

Further characterization of a 58 kDa *Plasmodium berghei* phosphoprotein as a cochaperone¹

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Abstract

Molecular chaperones are important for proper protein folding during protein biogenesis. This report describes a protein from *Plasmodium berghei* which is 30% identical and 40% similar to a recently described mammalian cochaperone, or heat shock protein 70 interacting protein. The *P. berghei* cochaperone accumulates throughout the trophozoite stage and decreases during the schizont stage. The stage specific expression is consistent with its presumed role in protein folding or protein–protein interactions. The largest difference between the *Plasmodium* and mammalian sequences is a more extensive domain of imperfect glycine-glycine-methionine-proline (GGMP) tandem repeats in the parasite's cochaperone sequence. Immunofluorescence studies show that the protein is an abundant cytosolic protein of the parasite. However, antibodies raised against the GGMP repeat domain, which is also found in other parasite chaperones, react with both the parasite and host erythrocyte membrane. The reactivity with the host membrane suggests that the parasite exports molecular chaperones into the infected erythrocyte. Copyright © 1996 Elsevier Science B.V.

Abbreviations: GGMP, tetrapeptide of glycine-glycine-methionine-proline; HBSS, Hanks' balance salt solution containing 5 mM HEPES, pH 7.4, and 0.25% glucose; Hip, HSP70 interacting protein; HSP70, member of the 70 kDa family of heat shock proteins; HSP90, member of the 90 kDa family of heat shock proteins; p48, progesterone receptor associated protein; Pbpp58, 58 kDa phosphoprotein from *Plasmodium berghei*; pBSIIKS+, plasmid Bluescript II KS+; SDS, sodium dodecyl sulphate.

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1. Introduction

Molecular chaperones, often referred to as heat shock proteins, assist in the proper folding of proteins during their biogenesis [1,2]. Two major groups of chaperones are the family of 70 kDa proteins, or HSP70, and the family of 60 kDa proteins, or chaperonins. Other families of chaperones which participate in protein biogenesis include HSP90, HSP104, the peptidyl-prolyl *cis-trans* isomerases (i.e. immunophilins), and protein disulfide isomerases. Molecular chaperones facilitate proper protein folding by transiently binding to nascent polypeptide chains or hydrophobic domains of unfolded proteins. This noncovalent interaction between the chaperone and the substrate protein blocks nonproductive protein–protein interactions. Chaperones also sequester folding intermediates allowing for an ordered folding of domains and assembly of oligomers.

Chaperones from the malarial parasite have also been identified and partially characterized. Frequently antibodies against heat shock proteins are generated as a result of parasite infection [3]. The best characterized among the *Plasmodium* chaperones are the members of the HSP70 family [4]. At least five different *P. falciparum* HSP70 genes have been identified [5,6]. In addition, *P. falciparum* homologues of chaperonin [7], HSP90 [8–10] and immunophilins [11,12] have been identified.

Recently a new chaperone, described as a cochaperone, has been identified [13]. Others have recently identified this same protein as being involved in the maturation of steroid hormone receptor complexes [14]. Cochaperones interact with other heat shock proteins and assist in the proper folding of substrate proteins. This newly described cochaperone is homologous to a previously described *P. berghei* gene [15], and as described herein, is the same as a 58 kDa *P. berghei* phosphoprotein [16,17].

2. Materials and methods

2.1. Cloning and sequencing

A λ gt11 expression library was prepared from *Plasmodium berghei* ANKA (clone HP8417) cDNA using a force ligation procedure [18,19]. Approximately 10^6 recombinant phage were screened with a mixture of mAb-b5, mAb-c7 and mAb-g12 [17]. Positive plaques were subjected to plaque purification using each of the mAb separately. Two identical recombinant λ gt11 clones recognized by mAb-b5 and containing inserts of approximately 1 kb were obtained. Insert DNA was excised with EcoRI and subcloned into pBSII-KS+ (Stratagene). The complete 1 kb fragment was sequenced by the dideoxy chain termination method, using internal restriction sites and unidirectional nested deletion mutants to generate smaller fragments.

