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Immunology

A repetitive antigen of *Plasmodium falciparum* that is homologous to heat shock protein 70 of *Drosophila melanogaster*

(malaria/cDNA clone/*dnaK* gene/immunoelectron microscopy)

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ABSTRACT **We describe an antigen of *Plasmodium falciparum*, defined by a cDNA clone designated Ag63. The antigen is an abundant, soluble cytoplasmic polypeptide of M_r 75,000 present in all stages of asexual development in the blood and in gametocytes, but not in sporozoites. The sequence of the cDNA clone revealed that, like many other antigens of *P. falciparum*, it contains tandemly repeated amino acid sequences, in this case Gly-Gly-Met-Pro. However, the rest of the sequence is 70% homologous at the amino acid level to the heat shock protein hsp70 of *Drosophila melanogaster*.**

A striking feature to emerge from studies on cloned antigens of plasmodia is that many of the antigens contain arrays of tandemly repeated amino acid sequences. These tandem repeats have been identified in gene products expressed by sporozoites and by blood-stage parasites and encode epitopes that are targets of the human immune response induced by infection (1–10). Although tandem repeats of amino acid sequences occur in a wide variety of proteins, their extent in malaria antigens suggests that they play a role in the interaction between the parasite and host. Such interactions could include receptor–ligand interactions (1, 9) and interactions between the parasite and the host immune system (11). Polypeptides involved in receptor–ligand interactions might be surface molecules, such as the circumsporozoite protein (1) and merozoite surface protein precursor (8), or secreted molecules, such as the S antigen and glycophorin-binding protein (2, 9). An important immunological consequence of repeats is that these antigens may produce a "smokescreen" effect that subverts effective host immune responses to critical epitopes on parasite antigens (10, 11). Extensive cross-reactions among the repeats have been suggested to delay the generation of high-affinity antibodies to critical epitopes, which are presumed to be important for host protection (11). Polypeptides that are internal to the parasite and released only on parasite rupture could be as important in this smokescreen as surface components because of the multiple cycles of infection experienced in endemic areas. Indeed, this situation of immunological selection due to antigenic competition might be expected to lead to the evolution of further repeats in additional genes (10).

We describe here a cDNA clone, corresponding to an antigen of *Plasmodium falciparum* that is abundant throughout the asexual blood cycle and is an internal cytoplasmic component of the parasite. Surprisingly, the cDNA sequence demonstrates that the polypeptide is homologous to the M_r 70,000 heat shock protein (hsp70) from *Drosophila melanogaster* (12). However, the *P. falciparum* antigen contains a tetrapeptide repeat sequence not found in the *D. melanogaster* heat shock protein.

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MATERIALS AND METHODS

Parasites. *P. falciparum* isolates FCQ27/PNG (FC27), NF7, and K1 were obtained as detailed (2). HB3 was obtained from W. Trager (Rockefeller University, New York); V1 was obtained from L. Miller (National Institutes of Health, Bethesda, MD). Isolates were maintained in culture and stage-specific life-cycle forms were obtained as described (13).

Cloned Parasite Antigens. The construction of a cDNA expression library from *P. falciparum* isolate FC27 in λgt11-Amp3 (λAmp3) has been described (14). Antigen-positive clones were detected by *in situ* colony immunoassay using sera from individuals exposed to malaria in Papua New Guinea (14).

Antibodies. Antisera against the fused polypeptide of Ag63 were prepared by immunizing rabbits (2). Human antibodies against Ag63 were isolated from a pool of plasma from individuals living in Papua New Guinea by affinity purification on an immunoadsorbent prepared with proteins from induced bacteria coupled to CNBr-activated Sepharose (13). Specificity of these antibody preparations was tested by colony immunoassay using an array of known antigen-positive clones (13).

Radiolabeling and Immunoprecipitation. Intact erythrocytes from synchronized cultures at 8–12% parasitemia, containing ring-, trophozoite-, or schizont-stage parasites were surface labeled with ^{125}I using lactoperoxidase (15). Freshly-collected merozoites were surface labeled with ^{125}I by a modified lactoperoxidase procedure (16).

