Expression of cloned cDNA for a major surface antigen of *Plasmodium falciparum* merozoites

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A cDNA library of *P. falciparum* was constructed. Using size-selected mRNA as a probe several clones were isolated which hybridized to mRNAs larger than 5 kilobases (kb). The cDNA insert of pFC 17, which hybridizes to 5.6-kb mRNA was expressed by fusion to anthranilate synthetase I in a plasmid expression vector. The expressed fusion protein was shown to contain epitopes of a 195-kDa protein which is the precursor to 3 major surface antigens of *P. falciparum* merozoites.

Human malaria *Surface antigen* *cDNA* *Fusion polypeptide*

1. INTRODUCTION

Malaria is an increasing health problem throughout the third world. Several hundred million people suffer from the disease and the most acute form, caused by the protozoan parasite *Plasmodium falciparum,* kills over a million children a year in Africa alone. Prevention of reinvasion of red blood cells by an effective immune response against the invasive form, the merozoite, should interrupt the multiplication cycles of the parasite in the bloodstream, thereby preventing the clinical symptoms of the disease. In a rodent malaria model it has been shown that vaccination with a purified antigen of 23 kDa can generate protective immunity against *P. yoelii* [1]. For *P. falciparum* an analogous protein of 195 kDa has been identified [2] which cross-reacts immunologically [3] with the 230-kDa protein of *P. yoelii*. The biosynthesis of this 195-kDa protein takes place in the mature intraerythrocytic forms, the schizonts, and the protein is subsequently processed into discrete fragments [2]. Three of these fragments of 83, 42 and 19 kDa are major surface antigens of merozoites and are strongly recognized by human immune serum [4,5]. For these reasons the 195-kDa protein of *P. falciparum* is a possible candidate for a blood stage malaria vaccine. We now report the cloning and expression of cDNA for the 195-kDa protein. The bacterially produced fusion protein is shown to share epitopes with the parasite-derived 195-kDa protein.

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2. EXPERIMENTAL

2.1. Cultures and RNA extraction

The West African strain of *P. falciparum* was maintained as cultures as in [2]. Parasites were synchronized with D-sorbitol [4] and 30–40 h after the last round of reinvasion total RNA, polyadenylated RNA and size-fractionated RNA were isolated as in [6].

2.2. cDNA library

cDNA was synthesized in 50 μl reaction mixture containing 6 μg polyadenylated RNA, 5 μg oligo-$dT_{(12-18)}$, 1 mM of each nucleoside triphosphate, 0.1 M Tris–HCl (pH 8.3), 10 mM $MgCl_2$, 140 mM KCl, 10 mM DTT and 30 units AMV reverse trans-

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criptase (Life Science Inc., St. Petersburg, FL) for 90 min at 42°C. Second strand synthesis was in 0.1 ml of 0.1 M Hepes (pH 6.9), 10 mM $MgCl_2$, 2.5 mM DTT, 70 mM KCl, 0.5 mM of each nucleoside triphosphate and 50 units *E. coli* DNA polymerase large fragment (Boehringer Mannheim) for 16 h at 15°C. After digestion with nuclease SI, 5 μg DNA recovered and 0.5 μg were inserted into the *PSt*I site of pUC8 [7] using homopolymeric G-C tails as in [8]. $CaCl_2$-treated [8] *E. coli* HB101 cells were used for transformation.

2.3. Colony hybridization

Replica filters carrying recombinants were prepared for hybridization as in [8]. Size-fractionated [6] mRNA (50 ng), with an apparent sedimentation coefficient of 33 S, was partially hydrolysed by incubation in 50 mM Na_2CO_3 for 20 min at 40°C and was subsequently 5'-end labeled with [γ-^{32}P]ATP using polynucleotide ligase (Biolabs). The probe (0.5×10^6 cpm) was hybridized to the replica filters as in [8].

2.4. Northern blots

Five μg *P. falciparum* RNA were denatured in DMSO and glyoxalated as in [9]. After electrophoresis through a 0.7% agarose gel blots [10] were prepared on nylon filters (Biodyne) which were probed with nick-translated [8] plasmid DNA.

