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A 75 kd merozoite surface protein of *Plasmodium falciparum* which is related to the 70 kd heat-shock proteins

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Proteins on the merozoite surface of the human malarial parasite *Plasmodium falciparum* are targets of the host's immune response. The merozoite surface location of p75, a 75 kd *P. falciparum* protein, was established by immunoelectron microscopy using antisera raised to the expressed product of a cDNA clone. Immunoprecipitation from protein extracts biosynthetically labeled during different periods of the asexual cycle showed that p75 is made continuously, although ring-stage parasites appear to synthesize larger quantities. p75 is conserved and invariant in size in eight isolates of *P. falciparum*. The 880 bp cDNA sequence encoding part of p75 reveals one open reading frame containing a repetitive sequence unit of four amino acids. The predicted reading frame is correct since antisera to a synthetic peptide corresponding to the repetitive region recognize p75 in immunoblots. The sequence of p75 is homologous with the sequences of proteins from the ubiquitous, highly conserved family of 70 kd heat-shock proteins, suggesting an important physiological function for p75. The cDNA fragment encoding part of p75 hybridizes with multiple genomic fragments, whose sizes are identical in DNA from nine *P. falciparum* strains, suggesting that the gene for p75 is well conserved and may be part of a gene family.

Key words: human malaria/*P. falciparum*/merozoite surface protein/cDNA sequence/70 kd heat-shock proteins

Introduction

The parasite *Plasmodium falciparum* is responsible for the most virulent form of human malaria. The intra-erythrocytic parasite matures inside the cell, passing through the ring and trophozoite stages, and then undergoes repeated nuclear division to produce a schizont. Each schizont gives rise to several merozoites that are free in the blood for a brief period before they re-invade fresh erythrocytes. The merozoite, therefore, is the only extracellular form of the parasite during its asexual development.

The extracellular merozoite is an important target of the host's immune response to the parasite (Langreth and Reese, 1979). Hence, it is essential to obtain an understanding of the macromolecular structures on the merozoite surface and their antigenic properties prior to defining those that might be used to induce protective immunity. A number of merozoite proteins have been identified (for a review, see Heidrich, 1986). Of these, the most well-characterized at the genetic and biochemical levels is the glycoprotein gp185, which is the precursor of at least two merozoite surface proteins (Holder and Freeman, 1984; MacKay *et al.*, 1985; Howard *et al.*, 1985).

One approach chosen by our group to identify and characterize specific merozoite proteins was to clone and express *P. falciparum* cDNA in *Escherichia coli*, starting with the mRNA from mature asexual-stage parasites (Ardeshir *et al.*, 1985). The *P. falciparum* cDNA library was screened by colony immunoassay with sera from owl monkeys immune to *P. falciparum*. These sera, which are known to be protective *in vitro* and *in vivo*, reacted with many clones in the expression library. Using rodent antisera against bacterial lysates of each clone, plasmodial proteins corresponding to these clones were identified by immunofluorescence, immunoblotting and immunoprecipitation assays with parasite antigens. Indirect immunofluorescence studies of extracellular merozoites showed that three of these parasite proteins appeared to be located on the merozoite surface.

In this paper, we clearly establish by immunoelectron microscopy the merozoite surface location of one of these, a 75 kd protein (p75) corresponding to the expressed product of the previously described cDNA clone C7 (Ardeshir *et al.*, 1985). This clone harbors the recombinant plasmid pfC7. In addition, we demonstrate that p75 is invariant in size in several geographically distinct parasite isolates, and that it is made throughout the asexual cycle. Also, we report the DNA sequence of the 880 bp cDNA insert from plasmid pfC7, which encodes part of p75. It contains one open reading frame whose predicted amino acid sequence includes a repetitive unit of four amino acids. Antisera raised against a synthetic peptide corresponding to the repetitive region recognize p75 in immunoblots of parasite proteins, demonstrating that the predicted reading frame is correct for the plasmodial protein. We also present the results of Southern blotting experiments with the 880 bp cDNA fragment as a probe, which suggest that the gene encoding p75 is highly conserved among *P. falciparum* isolates, and may be part of a multigene family.

Our most remarkable finding is that the predicted partial amino acid sequence for p75 shows strong homology with the sequences of proteins belonging to the highly conserved family of 70 kd heat-shock proteins (hereafter referred to as the hsp70 family). The expression of many of the proteins in this family is dramatically induced by heat and other stresses in a universal cellular response observed in species as evolutionarily distant as *E. coli* and man (reviewed in Craig, 1985; Lindquist, 1986). However, some proteins of the hsp70 family are constitutively expressed while some are subject to developmental control. The ubiquity and striking conservation of the 70 kd hsp, as well as their presence in normal cells, indicates their importance in cellular metabolism. The observation that the *P. falciparum* merozoite surface protein p75 is related to the hsp70 family suggests that p75 may perform an important physiological function and is consistent with the unusually strong conservation of this protein among parasite isolates.

