Parasite Immunology, 2005, **27**, 197–203

Research Note

Identification of karyopherin β as an immunogenic antigen of the malaria parasite using immune mice and human sera

ASIF MOHMMED, SHIVENDRA KISHORE, KAILASH P. PATRA, PALAKODETI V. N. DASARADHI, PAWAN MALHOTRA & VIRANDER S. CHAUHAN

International Centre for Genetic Engineering and Biotechnology, New Delhi 110 067, India

SUMMARY

A differential immunoscreening of the λgt11 Plasmodium falciparum *genomic expression library was carried out using anti-*P. yoelii *sera (convalescent-phase mouse sera) and immune sera collected from healthy adults, to identify novel cross-reactive and possibly protective antigens of the parasite. One clone, with an insert size of 1132 bp that reacted strongly with both the sera was selected. The insert was found to be a part of the* P. falciparum *karyopherin β (PfKβ) homologue. RT-PCR and Northern blot analysis confirmed the expression of PfKβ in the blood stages of the parasite. The ~110 kDa protein was localized in the cytoplasm at the ring and trophozoite, and in the parasitophorous vacuole at the schizont stage. Two large fragments of PfKβ representing the N- and C-terminal halves were expressed in* E. coli. *The recombinant proteins were highly immunogenic in mice, and also found to be the target for immune response in natural infections of* Plasmodium *spp. Anti-sera against the protein showed a low level of anti-parasitic activity. Immunization with recombinant PfKβ fragments was only partially protective against a heterologous challenge infection in mice. Our results show that the parasite releases a highly immunogenic, cytoplasmic protein into the host which may not contribute to the development of protective immunity.*

Keywords *immune sera, immunogenicity, karyopherin β,* Plasmodium falciparum

RESEARCH NOTE

Malaria remains as one of the leading causes of morbidity and mortality, and there are an estimated 300–500 million cases resulting in over a million deaths every year globally (1). To control malaria, improved tools must be developed and in this context a tool such as a vaccine is necessary because of the massive disease burden of malaria in the developing world. However, malaria vaccine development is a formidable challenge, due to poor understanding of the protective immune mechanism and lack of characterization of appropriate vaccine candidate antigens (2). Selection of parasite proteins as vaccine candidates is usually based upon their location in the parasite, immunogenicity and conservation among different *Plasmodium* species. However the high antigenic polymorphism in malarial parasite antigens and lack of a suitable animal model for human malaria parasites are major hurdles in the path of malaria vaccine development (2). Several of the currently used vaccine target antigens were first developed by characterizing homologues of *P. falciparum* in murine malaria, *P. berghei* and *P. yoelii*, and testing their efficacy as vaccine candidates in a mouse challenge model. The existence of conserved antigen analogues across the *Plasmodium* species has been demonstrated by cross-hybridization and by comparison of the amino acid sequences of different parasite proteins (3–6). Identification of such cross-reactive antigens that are conserved in different *Plasmodium* species may allow evaluation of their potential as vaccine candidates in the murine malaria model; lack of a validated animal model is one of the major hurdles in characterization of protective antigens of the human malarias, *P. falciparum* and *P. vivax*. The present study was undertaken with a view to characterize conserved malaria protein(s), which might be involved in the build-up of protective antibody response to *P. yoelii* infection in convalescent mice, and in healthy individuals living in a *P. falciparum* endemic area.

Asif Mohmmed, Shivendra Kishore and Kailash P. Patra contributed equally to this work.

Correspondence: Prof. V. S. Chauhan, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110 067, India (e-mail: virander@icgeb.res.in).

Received: 30 November 2004
Accepted for publication: 10 May 2005

An expression library of *P. falciparum* (3D7) genomic DNA was constructed in λgt11 following Snyder *et al*. (7). The rodent malaria parasites, *P. yoelii nigeriensis*, were maintained in BALB/c mice and the convalescent-phase mouse anti-*P. yoelii* serum was obtained after repeated *P. yoelii* infection and cure of the BALB/c mice (6). Human sera from healthy adults were obtained from residents of the Koraput district of Orissa, a highly *P. falciparum* endemic region of India (8). Serum samples were also collected from healthy individuals who had no known history of malaria and were used as negative control. The protocol for this study was approved by the Human Volunteer Research Ethical Committee of the International Centre for Genetic Engineering and Biotechnology, New Delhi, India.