2.5. Construction of trpE-cDNA fusions

The plasmid pATtrp was kindly provided by Dr A.J. Makoff (unpublished). It contains an antranilate synthatase 1 gene preceded by the tryptophan promotor-operator system, inserted into the *Eco*RI site of pAT 153 [11]. pATtrp DNA was digested with *Bss*HII, repaired with *E. coli* DNA polymerase large fragment and ligated to blunt end cDNA fragments. The cDNA fragments were generated by digestion of the cDNA insert of pFC17 (see text) with 0.1 units Bal 31 per μg DNA for 8 min at 37°C, followed by repair with DNA polymerase large fragment. Ligated DNA was used to transform *E. coli* MM 294.

2.6. Analysis of fusion polypeptides

Bacterial clones carrying pATtrp-cDNA fusions were grown overnight at 37°C in a medium consisting of M9 salts supplemented with thiamine, glucose and casamino acids but without additional tryptophan. One ml culture was added to 4 ml of the same medium, prewarmed and containing either 50 μg 3-β-indoleacrylic acid or 200 μg tryptophan, followed by 4 h of incubation at 37°C. Cells were collected by centrifugation and proteins from 0.1 A_{650} of bacteria were analyzed by SDS-polyacrylamide electrophoresis (SDS–PAGE) [12] followed by staining or western blotting.

Western blots were prepared as in [3]. After SDS–PAGE proteins were transferred electrophoretically to a nitrocellulose membrane, probed with a polyvalent rabbit antiserum raised against purified *P. falciparum* 195-kDa protein, and developed by reaction with ^{125}I-labelled protein A followed by autoradiography. Generation of the antiserum was performed as in [5]. Briefly, 195-kDa protein was purified from detergent extracts of *P. falciparum* schizonts by monoclonal antibody–Sepharose affinity chromatography, using monoclonal antibody 89.1 [1,2]. A rabbit was immunized with 100 μg purified protein in Freund's complete adjuvant and boosted with 100 μg protein in Freund's incomplete adjuvant on 3 subsequent occasions.

3. RESULTS

The isolation of functional messenger RNA from synchronous cultures of *P. falciparum* has been described [6]. Total polyadenylated RNA from late stage parasites was used for the synthesis and cloning of cDNA. A library of 3000 recombinants in the multicopy plasmid pUC8 [7] was constructed. On the basis of [^{35}S]methionine incorporation, the 195-kDa protein is estimated to represent at least 1% by mass of total parasite protein synthesized in schizonts. By inference the probability of the presence of a recombinant of interest in the cDNA library was estimated to be >95%.

The size of mRNA coding for a protein of 195 kDa was expected to exceed 5 kb. Accordingly, it has been possible to obtain a partially purified mRNA probe for the 195-kDa protein by size fractionation in sucrose gradients [6]. The mRNA for the 195-kDa protein, as detected by translation in vitro, was present in fractions containing RNA with an apparent sedimentation coefficient of 28–35 S. ^{32}P kinase-labelled RNA from the 33 S fraction was used as a probe in a colony hybridiza-

tion experiment. Of the 300 recombinant plasmids in the library, 60 were detected with the probe, and 12 of these showed strong hybridization signals. With these 12 recombinants 6 groups were formed on the basis of cross-hybridization of inserts. One member of each group was labelled and used as a probe on *P. falciparum* RNA Northern blots. Three recombinants, pFC15, pFC16 and pFC17 with cDNA inserts of 1.6, 2.3 and 1.1 kb, respectively, hybridized to mRNAs with a size larger than 5 kb. By extrapolation from the sizes of the ribosomal RNAs, the sizes of these mRNAs were estimated to be 9 kb for pFC15 (fig.1, track 1), 7.5 kb for pFC16 (fig.1, track 2) and 5.6 kb for pFC16 (fig.1, track 3). The 3 other plasmids detected mRNAs smaller than 5 kb.