Results

Localization of p75 on the merozoite surface

Antisera raised against bacterial lysates of the *P. falciparum* cDNA clone C7 (anti-C7 sera) have previously been shown to recognize a 75 kd parasite protein. Immunofluorescence exper-

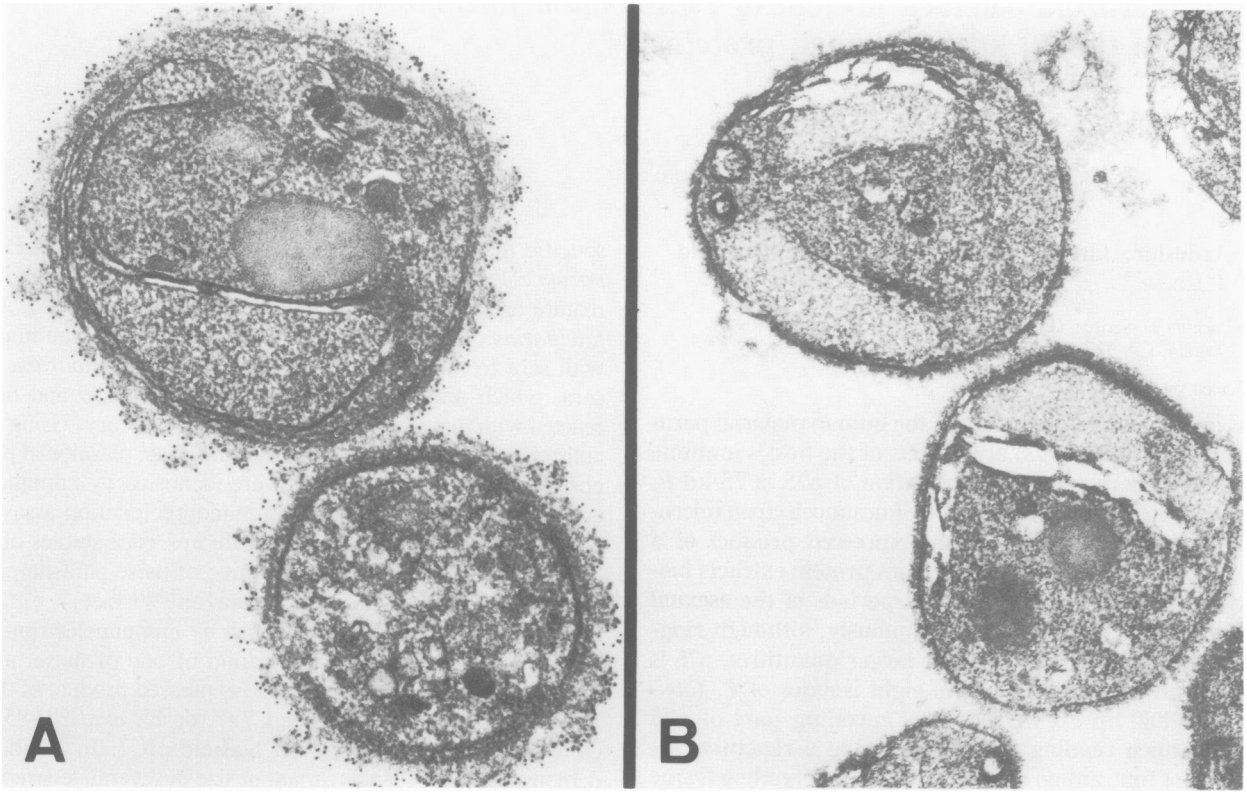

Fig. 1. Immunoelectron micrographs of extracellular merozoites of *P. falciparum*. Merozoites were incubated with **(A)** mouse antiserum to bacterial lysates of clone C7, **(B)** mouse antiserum raised to *E. coli* containing the cloning vector pUC8 (negative control). They were then treated as described in Materials and methods.

iments using these antisera on live extracellular merozoites suggested that this protein was located on the merozoite surface (Ardeshir *et al.*, 1985). Here we show that in immunoelectron microscopic studies on lightly fixed extracellular merozoites, mouse anti-C7 serum clearly reacts with a surface component (Figure 1, panel A), thus establishing that the C7 antigen is located on the merozoite surface.

Biosynthesis of p75 during asexual development

Immunofluorescence experiments (Ardeshir *et al.*, 1985) showed that the C7 antigen was not only found in merozoites but was also present in ring and trophozoite stage parasites. The p75 detected by these assays could either have been carried into the erythrocytes with the invading merozoite or synthesized *de novo* by the newly established ring.