The expression library was probed by immunoscreening with hyper-immune anti-*P. yoelii* sera following Snyder *et al*. (7). Twenty phage plaques that reacted with the sera were then immunoscreened with the pooled human sera collected from *P. falciparum* endemic areas. We identified one of the clones (KP1) that reacted strongly with both the sera, suggesting that it may represent some conserved, cross-reactive and immunogenic antigen(s). The phage DNA was prepared from the KP1 clone and digested with *Eco*RI to release the insert. The released insert (1132 bp) showed a continuous reading frame. In Southern blot analysis using KP1 insert as the probe, a hybridization band of ~1·1 kb was detected with *P. falciparum* DNA digested with *Eco*RI, whereas the same probe did not hybridize with human DNA. A ~4·4 kb band was detected in Northern blot analysis using total parasite RNA and the KP1 insert as probe. A fragment of 1·1 kb was amplified by RT-PCR using total RNA of *P. falciparum* with terminal primers corresponding to KP1 insert sequences (data not shown). Together, these results indicated that the cloned insert was derived from *P. falciparum* and is expressed during the blood stages of the parasites.

A BLAST search at GenBank, using a sequence of the KP1 clone showed that the deduced amino acid sequence had significant homology with karyopherin β proteins of different organisms. The cloned segment was found to be located on chromosome 5 of *P. falciparum* using the *Plasmodium* genome database at PlasmoDB (*www.plasmodb.org*). The ORF finder and gene finder programs identified a 3372 bp long gene sequence containing the sequences of the KP clone. This gene sequence encodes a putative translation product of 1124 aa and has been identified earlier as the *P. falciparum* homologue of karyopherin β (PfKβ) (9), which showed 24% identity and 21% similarity with the *Homo sapiens* karyopherin β3 protein, and 20% identity and 22% similarity with *Saccharomyces cerevisiae* karyopherin β3. A homologue of the PfKβ sequence was also found by BLAST search of the *P. yoelii* genome database on contig chrPy_1_cpy42. The gene finder program identified a 3363 bp long ORF on this contig. The deduced amino sequence showed 83% identity and 10% similarity with that of PfKβ. The complete PfKβ gene was PCR-amplified from *P. falciparum* genomic DNA and sequenced, and it matched fully with the sequence available in the database.

The full length PfKβ gene was cloned in pGEX and pQE30 vectors to express the recombinant protein as a GST fusion protein or with a histidine tag at the N-terminal, respectively. However, even after several attempts using different expression conditions we were unable to produce sufficient amounts of the full length recombinant purified protein. Therefore, the N-terminal (aa 1–576; PfKβ-N) and C-terminal (aa 524–1127; PfKβ-C) (Figure 1a) halves were cloned into pQE31 expression vector and the corresponding recombinant proteins were expressed with 6 × histidine tags in *E. coli*. For expression and purification of the recombinant proteins corresponding to PfKβ-N and PfKβ-C fragments, *E. coli* M15 cells containing the recombinant plasmids were grown in LB and induced for protein expression with 1 mM IPTG for 5 h at 37°C. The harvested cell pellet in each case was suspended in lysis buffer (10 mM Tris-HCl PH8·0, 5 mM benzamidine-HCl, 2 mM PMSF, 100 mM NaCl, 200 μg/mL lysozyme, 0·1% Tween 20) and lysed by sonication. In each case the recombinant protein was found to be expressed in the inclusion bodies. Inclusion bodies were collected by centrifugation of lysed cells at 12 000 ***g*** for 30 min at 4°C. The pellet was solubilized in 8 M urea in TBS (25 mM Tris-HCl pH 8·0, 500 mM NaCl) and the insoluble material was removed by centrifugation. The 6 × his-tagged protein in the supernatant was purified using nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen) and refolded by serial dialysis in a step gradient of urea in the presence of 1 mM of reduced and oxidized glutathione (GSH and GSSG). The refolded protein was further purified by ion exchange chromatography using a Q-sepharose column. Purity of the protein was judged by SDS-PAGE and protein concentration was estimated by using Bradford reagent (Bio-Rad Laboratories, CA). Two recombinant proteins of ~62 kDa and ~60 kDa sizes corresponding to PfKβ-N and PfKβ-C fragments, respectively, were purified to homogeneity (Figure 1b). Both purified protein fragments cross-reacted in an immunoblot assay with the convalescent mouse anti-*P. yoelii* sera as well as with the pooled human sera, which were used initially for the library screening (Figure 1c,d); no recognition was found with normal human and mice sera. These results confirm the presence of antibodies in these sera against the expressed proteins.