2.2. Immunoblotting

Parasites from *P. berghei* (K173 strain) were isolated by saponin lysis and differential centrifugation [16]. The parasites were hypotonically lysed in 10 volumes of 20 mM phosphate, pH 7.6, containing 1 mM EDTA. Particulate material was removed by centrifugation at $27\,000 \times g$ for 20 min. The parasite cytosol was subjected to SDS gel electrophoresis on 9% polyacrylamide gels using a single wide lane and proteins were transferred to Immobilon[®] membranes (Millipore). The membranes were cut into strips, probed with primary antibodies, incubated with alkaline phosphatase conjugated anti-mouse IgG (Sigma, A3562), and developed with the colorimetric substrates 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium. The primary antibodies used were mAb-b5 prepared from 2-dimensional gel purified protein [17] and two polyclonal mouse antibodies prepared against recombinant proteins [15]. One of the polyclonal antibodies was raised

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L21710  GAAGAAGCTGCAGATTTCTCAAATATATTTTTTACGATTTGTTCAATTTCGAACAAAATATATTATATCAATTGTTTATATGACATTTTAAAATCGTATATGTATAG 110
L04508  ATAAAATTCACAGATTTCTCAAATATATTTTTTACGATTTGTTCAATTTCGAACAAAATATATTATATCAATTGTTTATATGACATTTTAAAATCGTATATGTATAG 329
L21710  TAGTATGTATATATACATTATATATATATTAATTAGTTAATTTAAATACCTATTTGGTACTTTATTATTTTCCTTTGTCGCCATTTTTTTTTATAAAAATATTGTAA 220
L04508  TAGTATGTATATATACATTATATATATATTAATTAGTTAATTTAAATACCTATTTGGTACTTTATTATTTTCCTTTGTCGCCATTTCCCTTTT-ATAAAAATATTGTAA 438
L21710  CAATGCATATTGAAAAA-----TTGAAGATCTTAAAAAATTTGTCGCAA 238
L04508  CAATGCATATTGAAAAAGTAGTTTGAAGAAAAAATAATCGTATATATCTTATGTTATACATTTGAATTTACCAAATATAGTTTATATATAATAATTTTTTAA 548
L21710  -----TTGAAGATCTTAAAAAATTTGTCGCAA 265
L04508  CTTATAAAAAACAATTACATACAATCTATATATTTTTTGCATAACTCCACATTTTCCTTTCATTTATTTTTCATCCTTTAGTTGAAGATCTTAAAAAATTTGTCGCAA 658
L21710  GTTGTGAAGAAAACCCATCAATTTCTGCTCAAACAGAAATTATCGTTTTTAAGGATTTTATCGAAAGTTTTGGAGGAAAAATAAAAAAGATAAAATGGGTTATGAGAAA 375
L04508  GTTGTGAAGAAAACCCATCAATTTCTGCTCGGGCCAGAAATTATCGTTTTTAAGGATTTTATCGAAAGTTTTGGAGGAAAAATAAAAAAGATAAAATGGGTTATGAGAAA 768
L21710  ATGAAAAGCGAAGATAGTACAGAAGAAAAATCAGATGAAGAGGAAGAAGATGAAGAGGAAGAAGAAGAAGAAGAAGATGATGATCCTGAAAAATTAGAATTAATTTAA 485
L04508  ATGAAAAGCGAAGATAGTACAGAAGAAAAATCAGATGAAGAGGAAGAAGATGAAGAGGAAGAAGAAGAAGAAGAA...GATGATGATCCTGAAAAATTAGAATTAATTTAA 875

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Fig. 1. Nucleotide alignment of cDNA and genomic DNA clones. Nucleotides from the cDNA clone (L21710) and the previously described genomic clone (L04508) were aligned using the nalign program of PCGENE. The proposed start codon is double overlined and the -1 and -3 A residues are denoted with \uparrow 's. The consensus splice junction and branch sites are underlined. The previously proposed start codon [15] is double underlined. The remaining 3' overlap of the two sequences is identical (data not shown).

against a recombinant protein corresponding to amino acids 7–364 and included the 19 imperfect repeats of GGMP (designated α -19R). The other monoclonal sera was raised against a recombinant protein corresponding to amino acids 7–277 which was devoid of the GGMP repeats (designated α -NR).