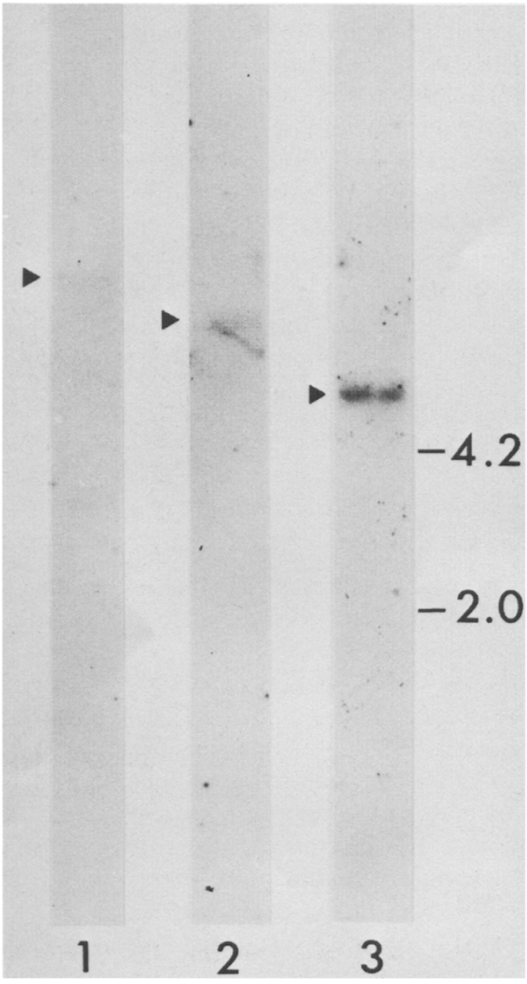

Fig.1. Cloned *P. falciparum* cDNA analysed on Northern blots. Five μg *P. falciparum* RNA was denatured in the presence of glyoxal [9] and transferred [10] to nylon membranes (Biodyne) after electrophoresis through a 0.7% agarose gel. Tracks: (1) hybridization with pFC15 DNA; (2) hybridization with pFC16 DNA; (3) hybridization with pFC17 DNA. The sizes of 4.2 and 2.0 kb indicate the position of the major ribosomal RNAs [6].

Further characterization of pFC17 was performed by expression of the cDNA insert. As pFC17 did not show detectable levels of expression of the cDNA insert sequence, isolated insert was treated with exonuclease Bal 31 to give randomized reading frames and was inserted into an expression plasmid carrying part of the tryptophan operon. DNA was inserted into the *Bss*HII site 13 amino acids from the carboxy terminus of the mature trpE-gene product, anthranilate synthetase I. In phase insertion of cDNA into this site will yield a fusion protein carrying 56 kDa of anthranilate synthetase I. The fusion protein can be distinguished from host proteins on the basis of induction of the gene by tryptophan starvation in the presence of 3-β-indoleacrylic acid. Fig.2 shows the induction

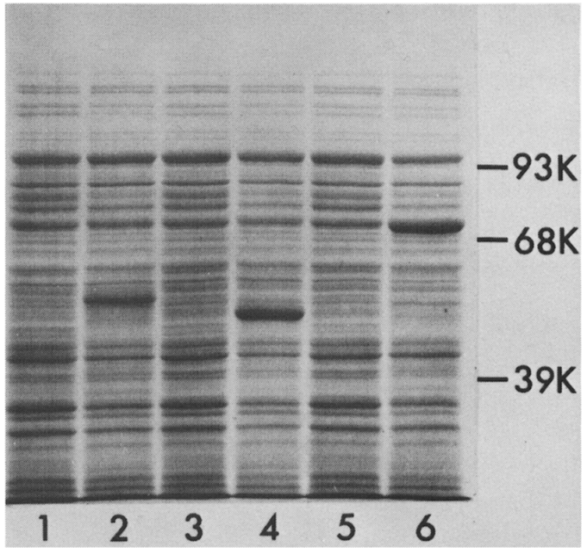

Fig.2. Induction of trpE-fusion polypeptides. Proteins from 0.1 A_{650} of bacteria were analyzed by electrophoresis in a 7.5% SDS-polyacrylamide gel [12]. Proteins synthesized in repressed cultures are in tracks 1, 3 and 5, and of induced cultures in tracks 2, 4 and 6. Tracks: (1,2) proteins synthesized in bacteria with a control plasmid carrying an insert in a closed reading frame; (3,4) proteins from cultures containing bacteria with a control plasmid without an insert; (4,5) proteins synthesized by bacteria carrying pFT1733.

