretch of hydrophobic amino acid residues (demarcated by arrowheads). The most striking structural feature is a histidine-rich domain (underlined) that contains 30 histidines within a span of 50 amino acids. The amino acid sequence following the histidine-rich domain does not have outstanding characteristics, with the exception of a notable sparsity of histidine.

Differential incorporation of amino acids into histidine-rich proteins has been used as one of the criteria for their characterization (8, 11, 12). Unlike the two small histidine-rich proteins of *P. falciparum* that have been described (8), KP incorporates substantial amounts of exogenous lysine (12). The deduced amino acid sequence of the histidine-rich domain of pfc43 (Fig. 3) contains very few lysine residues; however, sequence analysis of the complete 3′ half of the

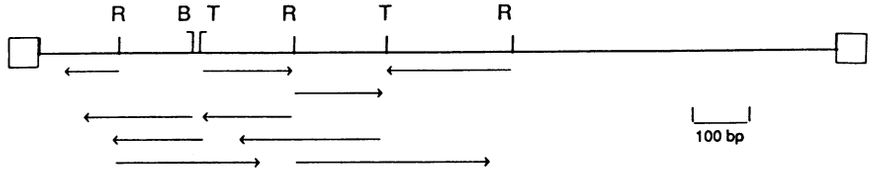

FIG. 2. Restriction map of pfc43 and the strategy for determining the partial nucleotide sequence. Arrows indicate the direction and extent of sequence determined. Letters above the line denote the restriction enzymes: R, *Rsa* I; B, *Bam*HI; T, *Taq* I. bp, Base pairs.

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                                                                  Met Lys Ser Phe Lys Asn Lys
TAATTATTTTTTAAAGGAAAAGTATTATAAAAGAAACAATATATATTATATATTTTACAAAATTTATATATTCGTACATAATTATTAGAGAATG AAA AGT TTT AAG AAC AAA   112

Asn Thr Leu Arg Arg Lys Lys Ala Phe Pro Val Phe Thr Lys Ile Leu Leu Val Ser Phe Leu Val Trp Val Leu Lys Cys Ser Asn Asn
AAT ACT TTG AGG AGA AAG AAG GCT TTC CCT GTT TTT ACT AAA ATT CTT TTA GTC TCT TTT TTA GTA TGG GTT TTG AAG TGC TCT AAT AAC  202

Cys Asn Asn Gly Asn Gly Ser Gly Asp Ser Phe Asp Phe Arg Asn Lys Arg Thr Leu Ala Gln Lys Gln His Glu His His His His His
TGC AAT AAT GGA AAC GGA TCC GGT GAC TCC TTC GAT TTC AGA AAT AAG AGA ACT TTA GCA CAA AAG CAA CAT GAA CAC CAT CAC CAC CAT  292

His His Gln His Gln His Gln His Gln Ala Pro His Gln Ala His His His His His His Gly Glu Val Asn His Gln Ala Pro Gln Val
CAC CAT CAA CAT CAA CAC CAA CAC CAA GCT CCA CAC CAA GCA CAC CAC CAT CAT CAT CAT GGA GAA GTA AAT CAC CAA GCA CCA CAG GTT  382

His Gln Gln Val His Gly Gln Asp Gln Ala His His His His His His His His His Gln Leu Gln Pro Gln Gln Leu Gln Gly Thr Val
CAC CAA CAA GTA CAT GGT CAA GAC CAA GCA CAC CAT CAC CAT CAT CAC CAC CAT CAT CAA TTA CAA CCT CAA CAA CTC CAG GGA ACA GTT  472

Ala Asn Pro Pro Ser Asn Glu Pro Val Val Lys Thr Gln Val Phe Arg Glu Ala Arg Pro Gly Gly Gly Phe Lys Ala Tyr Glu Glu Lys
GCT AAT CCT CCT AGT AAT GAA CCA GTT GTA AAA ACC CAA GTA TTC AGG GAA GCA AGA CCA GGT GGA GGT TTC AAA GCA TAT GAA GAA AAA  562

Tyr Glu Ser Lys His Tyr Lys Leu Lys Glu Asn Val Val Asp Gly Lys Lys Asp Cys Asp Glu Lys Tyr Glu Ala Ala Asn Tyr Ala Phe
TAC GAA TCA AAA CAC TAT AAA TTA AAG GAA AAT GTT GTC GAT GGT AAA AAA GAT TGT GAT GAA AAA TAC GAA GCT GCC AAT TAT GCT TTC  652