Alternatively, the polyclonal antibodies were used to immunoprecipitate the parasite cytosol followed by immunoblotting with mAb-b5, similar to the previously described enriched blotting [2]. Briefly, the polyclonal sera were incubated with or without parasite cytosol. The antigen-antibody complexes were collected with protein-G agarose (Pharmacia) and subjected to SDS gel electrophoresis and immunoblotting.

3. Immunofluorescence

P. berghei-infected erythrocytes were washed four times in HBSS, fixed with 0.025% glutaraldehyde and permeabilized with 0.1% Triton X-100 as described [21]. The samples were incubated with primary antibodies for 1 h at ambient temperature, washed three times and incubated for an additional hour with fluorescein conjugated anti-mouse IgG (Sigma, F6257). Following three additional washes, epifluorescence was detected under ultraviolet illumination.

3. Results

Monoclonal antibodies generated against *P. berghei* phosphoproteins [17] were used to screen an λ gt11 cDNA expression library resulting in the isolation of recombinant clones. Clone λ ppb1, recognized by mAb-b5, corresponds to a 58 kDa *P. berghei* phosphoprotein (Pbpb58). The 967 bp insert DNA was sequenced (GenBank™ AC# L21710) and submitted for a search of homologous sequences using the blastn program via the NCBI BLAST E-Mail server. Clone λ ppb1 is highly homologous to a *P. berghei* genomic DNA sequence (GenBank™ AC# L04508) described as an abundant *P. berghei* protein containing tandem GGMP repeats [15]. The most significant difference between these two sequences is a stretch of 174 nucleotides (nt) found in the genomic clone, but not found in cDNA clone (Fig. 1). These 174 nt probably correspond to an intron since consensus splice junction sites and a branch site [22] are present. The splice acceptor and donor sequences are homologous to those of other *P. falciparum* genes [23]. Other differences between the two *P. berghei* sequences are minor and appear to be of little consequence. The first 10 nt of the cDNA sequence are not homologous with the genomic DNA sequence. These 10 bases, however, are complementary to the next 11 bases

and therefore may represent a cloning artifact due to the formation of a hairpin loop during cDNA preparation. Therefore, we suspect that the genomic sequence is correct in this region. The only other differences between the two clones is a lysine codon instead of a glycine codon, and an additional glutamate codon in a stretch containing 18 consecutive acidic residues (Fig. 1).

The first ATG resulting in an open reading frame (ORF), considering the intron, is at position 223 in the cDNA clone (Fig. 1). The -1 and -3 positions are A residues as is typical of *Plasmodium* genes [24]. In addition, relatively long 5' untranslated regions are typical of *Plasmodium* genes [25]. Uparanukraw et al [15] had originally proposed that the start codon was at position 361 (Fig. 1). Removal of the intron results in an extended coding region that increases the previously reported protein sequence [15] by 46 amino acids. In addition, comparison with mammalian protein sequences (see below) suggest that the ATG at 223 is the true start codon.

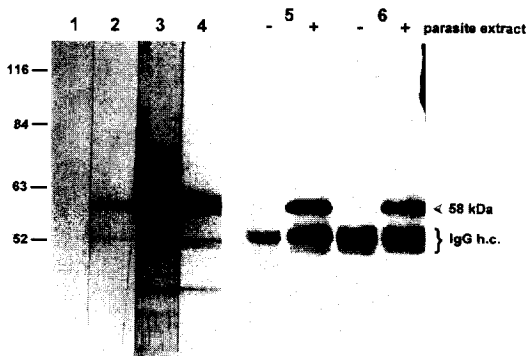


Fig. 2. Immunoblots demonstrating that the cloned gene corresponds to the 58 kDa phosphoprotein. Parasite cytosol was subjected to SDS gel electrophoresis and transferred to immobilon membranes. The membranes were cut into strips and incubated with either no primary antibody (lane 1), mAb-b5 hybridoma culture supernatant medium diluted 1:10 (lane 2), α -19R diluted 1:2000 (lane 3) or α -NR diluted 1:2000 (lane 4). Alternatively the parasite cytosol (+ lanes), or buffer controls (– lanes), were immunoprecipitated with either α -NR (lanes 5) or α -19R (lanes 6) followed by SDS gel electrophoresis and immunoblotting with mAb-b5. All three antibodies recognize the same 58 kDa protein (denoted with arrowhead). Molecular weight standards are indicated on the left and the heavy chain of IgG is indicated on the right.