To determine when this protein is synthesized during the 48-h erythrocytic cycle, and particularly if it is made during the ring stage, a parasite culture was synchronized so that the difference in the ages of the youngest and oldest ring was not more than 4–6 h. The synchronous parasite culture was divided into aliquots, each of which was biosynthetically labeled with [^{35}S]-methionine during a different period of the developmental cycle. The p75 made during each stage was then immunoprecipitated with anti-C7 rat serum (Figure 2). The amount of [^{35}S]methionine incorporated into p75 during each labeling period was quantitated by densitometric analysis of the autoradiographs of the radioimmunoprecipitates, taking into account the differences in labeling periods and relative volumes for each sample. Large amounts of p75 were synthesized during the ring and trophozoite stages (Figure 2, lanes 1, 2 and 3), and as the parasite matured, the apparent level of synthesis steadily decreased. During the last

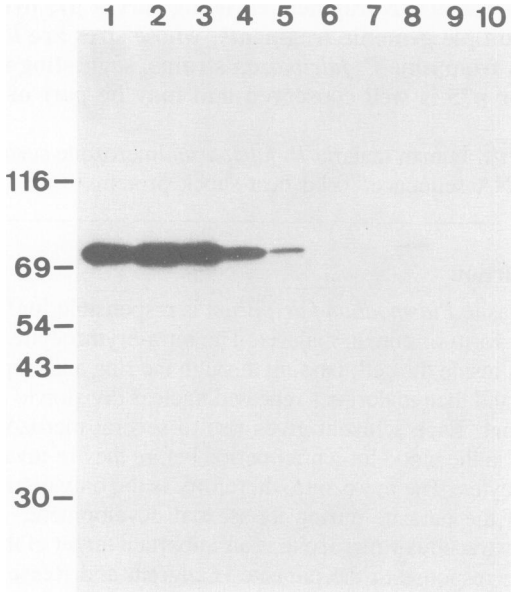

Fig. 2. Quantitative radioimmunoprecipitation of p75 labeled at various times during the asexual erythrocytic growth cycle. A synchronous culture of Honduras-isolate, ring-infected erythrocytes was divided into five aliquots. Aliquot 1 **(lanes 1, 6)** was labeled as early ring-stage parasites became large rings (10 h); aliquot 2 **(lanes 2, 7)** from large rings to young trophozoites (13 h); aliquot 3 **(lanes 3, 8)** from young trophozoites to large trophozoites (7 h); aliquot 4 **(lanes 4, 9)** from large trophozoites to schizonts containing four nuclei or less (6 h); aliquot 5 **(lanes 5, 10)** as nuclear division was completed and intracellular merozoites began to accumulate (4 h). Protein extracts were immunoprecipitated with either rat anti-C7 serum **(lanes 1–5)** or pre-immune rat serum **(lanes 6–10)**.