Radiolabeled parasites were solubilized in phosphate-buffered saline (PBS; 0.12 M NaCl/0.02 M phosphate, pH 7.3) containing 0.5% Triton X-100, 5 mM phenylmethylsulfonyl fluoride, 1 mM L-1-tosylamide-2-phenylethyl chloromethyl ketone, 2.5 mM EDTA, and 2 mM iodoacetamide. Immune complexes were recovered from immunoprecipitation using heat-killed and formalin-fixed *Staphylococcus aureus* and analyzed by $NaDodSO_4$/PAGE followed by autoradiography (2, 4). Merozoites and infected erythrocytes containing either stage-specific or asynchronous parasites were prepared for immunoblotting as described (13).

Indirect Immunofluorescence. Thin blood films of parasitized erythrocytes from asynchronous cultures of *P. falciparum* or patients infected with *Plasmodium vivax* were analyzed by indirect immunofluorescence as described (4).

Immunoelectron Microscopy. Samples of parasite culture were fixed with 0.25% glutaraldehyde and embedded in L. R. White resin, hard grade (London Resin, Basingstoke, England); and sections were prepared as described (17). The sections were incubated with antibodies followed by protein

Abbreviation: hsp, heat shock protein.

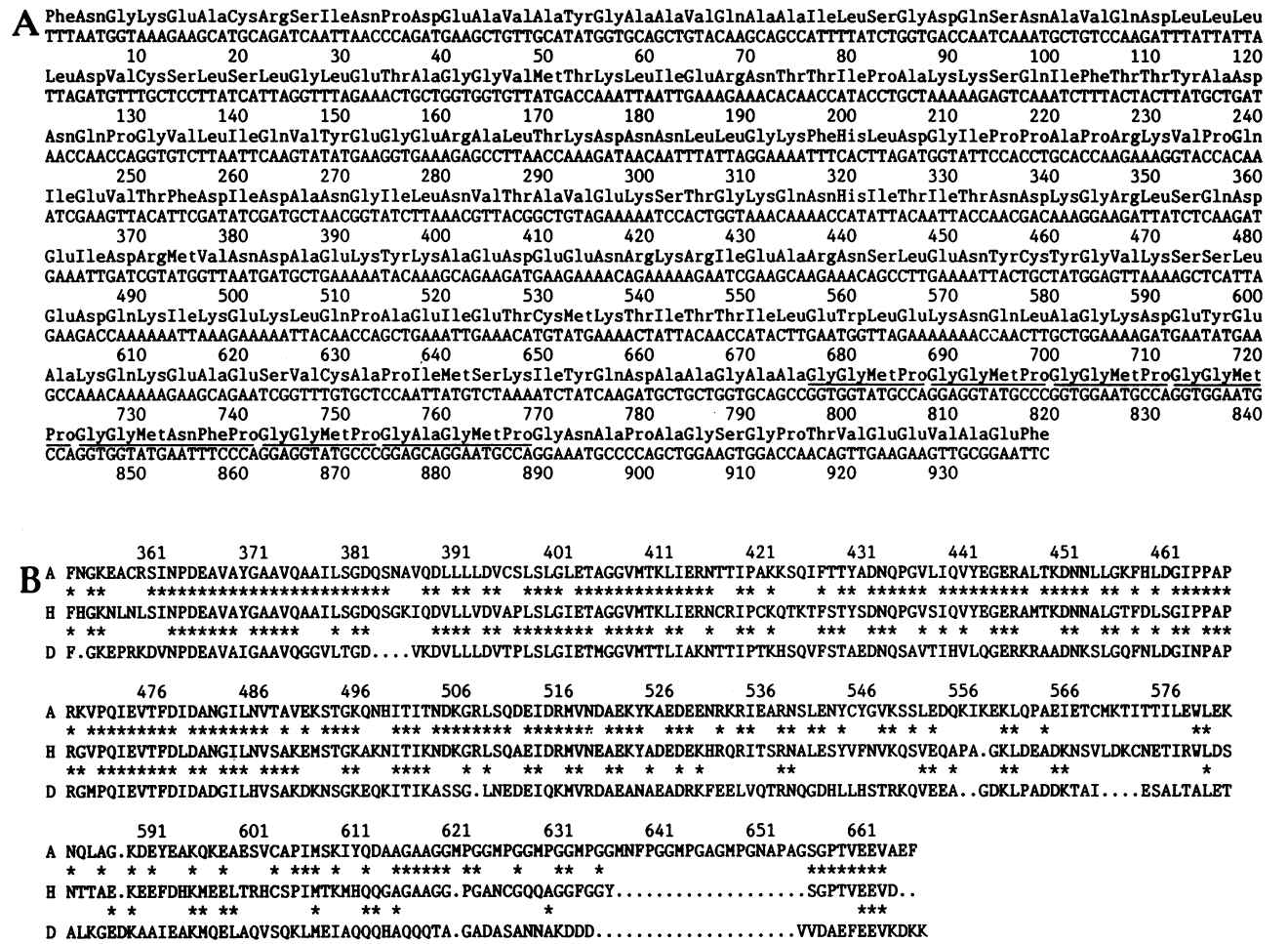

FIG. 1. Homology of the sequences of Ag63, *Hsp*70, and *dnaK*. The sequence of Ag63 was determined by the chain-termination procedure (19) on the insert of Ag63 inserted into M13 vectors (20). A search of the NBRF protein data bank using their SEARCH program (21) revealed homology of Ag63 with hsp70 from *D. melanogaster* (12). (*A*) Sequence of Ag63 with repeats underlined. (*B*) Alignment of the amino acid sequences of the products of Ag63 (A), *Hsp*70 (H) (12), and *dnaK* (D) (22) genes is shown. The numbering is for hsp70.