Antibodies raised against the recombinant protein and mAb-b5 were used in immunoblotting assays to confirm that the cloned gene is the 58 kDa phosphoprotein. Polyclonal sera were generated against either a recombinant protein consisting only of the non-repeat portion (α -NR) or against a recombinant protein which also contained the GGMP repeat domain (α -19R) [15]. All three antibodies react with a 58 kDa protein (Fig. 2). Furthermore, immunoprecipitation with α -NR or α -19R followed by immunoblotting with mAb-b5 confirms that the antibodies all recognize the same 58 kDa protein. The cloned gene, therefore, encodes the previously described 58 kDa phosphoprotein [16,17] which will be referred to as Pbpp58. α -19R also reacts with a protein doublet in the 70–75 kDa range. It was previously reported that α -19R immunoprecipitated a 75 kDa protein believed to be a member of the HSP70 family [15]. On other immunoblots (not shown) the upper band predominates suggesting that the lower protein in the doublet is a proteolytic fragment of the upper protein band. Similarly, the minor lower molecular bands recognized by α -NR and α -19R may also be proteolytic degradation products.

The complete amino acid sequence, deduced from the combination of L21710 and L04508, consists of 423 amino acids with a predicted molecular mass of 48 kDa. The difference between the observed and predicted molecular weights is likely due to the phenomenon that many *Plasmodium* proteins with tandem repeats migrate anomalously on SDS gels [26]. The predicted isoelectric point of 4.6 compares favorably with the observed isoelectric point of 4.3 [16].

A search for homologous sequences using the blastp program via the NCBI BLAST e-mail server revealed four additional sequences with significant similarity to the 58 kDa protein. One of the sequences was the analogous protein from *P. chabaudi* [27]. Another sequence was an unpublished partial sequence (GenBank™ AC# U16862) of a *P. falciparum* recombinant DNA which corresponds to the GGMP repeat domain and the immediate flanking areas. The *P. chabaudi* and *P. falciparum* sequences were virtually identical to the *P. berghei* sequence except that

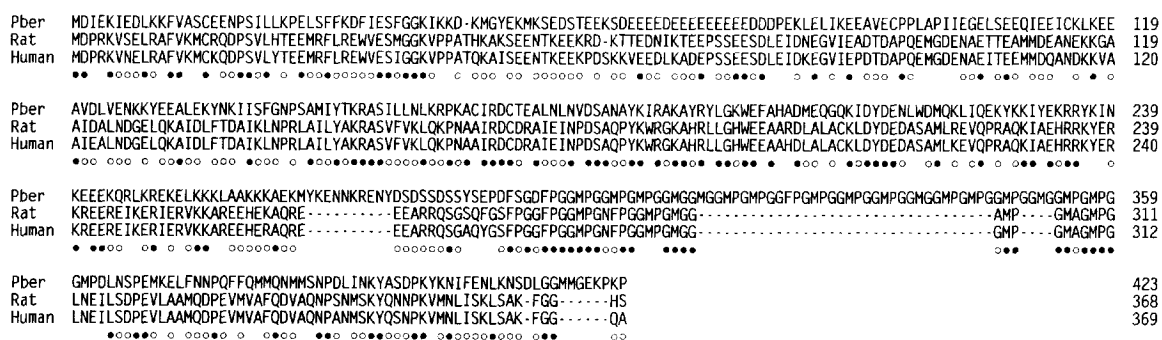


Fig. 3. Alignment of *P. berghei* 58 kDa protein with related mammalian proteins. The complete *P. berghei* 58 kDa protein sequence was aligned with the cochaperone sequence from either rat (AC # X82021) or human (AC # U28918) using the clustal program of PCGENE. Identical residues are indicated by ●, similar residues by ○ and gaps by –.

both contained more copies of the GGMP repeats (data not shown). The Pbpp58 sequence also exhibited homology to rat and human sequences (Fig. 3). The rat sequence is a recently characterized HSP70 interacting protein (Hip) [13]. The human sequence is a protein, designated p48, which associates with the progesterone receptor [14]. The rat and human sequences are greater than 90% identical (data not shown). Alignment of the *P. berghei*, rat and human sequences reveals that 30% of the residues are identical and another 40% are similar (Fig. 3). The most obvious difference between these proteins is the greater number of tandem GGMP repeats in the *P. berghei* sequence. The most conserved region of the protein is a central domain encompassing amino acids 150–220. This approximately 70 amino acid domain exhibits some homology to the tetratricopeptide repeat family of proteins [28]. Tetratricopeptide repeat domains are 34 amino acid motifs which form α -helices that fold back on each other resulting in a compactly folded domain. Presumably this ‘snap-helix’ is important for the structure of the protein.