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  2  ATTCCGGGTGACCAATCAAATGCTGTCCAAGATTTATTATTATTAGATGTT  52

 53  TGCTCCTTATCATTAGGTTTAGAAACTGCTGGTGGTGTTATGACCAAATTA 103
                                     MetThrLysLeu

104  ATTGAAAGAAACACAACCATACCTGCTAAAAAGAGTCAAATCTTTACTACT 154
     IleGluArgAsnThrThrIleProAlaLysLysSerGlnIlePheThrThr

155  TATGCTGATAACCAACCAGGTGTCTTAATTCAAGTATATGAAGGTGAAAGA 205
     TyrAlaAspAsnGlnProGlyValLeuIleGlnValTyrGluGlyGluArg

206  GCCTTAACCAAAGATAACAATTTATTAGGAAAATTTCACTTAGATGGTATT 256
     AlaLeuThrLysAspAsnAsnLeuLeuGlyLysPheHisLeuAspGlyIle

257  CCACCTGCACCAAGAAAGGTACCACAAATCGAAGTTACATTCGATATCGAT 307
     ProProAlaProArgLysValProGlnIleGluValThrPheAspIleAsp

308  GCTAACGGTATCTTAAACGTTACGGCTGTAGAAAAATCCACTGGTAAACAA 358
     AlaAsnGlyIleLeuAsnValThrAlaValGluLysSerThrGlyLysGln

359  AACCATATTACAATTACCAACGACAAAGGAAGATTATCTCAAGATGAAATT 409
     AsnHisIleThrIleThrAsnAspLysGlyArgLeuSerGlnAspGluIle

410  GATCGTATGGTTAATGATGCTGAAAAATACAAAGCAGAAGATGAAGAAAAC 460
     AspArgMetValAsnAspAlaGluLysTyrLysAlaGluAspGluGluAsn

461  AGAAAAAGAATCGAAGCAAGAAACAGCCTTGAAAATTACTGCTATGGAGTT 511
     ArgLysArgIleGluAlaArgAsnSerLeuGluAsnTyrCysTyrGlyVal

512  AAAAGCTCATTAGAAGACCAAAAAATTAAAGAAAAATTACAACCAGCTGAA 562
     LysSerSerLeuGluAspGlnLysIleLysGluLysLeuGlnProAlaGlu

563  ATTGAAACATGTATGAAAACTATTACAACCATACTTGAATGGTTAGAAAAA 613
     IleGluThrCysMetLysThrIleThrThrIleLeuGluTrpLeuGluLys

614  AACCAACTTGCTGGAAAAGATGAATATGAAGCCAAACAAAAAGAAGCAGAA 664
     AsnGlnLeuAlaGlyLysAspGluTyrGluAlaLysGlnLysGluAlaGlu

665  TCGGTTTGTGCTCCAATTATGTCTAAAATCTATCAAGATGCTGCTGGTGCA 715
     SerValCysAlaProIleMetSerLysIleTyrGlnAspAlaAlaGlyAla

716  GCCGGTGGTATGCCAGGAGGTATGCCCGGTGGAATGCCAGGTGGAATGCCA 766
     AlaGlyGlyMetProGlyGlyMetProGlyGlyMetProGlyGlyMetPro

767  GGTGGTATGAATTTCCCAGGAGGTATGCCCGGAGCAGGAATGCCAGGAAAT 817
     GlyGlyMetAsnPheProGlyGlyMetProGlyAlaGlyMetProGlyAsn

818  GCCCCAGCTGGAAGTGGACCAACAGTTGAAGAAGTTGATTAAACTAATAAA 868
     AlaProAlaGlySerGlyProThrValGluGluValAspEnd

869  AAAAAAAAAAA 879
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Fig. 3. Nucleotide sequence of the cDNA insert from plasmid pfC7 (top line); proposed amino acid sequence of the expressed bacterial protein (lower line).

labeling period, which covered the assembly of parasite components into discrete merozoites within the erythrocyte, the amount of incorporation into p75 was 10% or less than the incorporation during the ring stage. This biosynthetic pattern is different from that of other merozoite proteins for which data are available (Hall *et al.*, 1984; Brown *et al.*, 1985). The additional possibility mentioned earlier, that p75 could be carried into erythrocytes with invading merozoites, is currently under investigation.

Sequence of the cDNA

The nucleotide sequence of the 880 bp insert from the plasmid pfC7 was completely determined (Figure 3, top line). During the construction of the cDNA library, *Eco*RI linkers were attached to the ends of the cDNA molecules corresponding to the 5′ end of the mRNA and *Sal*I linkers to the ends corresponding to the 3′ end of the mRNA (Ardeshir *et al.*, 1985). The cDNA molecules were then ligated between the *Eco*RI and *Sal*I sites in the *lacZ* gene of the expression vector pUC8 (Vieira and Messing,

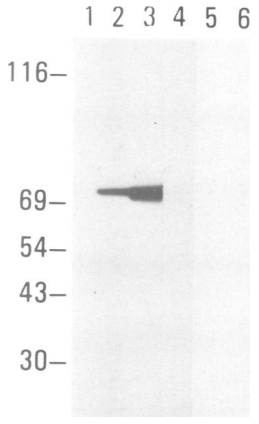

Fig. 4. Detection on immunoblots of p75 with antipeptide sera. Cultures of the Honduras I isolate were harvested as a mixture of trophozoites and schizonts, and the proteins were prepared for electrophoresis and immunoblotting as described **(lanes 1–4). Lanes 5** and **6** contain proteins from uninfected human erythrocytes. Antisera were used at a dilution of 1:100. Nitrocellulose strips were treated as follows: 1,5, prebleed from rabbit whose serum was used in **lane 2**; 2,6, rabbit antiserum to (Gly-Gly-Met-Pro)$_3$-Gly conjugated with KLH; **lane 3**, mouse anti-C7 serum; **lane 4**, mouse antiserum to *E. coli* containing pUC8 (negative control for **lane 3**). Strips 1, 2, 5 and 6 were then treated with ^{125}I-labeled goat anti-rabbit antibodies, strips 3 and 4 with ^{125}I-labeled rabbit anti-mouse antibodies.

1982). The pfC7 cDNA insert does have an *Eco*RI site at one end (nucleotide 2 is the second base of the *Eco*RI site), but it has lost the *Sal*I site at the other end, presumably by artifactual rearrangements during the cloning procedures.

Theoretically, the *lac* promoter and ribosome binding site should provide the apparatus for expression of DNA inserted in the multiple cloning site of pUC8 (Vieira and Messing, 1982), and the expressed proteins should be fused with five amino acids from the N terminus of β-galactosidase and two amino acids encoded by the linkers. However, the C7 cDNA sequence contains only one open reading frame, which is not in frame with β-galactosidase, but begins at an internal methionine codon (Figure 3, bp 92–94, underlined). Upstream of this methionine codon is the sequence GGTGG (Figure 3, bp 83–87, boxed), which could serve as a ribosome binding site in *E. coli* (Kozak, 1983). The derived amino acid sequence for the open reading frame is shown (Figure 3, lower line). The mol. wt of the predicted expressed protein is 27 646 daltons, which corresponds well with the size (~27 kd) of the protein that is expressed by bacteria carrying pfC7, as detected by immunoblotting with immune monkey sera (Flint *et al.*, 1986). The methionine codon at bp 92, which we propose is used as the initiation codon for the bacterially expressed protein, probably encodes an internal methionine residue of p75 in *P. falciparum* because the proposed reading frame is continuously open from the *Eco*RI site until the stop codon at bp 857. This stop codon probably signals the true terminus of p75 in *P. falciparum*, although we cannot rigorously exclude the possibility that it was artifactually created during cloning since we know rearrangements have occurred at the 3′ end of the pfC7 cDNA insert. Sixty-four per cent of the cloned coding region (bp 2–858) consists of A and T residues. This is consistent with the observation for other coding sequences of *P. falciparum* proteins; they are less A+T-rich (Cowman *et al.*, 1985) than the genome as a whole, which has an A+T content of 81% (Goman *et al.*, 1982).