Ser Glu Glu Cys Pro Tyr Thr Val Asn Asp Tyr Ser Gln Glu Asn Gly Pro Asn Ile Phe Ala Leu Arg Lys Arg Phe Pro Leu Gly Met
TCC GAA GAG TGC CCA TAC ACC GTA AAC GAT TAT AGC CAA GAA AAT GGT CCA AAT ATA TTT GCC TTA AGA AAA AGA TTC CCT CTT GGA ATG  742

Asn Asp Glu Asp Glu Glu Gly Lys Glu Ala Leu Ala Ile Lys Asp Lys Leu Pro Gly Gly Leu Asp Glu Tyr Gln Asn Gln Leu Tyr Gly
AAT GAT GAA GAT GAA GAA GGT AAA GAA GCA TTA GCA ATA AAA GAT AAA TTA CCA GGT GGT TTA GAT GAA TAC CAA AAC CAA TTA TAT GGA  832

Ile Cys Asn Glu Thr Cys Thr Thr Cys Gly Pro Ala Thr Ile Asp Tyr Val Pro Ala Asp Ala Pro Asn
ATA TGT AAT GAG ACA TGT ACC ACA TGT GGA CCT GCC ACT ATA GAT TAT GTT CCA GCA GAT GCA CCA AAT  904
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FIG. 3. DNA sequence of the 5′ end of pfc43. The deduced amino acid sequence is shown above the DNA sequence. Arrowheads delimit the hydrophobic stretch of amino acids. The histidine-rich domain is underlined. Numbers at right indicate the last nucleotide in each line.

corresponding gene isolated from a genomic DNA library shows an abundance of lysine residues (data not shown). These findings predict a differential distribution of histidine and lysine in different domains of the polypeptide. This prediction was tested by comparing the peptides generated from partial proteolysis of [^{14}C]histidine-labeled KP with those from [^{14}C]lysine-labeled KP. Despite some loss in resolution due to long exposure of the fluorogram, differential incorporation of these two amino acids in peptides of KP is clearly apparent (Fig. 4) and further supports the conclusion that pfc43 encodes the amino-terminal half of KP.

Although genetic variation is apparent in natural populations as well as laboratory isolates of *P. falciparum* (28), the knob$^-$ phenotype is clearly induced in the laboratory. To determine whether the change from knob$^+$ to knob$^-$ phenotype is due to a single mechanism, the genomic organization of pfc43 in four knob$^+$ variants (wild-type FCR-3 and clones A-2, C-3, and HB-3) was compared with that in two knob$^-$ variants (wild-type FCR-3 and clone D-4). DNA isolated from each variant was digested with *Dra* I (Fig. 5*a*) or *Hin*dIII (Fig. 5*b*). The samples were size-fractionated in agarose gels, transferred to nitrocellulose, and probed with pfc43. The gene was present in all knob$^+$ variants tested (lanes 1, 2, 4, and 6) as well as the wild-type knob$^-$ FCR-3 (lanes 3). No hybridization signal was detected in knob$^-$ clone D-4 (lanes 5); this clone is apparently a deletion mutant. In view of the importance of knobs for the survival of the parasite in the host, natural selection would eliminate deletion mutants such as clone D-4 from natural parasite populations.

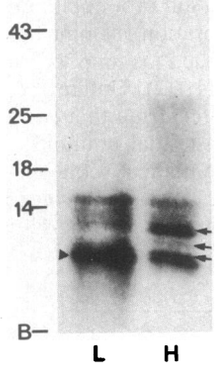

FIG. 4. Comparison of peptide map of KP labeled with [^{14}C]lysine (L) or [^{14}C]histidine (H). Positions of molecular weight markers (M_r × 10^{-3}) are at left. B, bromophenol blue front. Arrows denote peptides that are preferentially labeled with histidine. Arrowhead denotes a polypeptide preferentially labeled with lysine.