A-gold (Janssen Pharmaceutica, Beerse, Belgium; 10 nm in diameter) as described (17).

RESULTS

Isolation of Clone Ag63. Clone Ag63 was isolated from an expression library of *P. falciparum* cDNA sequences, cloned in *Escherichia coli* in the lysogenic phage vector λAmp3 (14). It reacted strongly with antibodies from Papua New Guinean adults living in areas endemic for malaria (18). Sibling analysis by hybridization of the Ag63 cDNA insert to a large set of antigen-positive clones isolated in this manner showed that about 3% of clones shared this sequence, suggesting that

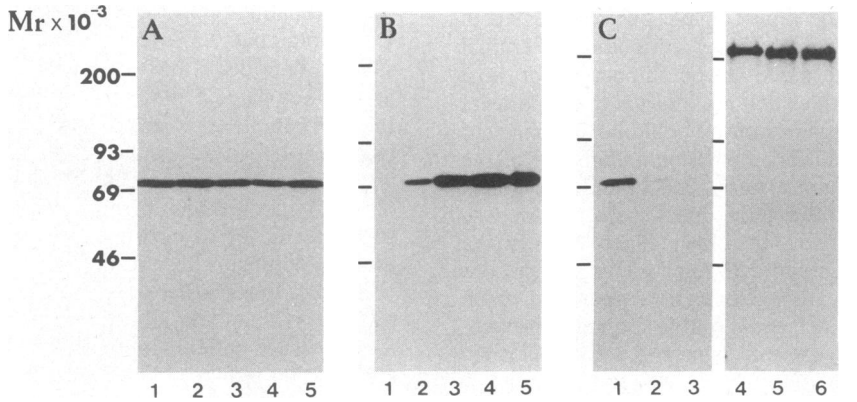

FIG. 2. Immunoblots with rabbit antibodies raised against Ag63. *P. falciparum* antigens were solubilized directly in electrophoresis sample buffer and fractionated by $NaDodSO_4$/PAGE on 7.5% gels. (*A*) Identification of Pf hsp70 in five different isolates grown in asynchronous culture is shown. Lanes: 1, NF7; 2, K1; 3, FC27; 4, V1; 5, HB3. (*B*) Detection of Pf hsp70 in various life-cycle stages of V1 is shown. Lanes: 1, uninfected erythrocytes; 2, 4- to 10-hr ring stages; 3, 26- to 32-hr trophozoites; 4, 38- to 44-hr schizonts; 5, released merozoites. (*C*) Examination of culture supernatants from FC27 for Pf hsp70 is shown. Lanes: 1 and 4, asynchronous parasite extract; 2 and 5, total culture supernatant proteins; 3 and 6, heat-stable, culture supernatant proteins. Lanes 1–3 were probed with anti-Ag63 antibodies. Lanes 4–6 were probed with antibodies against the S-antigen clone Ag16.

it represents an abundant mRNA. Ag63 produced an abundant β-galactosidase-fused polypeptide that was highly reactive with the human antibodies, and this polypeptide was useful for eliciting highly specific mouse and rabbit antibodies (18) or for affinity-purifying human antibodies (13).