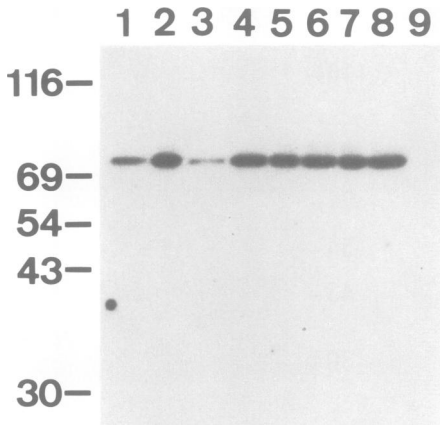

Fig. 5. Detection by immunoblotting of p75 in human erythrocytes infected with trophozoites and schizonts from eight different isolates of *P. falciparum*. **Lanes 1–8** contained proteins from different isolates in the following order: FVO (Vietnam), Geneva, Honduras I, Indochina, Kenya, Phillipines, Sierra Leone and Tanzania. **Lane 9** contained proteins from uninfected human erythrocytes. The total number of parasitized erythrocytes per sample was similar, although the proportions of trophozoites and schizonts in each may have been different. The blot was treated first with rat anti-C7 serum and then with ^{125}I-labeled goat anti-rat immunoglobulins.

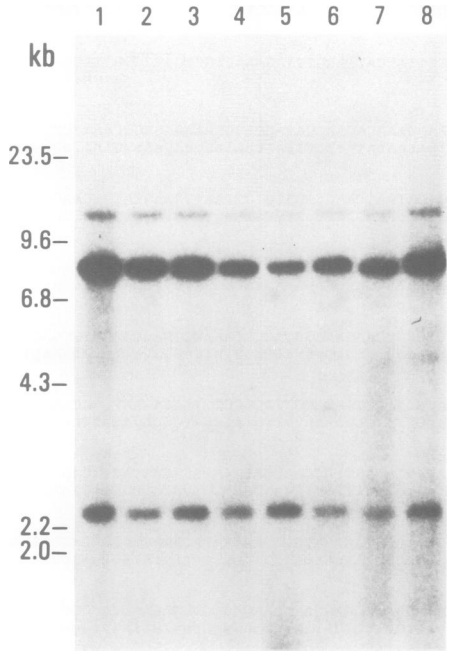

Fig. 6. Southern blot of *Eco*RI digests of genomic DNA of *P. falciparum* isolates and clones probed with C7 cDNA. **Lanes 1–8** contained DNA from the different parasite lines in the following order: Honduras I, FVO (Vietnam), Tanzania, Sierra Leone, Indochina, Geneva, clone 7G8 of the Brazilian IMTM22 strain, and clone A of the Malaysian Camp strain. DNA from the two cloned lines was a gift from Dr J.Weber of the Walter Reed Army Institute (Weber and Hockmeyer, 1985).

A notable aspect of the predicted amino acid sequences is a repeated sequence of four amino acids, -Gly-Gly-Met-Pro-, which begins at bp 719. There are five copies of this basic unit interspersed with four degenerate versions of the repeated sequence (Figure 3, bp 719–838, each unit is surrounded by a box). Repeating peptide sequences are common plasmodial antigens, though their function remains a mystery (Godson, 1985). In an attempt to confirm the predicted reading frame, the peptide $(Gly\text{-}Gly\text{-}Met\text{-}Pro)_3$-Gly was synthesized as an immunological model of this region. The peptide was conjugated to keyhole limpet hemocyanin (KLH), and the conjugate was used to immunize rabbits. The resulting antisera were found to be reactive against p75, the authentic parasite protein, by immunoblot assays, as shown in Figure 4. In addition, antipeptide antisera reacted specifically with parasite antigens in immunofluorescence assays. These results verify that the open reading frame in the cDNA sequence does indeed encode part of the *P. falciparum* protein p75.

Conservation of p75 and its gene(s) among parasite isolates

Immunoblotting experiments with anti-C7 sera show that eight distinct isolates of *P. falciparum* contain proteins which cross-react with p75 and are indistinguishable from it in size (Figure 5). To test whether the homogeneity of p75 at the protein level reflects genetic conservation, the 880 bp cDNA insert from the plasmid pfC7, which encodes ~35% of p75, was used as a probe in Southern blotting analyses. DNA from seven independent, non-clonal isolates of *P. falciparum* was fragmented by three different enzymes: *Eco*RI, *Hin*dIII and *Dra*I. In addition, *Eco*RI digests of two clonal lines of *P. falciparum* were analyzed. With each of the restriction enzymes, the sizes of the genomic fragments that hybridized to the C7 probe were identical in all the strains, suggesting a highly conserved gene structure for p75.