DISCUSSION

We have described the isolation of a cDNA clone that encodes the amino-terminal half of a polypeptide with all the known biochemical attributes of the KP of *P. falciparum*. Previous work showed that the KP of the FCR-3 isolates (*i*) is synthesized as a precursor polypeptide of M_r 75,000 molecular weight by knob$^+$ rings but not by knob$^-$ parasites, (*ii*) incorporates relatively large amounts of exogenous histidine as well as lysine, and (*iii*) is serologically crossreactive with the HRP of *P. lophurae* (7, 11, 12, 21, 25). Consistent with the above findings, pfc43 hybridized with a transcript that is present in knob$^+$ rings but not knob$^-$ parasites (Fig. 1); the polypeptide sequence deduced from the nucleotide sequence of pfc43 has a histidine-rich domain which contains

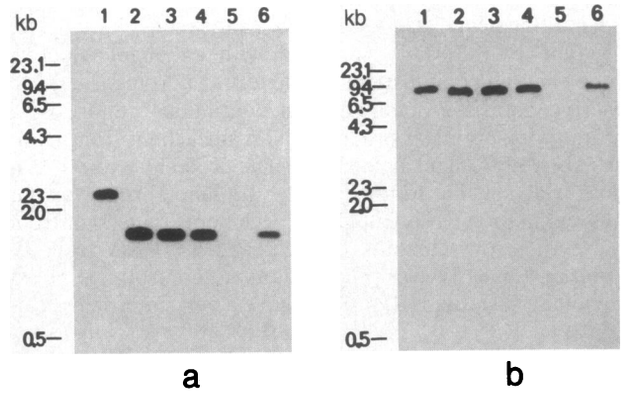

FIG. 5. Southern blot analysis of genomic DNA digested with *Dra* I (*a*) or *Hin*dIII (*b*) and hybridized with nick-translated pfc43. The *P. falciparum* isolates were knob$^+$ clone HB-3 (lanes 1), wild-type knob$^+$ FCR-3 (lanes 2), wild-type knob$^-$ FCR-3 (lanes 3), knob$^+$ clone A-2 (lanes 4), knob$^-$ clone D-4 (lanes 5), and knob$^+$ clone C-3 (lanes 6). Positions of *Hin*dIII fragments of bacteriophage λ DNA, used as size markers, are shown at left.

polyhistidine sequences similar to the HRP (Fig. 3). Furthermore, consistent with the differential incorporation of histidine and lysine in the peptides of KP (Fig. 4), pfc43 shows an uneven distribution of histidine (Fig. 3).

Although comparative studies on knob$^+$ and knob$^-$ variants from different geographical isolates have shown differences in the electrophoretic mobility of KP, they have not revealed any additional polypeptides that can be correlated with the induction of knobs or the knob$^+$ phenotype (8, 9). Therefore, KP is the only known polypeptide of *P. falciparum* that has the same characteristics as the polypeptide encoded by pfc43.

In addition to KP, ring stages of *P. falciparum* synthesize two smaller polypeptides that incorporate high levels of exogenous histidine but are present in both knob$^+$ and knob$^-$ parasites (8, 12). The primary structure of one of these, a small histidine- and alanine-rich polypeptide (SHARP) was determined recently (31). This polypeptide contains tandem repeats of hexapeptide and pentapeptide sequences that include two histidine residues. pfc43 does not contain repeated sequences, with the exception of the three polyhistidine stretches (Fig. 3). With our current knowledge of the presence of tandemly repeated sequences composed of a few amino acids in several polypeptides of malaria parasites, it is not surprising that polypeptides that contain histidine in a repeated unit would be histidine-rich. The physiological functions of HRP and SHARP are not known, and there does not appear to be any evolutionary relationship between these two polypeptides (31). KP is clearly essential for the induction of a specific structural complex, the knobs, that is important for the survival of the parasite in the host. The specific interaction of KP with the host erythrocyte membrane is not known. Current evidence indicates that in addition to the synthesis of KP, other factors are involved in the formation of a functionally complete structure that can mediate the adhesion of infected erythrocytes to endothelial cells (29). The elucidation of the complete structure of KP would permit studies on its chemical interactions with the host membrane that lead to the formation of knobs. Since, regardless of size differences, the KP of all geographical isolates incorporates relatively large amounts of exogenous histidine, some sequences of the histidine-rich domain must have been conserved. It remains to be seen whether polyhistidine sequences are essential for the induction of knobs.

The apparent lack of transcription of the KP gene in the two knob$^-$ variants studied is due to different mechanisms. Although the gene is present in wild-type knob$^-$ FCR-3, it has been deleted in knob$^-$ clone D-4. Adaptation of natural populations of parasites to *in vitro* culture conditions most likely involves several selective pressures. In culture, knob$^-$ variants can arise from wild-type as well as cloned isolates (4, 9). There has been one report of the reversion of a knob$^-$ variant to a knob$^+$ phenotype (9). With increasing evidence for the fluidity and rapid change of the genomic organization of *P. falciparum* (28), the expression of knob$^+$ or knob$^-$ phenotype may not necessarily reflect any one specific regulatory mechanism.

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