Ag63 Is Homologous to hsp70 but Contains a Repetitive Region. The sequence of the cDNA insert from Ag63 was determined by the chain-termination procedure (19). It contained a single open reading frame extending over the entire length of the cDNA clone. Computer-assisted analysis of this sequence revealed two notable features (Fig. 1). First, a considerable portion of the sequence was homologous to the sequence of the major heat shock protein from *D. melanogaster* hsp70 (12). Over the entire length of clone Ag63, homology at the amino acid level was 61%. However, in the region corresponding to amino acids 360–520 of hsp70, 128 (80%) of the amino acids were identical. Allowing conservative replacements, the homology of this region becomes 87%. Second, a segment of the nonhomologous region consisted of repeats. In Ag63, commencing at the position corresponding to amino acid 621 of hsp70 (Fig. 1), there were seven tandem repeats. Repeats 1–4 and repeat 6 consisted of the tetramer Gly-Gly-Met-Pro, repeat 5 consisted of the related hexamer Gly-Gly-Met-Asn-Phe-Pro, and repeat 7 of the pentamer Gly-Ala-Gly-Met-Pro. Homology between the two sequences was minimal in this region. However, insertion of a 20-amino acid gap into the hsp70 sequence resulted in a match of eight out of eight possible residues from the C-terminal region (Fig. 1). hsp70 from *D. melanogaster* has a homologue in *E. coli*, the *dnaK* gene product (22). Alignment of this sequence (Fig. 1) shows homology between Ag63 and hsp70 is greater than that of either with the *dnaK* gene product.

It was possible that the occurrence of tandem repeats typical of *P. falciparum* antigens in an otherwise conserved hsp70 sequence was caused by the adventitious joining of two unrelated cDNAs during cloning. To exclude this possibility, we examined three independent Ag63 cDNA clones obtained from a different isolate, NF7. Nonoverlapping probes generated from the Ag63 cDNA insert cloned in M13mp18 and 19 by priming with synthetic oligonucleotides, encompassing the *Hsp*70-homologous region (primer CAAGGCTGTTTCTTGC, nucleotides 553–568) and the repetitive region (primer CCAACTTGCTGGAAAAG, nucleotides 693–709) hybridized to the three NF7 clones (data not shown). Sequencing over 300 bases on one of the NF7 clones using both of the above primers demonstrated that the *Hsp*70 and repetitive regions are contiguous in NF7. We conclude that the sequences were not artifactually joined.

Identification of the hsp70 Polypeptide in *P. falciparum*. Rabbit antibodies raised against the purified, fused polypeptide of Ag63 reacted with a polypeptide of M_r 75,000 in immunoblots of asynchronous parasite extracts (Fig. 2). This polypeptide, designated Pf hsp70, was present in all isolates tested and showed no variation in apparent molecular weight (Fig. 2*A*). Similar results were obtained from immunoblots probed with affinity-purified human antibodies (data not shown) and from immunoprecipitation studies on *P. falciparum* polypeptides labeled *in vitro* with [^{35}S]methionine (18).

In parasite extracts prepared from various stages of the asexual blood cycle, immunoblots revealed a progressive increase in the abundance of Pf hsp70 from rings through to schizonts (Fig. 2*B*). Levels of Pf hsp70 were also high in released merozoites, but the numbers of merozoites that were extracted in sample buffer were not directly comparable with the numbers of intracellular parasites used. Only traces of the antigen were detected in culture supernatants collected during the release of merozoites from synchronized populations of schizonts (Fig. 2*C*). As a control for detection of released antigens, the same batches of culture supernatant were incubated with antibodies to the S antigen clone Ag16 (2). As expected, the supernatants were found to contain large amount of S antigen (Fig. 2*C*), a protein known to be liberated from hos cells at the time of merozoite release (2). Analysis of cultur supernatants collected at various times throughout the asexua cycle failed to demonstrate the release of the antigen into th medium at any stage (data not shown).