The cDNA sequence of the 880 bp fragment contains no internal *Eco*RI, *Hin*dIII or *Dra*I sites. Nevertheless, the cDNA probe hybridized with three fragments (13.5 kb, 8.5 kb and 2.4 kb) in the *Eco*RI digests from the genomic DNA of six isolates and two cloned parasite lines (Figure 6). The 8.5 kb band produced the most intense signal in each case. In *Hin*dIII digests of the DNA from seven isolates, the 880 bp probe also hybridized to three fragments, >23.5 kb, ~20 kb and 4.5 kb, respectively, of which the 20 kb band produced the most intense signal (data not shown). Finally, under the same stringency conditions for hybridization and washing, the cDNA fragment hybridized with two *Dra*I fragments (1.5kb and 0.55kb) in genomic DNA from each of the seven isolates. Each of the *Eco*RI and *Hin*dIII genomic fragments that hybridizes with the cDNA is theoretically large enough to contain the entire coding sequence for p75. Possible explanations for the multiple genomic fragments in each digest that hybridize with the cDNA are considered in the Discussion.

Homology of p75 with the 70 kd heat-shock proteins

During a systematic computer search through libraries of published protein sequences, we discovered that the predicted protein sequence of the recombinant peptide from clone C7 has a remarkable similarity to the sequences of proteins belonging to the universal and extremely conserved family of 70 kd heat-shock proteins. For example, the predicted amino acid sequence of the recombinant peptide shows 61% identity with both hsp70A from *Xenopus laevis* (Bienz, 1984) and a constitutive hsp-related rat protein (O'Malley *et al.*, 1985), 59% with human hsp70 (Hunt and Morimoto, 1985), 55% with the *Drosophila* major hsp70 (Ingolia *et al.*, 1980), and 53% with the rat hsp-related protein GRP78 (Munro and Pelham, 1986), whose synthesis is induced by glucose starvation but not by heat shock. An alignment of the predicted amino acid sequence of the recombinant peptide with part of the sequence of *X. laevis* hsp70A (Bienz, 1984) is shown in Figure 7. Over the total compared length of 255 resi-

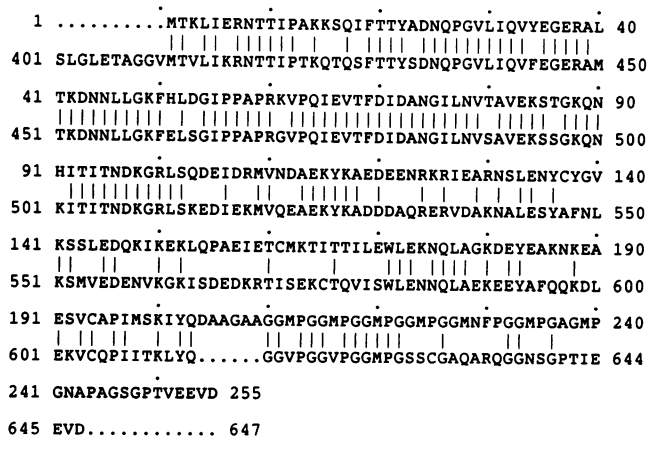

Fig. 7. Sequence comparison between the predicted recombinant protein (top line) and the C-terminal portion of the *X. laevis* 70 kd heat-shock protein. The GAP program (Devereux *et al.*, 1984) was used to align the sequences. Identical amino acid positions in the two proteins are marked by vertical lines. Gaps are inserted as dots.

dues, 61% are identical, and only one gap of six residues has been inserted. At the nucleotide level (not shown), the similarity is 60%. Moreover, if the aligned sequences are divided into two domains, 85% of the first hundred residues are identical, while the C-terminal domain (155 residues) is only 46% identical. This pattern of a higher degree of conservation toward the amino terminus has previously been observed with other members of the hsp family (Hunt and Morimoto, 1985). Interestingly, the last four amino acids, Glu-Glu-Val-Asp (EEVD), are conserved in the predicted sequence of the recombinant protein and in four of the five proteins mentioned above. The similarities in the pattern of conserved residues, as well as molecular size, suggest that our predicted amino acid sequence corresponds to the C terminus of p75 and that p75 is a member of the family of 70 kd heat-shock proteins.

Discussion

We have shown that p75, a protein from the asexual stages of *P. falciparum*, is conserved among all the parasite isolates examined. This result is consistent with our observation that the predicted amino acid sequence of p75 is homologous with the sequences of proteins from the strikingly conserved hsp70 family. The invariance of p75 among parasite isolates distinguishes this protein from other well-characterized *P. falciparum* proteins of the asexual stages, most of which are polymorphic, such as gp185 (McBride *et al.*, 1985), the S-antigen (Cowman *et al.*, 1985), the falciparum-interspersed repeat antigen (Stahl *et al.*, 1985) and gp56 (Stanley *et al.*, 1985). p75 thus possesses a unique combination of properties that make it an excellent candidate for use in immunodiagnostic assays for malaria: it is strongly conserved among parasite isolates, and it is produced in relatively large amounts in the ring stages, the only developmental form of *P. falciparum* that is easily found in peripheral blood. In addition, because of its conserved nature together with its location on the surface of extracellular merozoites, p75 should be studied further as a possible vaccine component. Although there is currently no information on whether antibodies to p75 afford protective immunity to the host, squirrel monkeys have been successfully immunized against *P. falciparum* with partially purified protein fractions eluted from the 75 kd region of SDS–polyacrylamide gels (Dubois *et al.*, 1984). It seems likely that these preparations did contain the 75 kd protein described here. It has previously been shown that immune sera from monkeys and humans react with the expressed product of the cDNA plasmid pfC7, which encodes part of p75 (Flint *et al.*, 1986). The expressed recombinant protein is also very immunogenic in rodents (unpublished results). Thus, the *P. falciparum* protein p75 definitely appears capable of eliciting an antibody response in monkeys, humans and rodents. However, the potential use of the antigen in immunodiagnosis or immunoprophylaxis of malaria is complicated by its close relationship with the evolutionally conserved hsp70 family. Since other members of this family are synthesized by the human host of the parasite, problems might arise due to possible cross-reaction between the human and plasmodial proteins. Nevertheless, since it is known that the C-terminal portion of the hsp70s is relatively poorly conserved among organisms (Hunt and Morimoto, 1985), we are investigating whether this region of p75 from *P. falciparum* contains antigenic determinants that are conserved among *P. falciparum* isolates but are not shared with the hsp70s of the human host.