Twelve of the 20 top scoring homologies in the blast search were nucleolins. However, these scores were considerably less than the chaperone related proteins discussed above. In addition, analysis of the *P. berghei* sequence by the PCGENE prosite program revealed two overlapping bipartite nuclear targeting consensus sequences [29], thus suggesting that this *P. berghei* protein

may be localized to the nucleus. Immunofluorescence studies with α -NR and α -19R, however, show that this protein is localized to the cytoplasm (Fig. 4). Interestingly the α -19R anti-sera also reacts with the host membrane of infected erythrocytes (Fig. 4B). The antibody did not react with uninfected erythrocytes. The reaction with the host erythrocyte membrane may be due to heat shock proteins, or other proteins, containing the GGMP repeats. Infected erythrocytes were fractionated into isolated parasites and erythro-

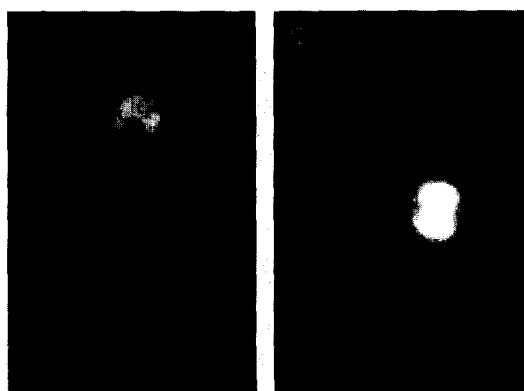


Fig. 4. Immunofluorescence staining with sera raised against recombinant protein. Infected erythrocytes were fixed with glutaraldehyde and permeabilized with Triton X-100 as described [21]. Samples were incubated with either α -NR (A) or α -19R (B) as the primary antibodies and fluorescein conjugated anti-mouse IgG as the secondary antibody. The α -NR serum reacts specifically with the parasite, whereas the α -19R serum reacts with the parasite and the erythrocyte membrane.

cyte membranes as described [30] and analyzed by immunoblotting. No unique proteins exclusively fractionating with the erythrocyte membrane were identified (data not shown). The presumed *P. berghei* HSP70 did fractionate with both the parasites and the erythrocyte membrane. However, it was difficult to determine whether the erythrocyte membrane associated HSP70 was due to parasite contamination or actually represented an erythrocyte membrane form of *P. berghei* HSP70.

4. Discussion

This report describes a *P. berghei* homologue to the recently characterized cochaperone [13,14] and extends the protein sequence of a previously reported *P. berghei* gene [15] by 46 amino acids. This cochaperone interacts with members of the HSP70 family and has been designated as either Hip [13] or p48 [14]. Hip binds to the ATPase domain of HSP70 and stabilizes the ADP state of HSP70 [13]. Stabilization of the ADP state increases the affinity of HSP70 for its protein substrate. In addition, Hip, through its own chaperone activity, may participate in the interaction of HSP70 with substrate proteins and may assist in protein folding. p48 is identical to Hip, but is described as a protein associated with the immature progesterone receptor complex [14].

The *P. berghei* analog of Hip/p48 was first characterized as a 58 kDa phosphoprotein [16,17] and more recently as a 56 kDa protein with GGMP repeats [15]. The 58 kDa phosphoprotein, Pbpp58, and the 56 kDa protein are the same as evidenced by antibody crossreactivity (Fig. 2). Pbpp58 is a relatively abundant cytosolic protein [15,16] as confirmed by immunofluorescence (Fig. 4). Mammalian Hip/p48 is also a major cytoplasmic protein which probably exists as a tetramer [13]. Non-denaturing gel electrophoresis indicates that the mobility of Pbpp58 is substantially less than expected for its subunit mass [16] suggesting that Pbpp58 is also an oligomer. In summary, this report describes a *P. berghei* protein with sequence homology to a recently described cochaperone. No role has previously been ascribed to the Pbpp58, but because of its similarity to cochaper-

ones, Pbpp58 probably plays a role in the HSP70 assisted folding of proteins or in protein-protein interactions.