Localization of Pf hsp70 in *P. falciparum*. Experiment designed to test whether Pf hsp70 was on the surface of the parasite or the host cell, by means of surface radioiodinatior and immunoprecipitation, clearly demonstrated that it wa not available to labeling on living parasites at any stage in the asexual blood cycle (Fig. 3). Rabbit antibodies against Ag6 precipitated enough of Pf hsp70 from extracts of surface-labeled trophozoites and schizonts to be detected by Coomassie blue staining on NaDodSO$_4$/PAGE gels as a discrete band migrating at M_r 75,000 (Fig. 3*A*). However, this band was not seen on autoradiographs exposed for over a week with an intensifying screen (Fig. 3*B*), indicating that the protein had not been radioiodinated. No specific radiolabeled bands were detected in immunoprecipitates of anti-Ag63 antibodies reacted with proteins from surface-iodinated

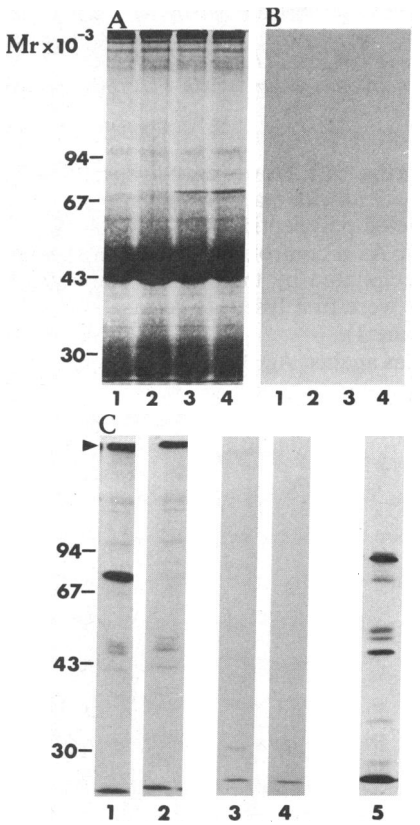

FIG. 3. NaDodSO$_4$/PAGE gels of immunoprecipitates of ^{125}I-labeled *P. falciparum* proteins with anti-Ag63 antibodies are shown. (*A*) Coomassie-stained gel of immunoprecipitates of rabbit anti-Ag63 antibodies with extracts from surface-labeled ring- (lane 2), trophozoite- (lane 3), or schizont- (lane 4) infected erythrocytes. A protein band corresponding with Pf hsp70 (M_r 75,000) is visible in lanes 3 and 4. Lane 1 shows an immunoprecipitation with uninfected erythrocytes. (*B*) Autoradiograph of *A* exposed for 1 week indicating that the protein reacting with the antibodies was not radiolabeled. (*C*) Autoradiographs of immunoprecipitates of rabbit antibodies against Ag63 (lanes 1 and 3) or against β-galactosidase (lanes 2 and 4) with proteins from merozoites. Lanes 1 and 2 contain proteins extracted from merozoites and then radiolabeled, and lanes 3 and 4 contain proteins from surface-labeled, live merozoites. Lane 5 shows an immunoprecipitate of polyspecific human antibodies from a malaria patient with proteins from the surface-labeled merozoites as a control.

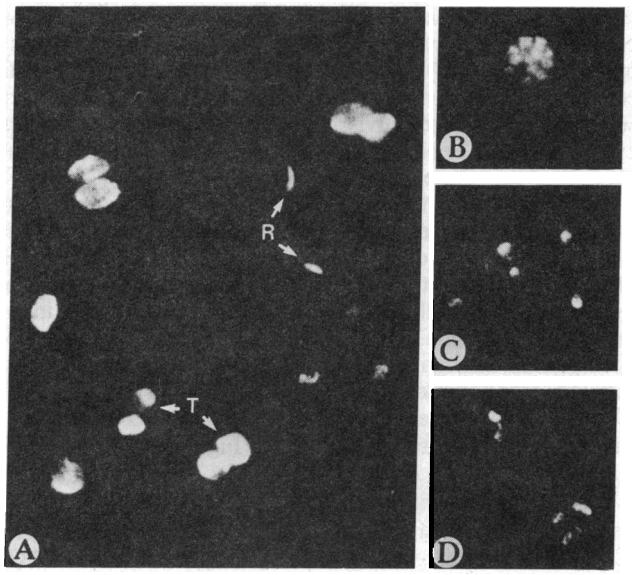

FIG. 4. Indirect immunofluorescence of acetone-fixed asexual blood-stage parasites labeled with rabbit antibodies against Ag63. (*A*) Rings (R) and trophozoites (T) of *P. falciparum* isolate FC27. (*B*) Well-segmented schizont of *P. falciparum* isolate FC27. (*C*) Released merozoites of *P. falciparum* isolate FC27. (*D*) *P. vivax* ring-stage parasites.

merozoites (Fig. 3*C*). However, polyspecific human antibodies from a patient with malaria immunoprecipitated a number of radiolabeled polypeptides in the same preparations (Fig. 3*C*, lane 5). As a control, Pf hsp70 was radioiodinated and immunoprecipitated by the method employed above if the merozoites were first lysed, to release internal components (Fig. 3*C*, lane 1).