Antibody response against PfKβ was analysed in human sera samples from *P. falciparum* endemic areas, using the recombinant proteins by enzyme-linked immunosorbent assay (ELISA). All the 20 sera samples analysed showed reactivity at 1 : 200 dilutions with both PfKβ-N and PfKβ-C (Figure 1e).

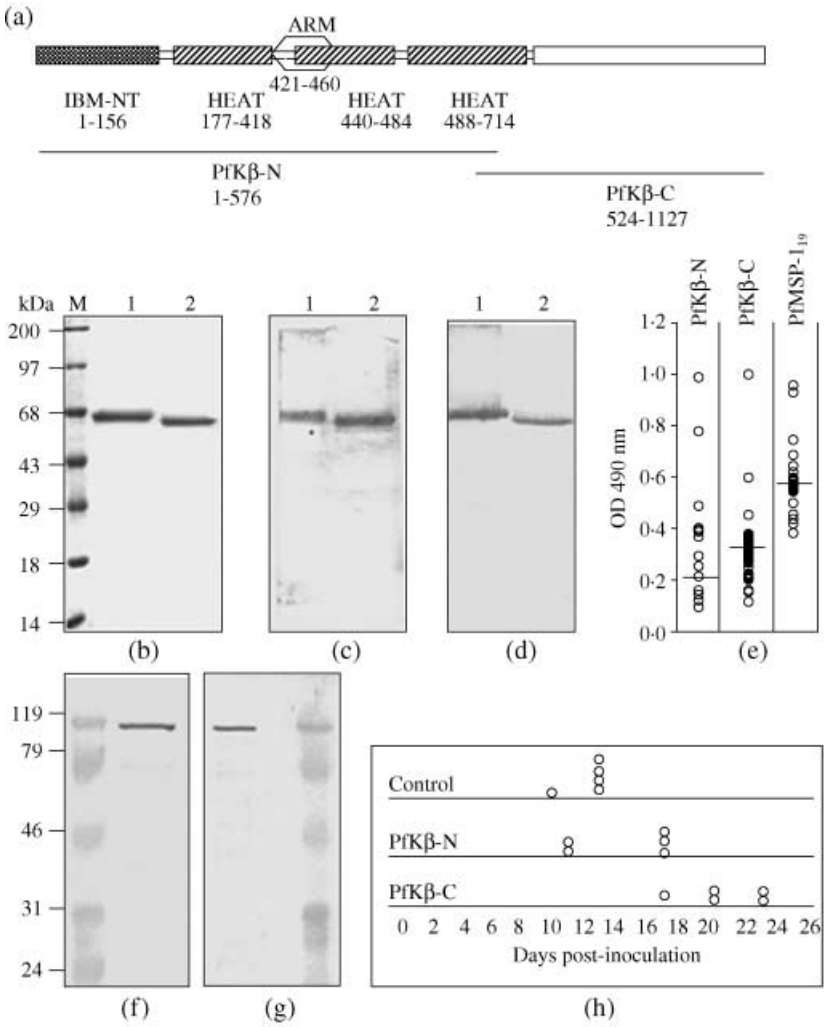

Figure 1 (a) Schematic representation of the full PfKβ gene, different functional domains [Importin beta N-terminal region (IBN-NT), HEAT and armadillo (ARM) repeats] and respective amino acid positions are indicated (9); the N-terminal (PfKβ-N) and C-terminal (PfKβ-C) regions expressed as recombinant proteins are also marked. (b) Purified recombinant PfKβ-N (lane 1) and PfKβ-C (lane 2) proteins; Western blots of the gel run in parallel with (c) endemic human sera and (d) convalescent mice anti-*P. yoelii* sera. (e) Scatter plot representing ELISA results using sera from individuals residing in *P. falciparum* endemic areas; each serum was diluted 1 : 200 and tested in triplicate against recombinant PfKβ-N and PfKβ-C, recombinant MSP1$_{19}$ was kept as positive control. The cross bars indicate median of the reactivity with different sera. (f–g) Western blot analysis of (f) *P. falciparum* and (g) *P. yoelii* parasite lysate showing karyopherin β protein recognized with mice anti PfKβ sera. (h) Survival period of mice in different immunization groups challenged intravenously with $1 \times 10\,000$ *P. yoelii nigeriensis* parasitized erythrocytes; each dot represents the time point of the death of a mouse.