The conserved nature of p75 extends to the genetic level. A cDNA fragment encoding approximately one-third of p75 hybridizes with multiple fragments whose sizes are identical in genomic digests of *P. falciparum* DNA from several isolates and clones. Since an 880 bp fragment without internal *Eco*RI or *Hin*dIII sites hybridizes under stringent conditions to three larger fragments in digests of genomic DNA with these enzymes, there must be at least three stretches of DNA sequence in the *P. falciparum* genome that are homologous to the mRNA from which pfC7 was produced. The possibility that the multiple bands represent allelic variants is ruled out since the two cloned parasite lines (Figure 6, lanes 7 and 8) each shared with the nonclonal isolates the same pattern of three hybridizing fragments in the *Eco*RI digests.

The multiple fragments may contain identical copies of the gene with the restriction site changes occurring outside the gene. In this case, the increased intensity of the 20 kb *Hin*dIII fragment and the 8.5 kb *Eco*RI fragment would result from hybridization to multiple identical copies of the gene contained either within a single genomic fragment or on several co-migrating genomic fragments. An alternative explanation for the multiple fragments is the presence in the genome either of unexpressed although homologous DNA sequences or of related genes producing proteins different from p75. In this case, the differences in hybridization intensities between bands could be ascribed to the different degrees of homology of each fragment with the probe. We are presently attempting to distinguish among these possibilities. Since the genes encoding the 70 kd hsps in eukaryotes are members of multigene families that encode several proteins of similar size and structure within each organism (Craig, 1985), it would not be surprising if the multiple sequences in the parasite genome that are homologous to the p75 cDNA did indeed encode related proteins from the hsp70 family.

We are currently investigating whether the expression of p75 is regulated by heat and other stresses or is developmentally controlled during the complex life cycle of *P. falciparum*. The biosynthetic pattern of p75 indicates it is constitutively synthesized in cultured asexual-stage parasites. However, at certain points during the parasite's life cycle, dramatic temperature changes occur; firstly, when parasites are transferred from mosquitoes to their human hosts and, secondly, during the fever episodes associated with the lysis of parasitized erythrocytes. The parasite also experiences hypoxia and glucose deprivation, both stresses that are known to induce hsp70s (Nover, 1984). The pattern of expression of p75 and any related proteins in *P. falciparum* should

therefore prove interesting. The temperature induced synthesis of a 70 kd protein has been demonstrated in other protozoan parasites (Lawrence and Robert-Gero, 1985) and it has recently been suggested that heat-shock gene expression might regulate the differentiation of the related protozoan parasite *Leishmania major* from the insect-adapted form to the mammalian host-adapted form (van der Ploeg *et al.*, 1985).

The relationship of p75 with the hsps implies that p75 either performs an important constitutive function or plays a role in the cellular response to stress. Unfortunately, with one exception, there has been no assignment of definite biochemical functions to proteins of the hsp70 family. The exception is the constitutively expressed uncoating ATPase, a bovine protein that releases clathrin cages from coated vesicles in an ATP-dependent reaction (Chappell *et al.*, 1986). In fact, ATP binding appears to be a highly conserved property among members of the hsp70 family (Welch and Feramisco, 1985; Chappell *et al.*, 1986). Based on the common characteristics of family members, it has been postulated that all hsp70s perform similar functions, involving either the disruption of hydrophobic aggregates in an ATP-dependent fashion or prevention of aggregate formation (Munro and Pelham, 1986; Schlesinger, 1986), with the specific substrate being different for each protein. For example, clathrin cages are the substrate for the uncoating ATPase (Chappell *et al.*, 1986), a nuclear ribonucleoprotein complex for the *Drosophila* hsp70 (Lewis and Pelham, 1985) and immunoglobulin heavy chains for GRP78 (Munro and Pelham, 1986). Such a function may be required by the merozoite during its invasion of erythrocytes or for evasion of the immune response and would be consistent with the location of p75 on the merozoite surface. On the other hand, p75 is made throughout the asexual cycle and may also play some role in intraerythrocytic development. Clearly, the first step toward defining a biochemical function for p75 is to examine whether the parasite protein shares the ATP binding property of the hsp70 family. In view of its merozoite surface location, it will also be relevant to test the binding capacity of p75 for fatty acids and immunoglobulins, since other hsp70s are known to bind these substrates (Guidon and Hightower, 1986; Munro and Pelham, 1986).