of the fusion protein encoded by one of the resulting recombinants pFT1733 (tracks 5,6). As controls, a plasmid with the insert in an apparently closed frame (tracks 1,2) and a plasmid without any insert (tracks 3,4) were used.

Bacterial extracts and an extract of *P. falciparum* schizonts were subjected to SDS–PAGE, transferred to nitrocellulose and then probed with a polyvalent rabbit serum specific for the 195-kDa protein [5]. The fusion protein of pFT1733 (fig.3, track 2) clearly reacted with the antiserum. The specificity of the antiserum is shown in track 1, where from a total *P. falciparum* schizont extract only the 195-kDa protein is detected. There was no reaction (track 2) with a bacterial extract containing an 80-kDa fusion protein consisting of the trpE-gene product and a foot and mouth disease virus VP1 protein. In addition, the control using normal rabbit serum was negative (not shown). Thus, pFC17 encodes some of the antigenic determinants of the *P. falciparum* 195-kDa protein. The fusion polypeptide coded by pFT1733 has a molecular mass of 72 kDa and therefore carried an additional 16 kDa encoded by the cDNA insert. pFT1733 contains a cDNA insert of 800 nucleotides, about twice the size needed to code for 16 kDa, indicating that the cDNA may cover the stop codon of the mRNA.

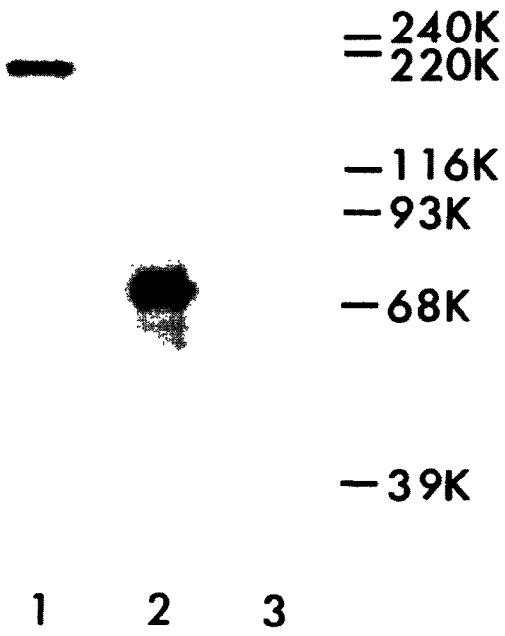

Fig.3. Western blot analysis of induced trpE-fusion proteins. Proteins from induced bacteria (see fig.2) and *P. falciparum* schizont proteins were analyzed on a Western blot. Tracks: (1) SDS-solubilized proteins of *P. falciparum* schizont infected erythrocytes; (2) extract of bacteria containing pFT1733; (3) extract of bacterial expressing a trpE-FMDV fusion protein (a kind gift from Dr A.J. Makoff).

4. DISCUSSION

As outlined in section 1, the 195-kDa protein probably plays an important role in the generation of protective immunity against malaria, and the cloning of part of its gene therefore constitutes a step towards the development of a merozoite based malaria vaccine. Expression of cloned cDNA will provide large amounts of pure antigens for vaccination studies.

It will be of interest to use pFC17 as a probe to isolate further cDNA clones covering the complete mRNA for the 195-kDa protein and to study the structure of its gene. Is is likely that the 195-kDa protein exhibits a degree of antigenic diversity [13,14]. It will now be possible to use cloned probes to study antigenic diversity in *P. falciparum* at the level of DNA sequence. Also, the organization of the processed fragments within the 195-kDa precursor protein can be investigated using antisera against fusion proteins of known cDNA fragments.

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