The sera from individuals who have never been exposed to malaria showed no or insignificant antibody response. Antibody response in sera from mice infected with *P. yoelii* and *P. berghei*, and rhesus monkeys infected with *P. cynomolgi*, were also analysed. Both anti-*P. yoelii* and anti-*P. berghei* sera showed significant response at 1 : 200 dilution with PfKβ-N and PfKβ-C as capture antigen (0·6–1·0 OD). Monkey anti-*P. cynomolgi* sera showed somewhat lower reactivity (0·35–0·4 OD). These results suggested that the conserved karyopherin β is also a target of antibody response during the infections with these plasmodia species.

In order to analyse immune response of the recombinant PfKβ fragments in animals, two groups of female BALB/c mice (6 weeks old, five mice per group) were immunized intraperitoneally with 30 µg of purified PfKβ-N and PfKβ-C recombinant proteins emulsified in complete Freund's adjuvant. Two booster immunizations with the same antigen in incomplete Freund's adjuvant were given after 21 and 42 days. One set of five mice was also immunized with adjuvant alone, according to the same schedule, to act as control. Sera samples were collected from these mice on days 0, 14, 28, 42 and 56. Sera from immunized mice (after the second booster) gave end-point titres of 1 : 205 600 and 1 : 411 200 for PfKβ-N and PfKβ-C, respectively, suggesting that both the recombinant proteins are highly immunogenic, and harbour B and T cell epitopes. These PfKβ antibodies also cross-reacted with the *P. falciparum* and *P. yoelii* parasite lysate on the immunoblot and detected the ~110 kDa protein band (Figure 1f,g). No cross-reaction of the parasite lysates was observed with pre-immune sera.

A large number of surface proteins of the malaria parasite or of the infected erythrocytes have been shown to be immunogenic and able to generate protective immunity in animal models of malaria (reviewed in 10,11). Identification of a presumably cytoplasmic protein like PfKβ, as a parasite antigen that induces high immune response during the course of