Antibodies against Ag63 applied to acetone-fixed parasites in indirect immunofluorescence assays reacted with all blood stages of *P. falciparum* (Fig. 4 *A*, *B*, and *C*), paralleling the findings from stage-specific immunoblots. Ring-stage parasites of *P. vivax* were also stained with these antibodies (Fig. 4*D*). The intensity of staining of *P. falciparum* at a given antibody dilution increased progressively from ring stages, through trophozoites to schizonts. Staining was localized to the parasite and not the host cell, and in the most advanced schizonts was clearly associated with the individual merozoites (Fig. 4*B*). Free merozoites in acetone-fixed smears were also intensely labeled (Fig. 4*C*). However, no staining occurred when anti-Ag63 antibodies were applied to glutaraldehyde-fixed and air-dried monolayers of infected erythrocytes, or to unfixed suspensions of parasitized erythrocytes and merozoites. Hence, we obtained no evidence of a surface location for this molecule. Similar results were obtained in immunofluorescence assays performed with rabbit, mouse, and human antibodies against Ag63-family clones (data not shown).

Expression of the Pf hsp70 in sporozoites from salivary glands removed from wild-caught mosquitoes collected in Papua New Guinea was examined using indirect immunofluorescence. *P. falciparum* sporozoites were identified by a readily detectable fluorescence with a rabbit antibody raised against an oligomer of the sporozoite coat protein of *P. falciparum*; $(Asn\text{-}Ala\text{-}Asn\text{-}Pro)_3$ (3). There was no detectable fluorescence of sporozoites using rabbit anti-Ag63 antibodies whereas the same antibody preparation gave strong fluorescence against asexual blood stages. Incubation of infected mosquitoes at 37°C for 4 or 18 hr did not result in production of detectable levels of Pf hsp70 in the sporozoites, even though the sporozoites were still viable after 4 hr.

Pf hsp70 was observed by immunoelectron microscopy to be localized within the parasite cytoplasm (Fig. 5). Labeling was heaviest in erythrocytes containing mature trophozoites and schizonts, but occurred in all blood stages, including free merozoites. There was no indication of specific labeling