Pbpp58 is synthesized during the late ring and early trophozoite stages with the maximum rate of synthesis occurring 6–9 h post merozoite invasion [17]. Pbpp58 gradually accumulates throughout the trophic period. During this period the parasite is rapidly increasing its mass by digesting host hemoglobin and using the amino acids for its own protein biogenesis. The high level of protein synthesis that occurs during the trophic period implies that chaperones are also needed at high levels to insure proper protein folding. *P. falciparum* HSP70 [31,32] and immunophilin [12] are also expressed at higher levels during the late ring and early trophozoite stages. The amount of Pbpp58 begins to decrease at approximately 18 h post invasion and continues to decrease until it is completely depleted at the time of merozoite release [17]. Coinciding with this decrease in Pbpp58 is the beginning of DNA synthesis and nuclear division and a decrease in the overall rate of protein synthesis [33]. The synthesis and accumulation of the Pbpp58 throughout the trophozoite stage and its decrease during the schizont stage are consistent with the presumed chaperone function of Pbpp58.

The major *Plasmodium* phosphoproteins, including Pbpp58, are phosphorylated at a maximum rate during the late trophozoite and early schizont period [17]. Members of the HSP70 family from *P. falciparum* are also phosphorylated at a maximum rate during the late trophozoite and early schizont stage [34]. These results suggest that phosphorylation may function to down regulate the cochaperone and chaperone activities since the rate of protein synthesis is decreasing during the late trophozoite and schizont stages. The phosphorylation status and regulation of mammalian Hip/p48 are unknown. The protein kinase activity, which phosphorylates Pbpp58 and the other major phosphoproteins, is most similar to the type I casein kinases [16,35]. The function of the type I casein kinase is not known, but is speculated to play some role in the regulation of translation [36]. The proper folding of newly translated proteins would certainly be considered part of the

protein translation process. Proteolysis of a 34 kDa phosphoprotein and its potential role in the regulation of the *Plasmodium* casein kinase activity has been recently discussed [37].

The *Plasmodium* analogue of Hip/p48 contains several more tandem repeats of the GGMP motif than mammalian Hip/p48. *Plasmodium* HSP70 also contains several more GGMP repeats than HSP70 from most other organisms [4]. Other organisms that have extensive GGMP repeat domains include *Trypanosoma brucei* [38], *T. cruzi* [9], and a fungus *Bremia lactucae* [40], suggesting that this might be a feature of lower eukaryotes.

It is not clear why lower eukaryotes have more copies of the GGMP repeats. A 34 kDa *P. berghei* phosphoprotein contains numerous repeats composed predominantly of glycine-glycine-methionine that are similar to those in chaperonins [41]. It has been speculated that these glycine-glycine-methionine repeats may play a role in protein-protein interactions. Alternatively, these simple repeats may play a role in immune evasion as suggested by Bianco et al. [42].

Another interesting observation in this study is the binding of α -19R to the host membrane from infected erythrocytes. This suggests that the malarial parasite exports a protein(s) with GGMP repeats to the erythrocyte membrane. However, we were unable to biochemically identify the protein responsible for the erythrocyte membrane-associated fluorescence. Thus far, *Plasmodium* proteins containing GGMP repeats are either chaperones (i.e. HSP70) or as cochaperones (i.e. Hip/p48). A mAb raised against the C-terminal GGMP repeat portion of *P. falciparum* HSP70 reacts with the host membrane of infected hepatocytes [43], suggesting that a similar phenomenon occurs during the exoerythrocytic stage of the life cycle. It is feasible that the parasite exports chaperones to assist in protein folding or the assembly of molecular complexes found within the infected erythrocyte. HSP70 has been subdivided into four families based upon sequences [44]. Interestingly, these four families display different localization patterns (i.e. cytosol, endoplasmic reticulum, mitochondria and chloroplast). The localization of HSP70 epitopes to the host membrane in both infected erythrocytes and hepatocytes suggests

that the malarial parasite has evolved an exported chaperone to assist in the remodeling of the parasitized host cell.

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