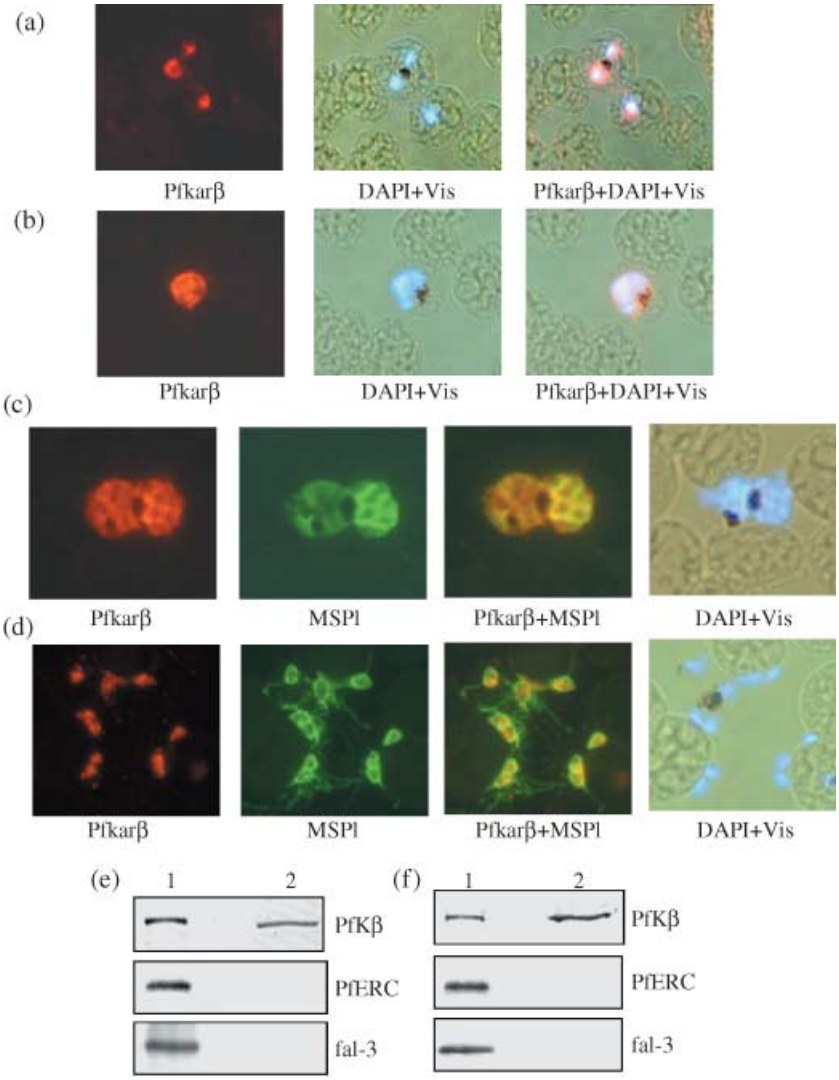

Figure 2 Localization of PfKβ by immunofluorescence assay. *Plasmodium falciparum* parasites at (a) ring, (b) trophozoite, (c) late schizont and (d) merozoite stages were immunostained with mouse anti-PfKβ sera (red) and visualized with a confocal microscope. Schizonts and merozoites were also immunostained with anti-MSP1 antibodies (green); parasite nuclei were stained with DAPI (blue). (e) Localization of PfKβ in infected erythrocyte fractions by immunoblot assay. Infected erythrocytes were lysed by saponin, parasite (lane 1) and supernatant (lane 2) fractions were separated on SDS-PAGE and analysed on immunoblot using anti-PfKβ or anti-PfERC or antifalcipain-3 antibodies. (f) Localization of PfKβ in culture supernatant by immunoblot assay. The parasite pellet (lane 1) and the culture supernatant (lane 2) collected from schizont/merozoite-stage parasite culture, were analysed on an immunoblot using anti-PfKβ, anti-PfERC or anti-falcipain-3 antibodies.

natural infection, may appear somewhat surprising, and in contrast to the above studies. However, in a study somewhat similar to the present one, Lobe *et al*. (12) screened the cDNA expression library of *P. falciparum* using sera from malaria-immune persons and identified a ribosomal phosphoprotein P0 of the parasite as one of the major antigens inducing high antibody titres in these sera. Ribosomal phosphoprotein P0 is expected to be an internal protein with a role in ribosomal assemblage (13). However, a transient surface localization of P0 on the merozoite surface has been implicated (14).

Karyopherin β plays a central role in nuclear transport; proteins bearing nuclear localization sequences (NLS) bind to the heterodimer consisting of karyopherin α and β for translocation through the nuclear pore complex (NPC) (15,16). We have recently shown interaction of PfKβ with the karyopherin α homologue of the parasite, suggesting its conserved role in nuclear transport within the parasite (9). How can PfKβ, which is expected to be a cytoplasmic protein, generate antibody response during the course of malaria infection with different plasmodia species? To address this question we carried out localization studies using anti-PfKβ antibodies. Results of IFA showed that the protein was localized in the cytoplasm at the ring and trophozoite stages (Figure 2a,b), whereas at the schizont stage it was mostly localized in the parasitophorous vacuole (Figure 2c). To further confirm the localization of the protein in the parasite Western blot analysis was carried out with fractionated infected erythrocytes and schizont/merozoite-stage culture supernatant. Infected erythrocytes (2×10^7) were fractionated by treatment with saponin (0·15%), and both the supernatant and parasite pellets were separated on SDS-PAGE and