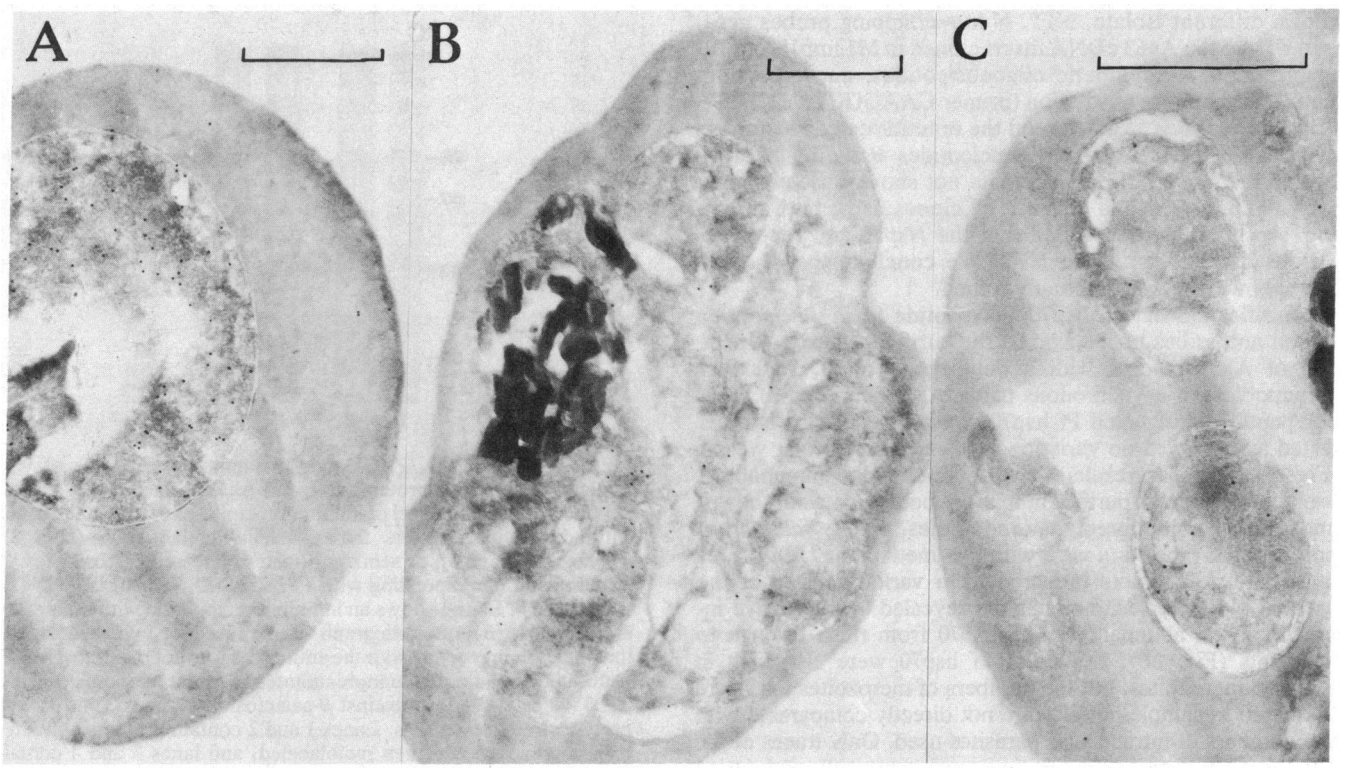

FIG. 5. Immunoelectron micrographs showing sections of *P. falciparum*-infected erythrocytes that have been incubated with affinity-purified rabbit anti-Ag63 followed by protein A-gold (10 nm) to show the location of Pf hsp70. Trophozoite-(*A*) and schizont-(*B* and *C*) infected erythrocytes. (Bars = 0.5 μm.)

associated with defined structures or organelles that could be distinguished in these preparations, or with the surface of any stage examined. The specificity of the labeling patterns observed was demonstrated by the distinct patterns of staining that were obtained when antibodies against a series of unrelated cloned antigens of *P. falciparum* were applied to sections cut from the same blocks (e.g., ref. 17).

DISCUSSION

The data presented here show that a *P. falciparum* protein of M_r 75,000 is homologous with hsp70 of other organisms but contains a region of tandemly repeated amino acid sequences, a characteristic of many malaria antigens. The repeats are not present in the corresponding sequences from *D. melanogaster* or *E. coli* sequences that are otherwise closely related. Heat shock genes are present in a wide variety of organisms and are believed to constitute a protective response to stress, including heat shock (23). In some organisms heat shock genes constitute multigene families, only some of which are thermally inducible (24, 25). In the protozoan parasite *Leishmania*, which has life-cycle stages in its sandfly vector at ambient temperature and in the mammalian host at 37°C, the heat shock genes are expressed only in the higher temperature environment (26–28). Culture of promastigotes, characteristic of the insect vector, at 37°C *in vitro* rather than at 25°C results in differentiation of promastigotes into amastigotes (25, 26). Hence the heat shock response may be an important *in vivo* signal in differentiation during the parasite life cycle. The data here show that Pf hsp70 is expressed at high levels in *P. falciparum* blood stages cultured *in vitro* at 37°C in human erythrocytes. This is also true for blood stages of *P. falciparum* and *P. vivax* in natural infections. Gametocytes of *P. falciparum* also react strongly with antibodies to Ag63 (A.E.B., unpublished observations). Our experiments with sporozoites from mosquitoes infected with *P. falciparum* indicate that this gene is indeed turned off in the mosquito stages as expected. However, we have not succeeded in inducing it in sporozoites. Whether it can be induced in other metabolically more active mosquito stages is not known.

Although Ag63 clearly encodes the *P. falciparum* homologue of *Hsp*70, the functional significance of this is not clear. Because of the high degree of homology with the sequence from *D. melanogaster*, we assume that this gene retains important elements of its function. Chappell *et al.* (29) have presented evidence that the conserved portion is an ATPase. We, therefore, propose that the repeats do not interfere with this function because they have been elaborated in a less conserved region (see Fig. 1) of the molecule where structural variation has little effect on function. As Pf hsp70 is an abundant cytoplasmic protein, these repeats may further augment an antibody response that is of no protective value to the host.

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