The location of p75 on the merozoite surface is unusual for an hsp-related protein, as most of these are normally intracellular (Nover, 1984). The predicted sequence for the C-terminal third of p75 does not contain any hydrophobic regions which might be transmembrane, and thus, at least this portion of the molecule is not likely to be integrally membrane-bound. p75 is synthesized early in the developmental cycle much before discrete merozoites are observed. There are several ways by which the protein might arrive at the merozoite surface. Prior to merozoite formation, p75 may become associated with incipient membranes destined to compose the merozoite surface. Alternatively, p75 may be cytoplasmic during the early stages, and might adhere to the surface of merozoites after they are formed, or during cell lysis. At present we cannot distinguish among these possibilities.

Materials and methods

Parasites

Isolates of *P. falciparum* were cultured in human erythrocytes by a modification of the method of Trager and Jensen (1976). Cultures were synchronized, and concentrated late-stage parasites were separated from ring-stage parasites with Physiogel (Reese *et al.*, 1979).

Immunoelectron microscopy

Merozoites were harvested and lightly fixed as described previously (Ardeshir *et al.*, 1985). They were first incubated with mouse anti-C7 serum or mouse anti-pUC serum (negative control) and then incubated sequentially with biotinylated antimouse immunoglobulin and streptavidin-conjugated to colloidal gold (courtesy of Dr J.Wencel-Drake, SCRF). Merozoites were fixed and prepared for electron microscopy as described (Langreth *et al.*, 1978).

Biosynthetic labeling and immunoprecipitation

A synchronous culture of Honduras-isolate, ring-infected erythrocytes (9% parasitemia) was divided into five aliquots. Each aliquot was labeled during a different period of the asexual cycle by growth in methionine-free medium supplemented with 0.1 mCi/ml [^{35}S]methionine (Amersham). Cultures were harvested, and the sample volumes were adjusted to contain equal amounts of radioactivity before immunoprecipitation. Immunoprecipitation, gel electrophoresis and autoradiography were performed as described (Ardeshir *et al.*, 1985). Densitometric analysis was done with a Zeinek soft laser scanning densitometer.

Immunoblotting

Cultures of erythrocytes infected with parasite isolates were harvested as a mixture of mature trophozoites and schizonts. The procedures for sample preparation, electrophoresis, pre-adsorption of antisera and immunoblotting have been described (Ardeshir *et al.*, 1985).

DNA preparation and Southern blots

Genomic DNA was prepared from concentrated late-stage parasites by the method described in Brison *et al.* (1982). Restriction enzymes were purchased from Boehringer Mannheim Biochemicals and used according to the manufacturer's directions. Approximately 3 μg of genomic DNA was digested per sample. To ensure completion of the digest, 4-fold enzyme excess was used. Fragments were resolved by electrophoresis on a 0.8% agarose gel and then transferred (Southern, 1975) to a nylon membrane (Biodyne, New England Nuclear). The 880 bp cDNA insert from pfC7 was labeled by nick translation and hybridized to the blots under standard conditions (Maniatis *et al.*, 1982). Blots were washed at 50°C in 0.1% SDS, 15 mM sodium chloride and 1.5 mM sodium citrate (0.1 × SSC).

DNA sequencing

The 880 bp cDNA fragment from pfC7 was purified on NA45 membranes (Schleicher and Schuell), self-ligated, and randomly sheared by sonication. The pieces were subcloned into M13mp8 and sequenced by the chain termination method as detailed by Bankier and Barrell (1983) using [^{35}S]thio dATP and gradient gels (Biggin *et al.*, 1983). Portions of the DNA were also sequenced by the chemical degradation method (Maxam and Gilbert, 1977). Both strands of the fragment 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 align the nucleotide sequences and to analyze the data.

Peptide synthesis

The peptide (Gly-Gly-Met-Pro)$_3$-Gly was synthesized using solid-phase methodology (Merrifield, 1963) on 4-methylbenzhydrylamine resin in a SamII (Biosearch) peptide synthesizer. Each coupling was checked for completeness by the quantitative ninhydrin procedure, and couplings below 99.6% were repeated. Capping with acetylimidazole was performed after efficient addition of each residue. Final cleavage of the peptide was carried out in liquid hydrogen fluoride with anisole, dimethylsulfide and ethanedithiol as scavengers. The peptide was purified to >93% purity by reverse-phase h.p.l.c. using a gradient of water:acetonitrile containing 0.1% trifluoroacetic acid and was subjected to amino acid analysis before conjugation. The peptide was conjugated to KLH with 1% glutaraldehyde followed by reduction with sodium borohydride yielding substitutions of 116–174 nmol of peptide per mg of protein. Quantities of conjugate corresponding to 200 μg of peptide in Freund's complete adjuvant were used to immunize rabbits by s.c. injection, with later boosts administered in incomplete Freund's adjuvant.

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