analysed on immunoblot using anti-PfKβ antibodies. Schizont/merozoite-stage culture supernatant was prepared from *P. falciparum* 3D7 culture following Goel *et al*. (17) and also analysed on immunoblot. These Western blot analyses again showed the presence of PfKβ protein in the parasitophorous vacuole at the schizont stage, as well as in the culture supernatant collected after erythrocyte rupture (Figure 2e,f). Antibodies against endoplasmic reticulum resident protein (PfERC) and a cysteine protease (falcipain-3) were used as negative controls; bands corresponding to PfERC and falcipain-3 were detected in the parasite fraction with these antibodies but were absent in the parasitophorous vacuole and the culture supernatant (Figure 2e,f). It may be that the PfKβ present in the parasitophorous vacuole during the schizont stage is released in the host environment during schizont rupture and is exposed to the immune system of the host, which leads to generation of an immune response against it. It remains to be analysed if other proteins involved in nuclear transport are also released from the parasite in a similar manner. Histidine rich protein II (HRPII), a major blood-stage *P. falciparum* protein, is also known to be released in large amounts in the host plasma (18). The HRPII protein is transported to the RBC cytoplasm and is released after host cell rupture (19). Involvement of cytoplasmic functional proteins in immunity against pathogens is not limited to *P. falciparum* alone. For example, a cytoplasmic urease enzyme and an associated protein, HspB, in *Helicobacter pyroli* have been shown to be the targets of immune recognition in patients (20). The urease is released by the bacteria in the host environment in a programmed manner (21). The recombinant urease and HspB were found to be immunogenic in animals and are being considered as major vaccine candidate antigens (22–24).

After confirming the immunogenicity of PfKβ in mice by immunization and in human as inferred from immunological observations from clinical isolates, we made an attempt to assess anti-parasitic activity of anti-PfKβ-N and anti-PfKβ-C antibodies *in vitro* culture following Kennedy *et al*. (25). *Plasmodium falciparum* (3D7) parasite cultures were synchronized by sorbitol treatment (26) and the parasitaemia was adjusted to 1%. These synchronized parasites at late trophozoite/schizont stages were cultured in 96-well plates with 5%, 10% and 20% of the immune sera from mice immunized with the antigens or with the adjuvant alone, in triplicate. After 24 h thin blood smears were prepared and stained with Giemsa stain and parasitaemia was determined microscopically. Antisera from both the group of immunized mice showed only 25–37% growth inhibition of parasite *in vitro*; these levels of inhibition are considered as insignificant in these assays. This marginal growth inhibition by anti-PfKβ-N and anti-PfKβ-C antibodies suggested that antibodies against theses regions of PfKβ may not be involved in protective immunity against blood-stage malaria. It has been shown that antibodies against several malarial proteins or protein domains do not contribute to protective immunity (27,28).

To assess the protective efficacy of recombinant proteins PfKβ-N and PfKβ-C against parasite challenge, mice immunized with these antigens were challenged with murine malaria parasite *P. yoelii*. The immunized mice (five for each group) were challenged intravenously with $1 \times 10\,000$ *P. yoelii nigeriensis* parasitized erythrocytes. Mice immunized with adjuvant alone were used as control. Starting from day 1 after the challenge infection, parasitaemia was monitored every alternate day by microscopic examination of stained blood films, counting at least 10 000 RBC. All the mice in control group showed continuous increase in parasitaemia from days 3–10 post-infection, eventually leading to death of the animal (Figure 1h). The mice immunized with PfKβ-N and PfKβ-C and challenged with *P. yoelii* infection did not survive the challenge, although there was a considerable increase in the survival period as compared with the control mice (Figure 1h), indicating only a partial protection against the heterologous challenge infection. Taken together these results suggest that anti-PfKβ antibodies, generated during the course of malaria infections, may have moderate anti-parasitic abilities and may contribute to the overall immunity against malaria. However, if so, then the question of immune response to a parasite protein somewhat homologous to the human counterpart (~24% homology), leading to autoimmune response may become relevant. The role of autoimmunity in immune protection against malaria has been a subject of debate (29). Presence of serum autoantibodies was first described by Shaper *et al*. (30) in malaria-infected individuals, and by Kreier and Dilley (31) in experimental rat infections. Since then a number of studies have reported the presence and reactivity of autoantibodies to DNA, erythrocyte, lymphocyte, phospholipids, ribonucleoprotein, etc., during the course of acute disease in humans or in experimental infection in animals (reviewed in 32). It has been suggested that parasites protect themselves from host immune response by employing proteins that are either homologous or are able to mimic host cellular proteins. The works presented in this report and by earlier workers point towards generation of immune responses to some malaria proteins that are homologous to the counterpart host proteins. It is also known that a number of malaria protein antigens induce non-protective immune responses, and also that the malaria parasite generates highly immune evasive 'smoke-screen epitopes' to keep the host immune response busy (27). Such immune responses may not control the parasite levels effectively and an effective control occurs only when immune responses against crucial epitopes are generated. Presence of such highly immunogenic molecules on the parasite surface or release of such molecules during its life

cycle could be one of the immune evasion strategies that the parasite has developed.

Although the findings of the present study that a conserved and essentially a cytoplasmic protein is involved in the build-up of the immune response during malaria infection may be useful in understanding malaria immunity, the approach of differential immuno-screening used here may not always lead to the characterization of malaria proteins that are involved in protective immunity. At the same time it is also clear that immune response to malaria proteins is cross-reactive among the malaria species and may not be limited to the parasite surface proteins only.

ACKNOWLEDGEMENTS

We thank AF Cowman and P Rosenthal for providing antibodies for PfERC and falcipain-3, respectively. We thank PK Das for helpful discussions on the manuscript and for making it possible for KPP to work at ICGEB, and F. Kironde for initiating work in this direction. PVND was supported by CSIR research fellowship.

REFERENCES

1 World Health Organization. *Report* 2002; *http://www.who.int/infections-disease-report/2002/index.html.*
2 Doolan DL, Aguiar JC, Weiss WR, *et al.* Utilization of genomic sequence information to develop malaria vaccines. *J Exp Biol* 2003; **206**: 3789–3802.
3 Carlton JM, Angiuoli SV, Suh BB, *et al.* Genome sequence and comparative analysis of the model rodent malaria parasite *Plasmodium yoelii yoelii*. *Nature* 2002; **419**: 512–519.
4 Del Portillo HA, Longacre S, Khouri E & David PH. Primary structure of the merozoite surface antigen 1 of *Plasmodium vivax* reveals sequence conserved between different *Plasmodium* species. *Proc Natl Acad Sci* 1991; **88**: 4030–4034.
5 Kaslow DC, Syin C, McCutchan TF & Miller LH. Comparison of the primary structure of the 25 kDa ookinete surface antigens of *Plasmodium falciparum* and *Plasmodium gallinaceum* reveals six conserved regions. *Mol Biochem Parasitol* 1989; **33**: 238–288.
6 Ray P, Sahoo N, Singh B & Kironde FAS. Serum antibody immunoglobulin G of mice convalescent from *Plasmodium yoelii* infection inhibits growth of *Plasmodium falciparum in vitro*: blood stage antigens of *P. falciparum* involved in interspecies cross-reactive inhibition of parasite growth. *Infect Immun* 1994; **62**: 2354–2361.
7 Snyder M, Elledge S, Sweetser D, Young RA & Davis RW. λgt11, gene isolation with antibody probes and other applications. *Method Enzymol* 1987; **154**: 107–128.
8 Rajagopalan PK, Das PK, Pani SP, *et al.* Parasitological aspects of malaria persistence in Koraput district Orissa. *India Indian J Med Res* 1990; **91**: 44–51.
9 Mohmmed A, Kishore S, Dasaradhi PVN, Patra K, Malhotra P & Chauhan VS. Cloning and characterization of *Plasmodium falciparum* homologs of nuclear import factors, karyopherin α and karyopherin β. *Mol Biochem Parasitol* 2003; **127**: 199–203.
10 Kumar S, Epstein JE & Ricie TL. Vaccine against asexual stage malaria parasites. *Chem Immunol* 2002; **80**: 262–286.
11 Chauhan VS & Bhardwaj D. Current status of malaria vaccine development. *Adv Biochem Eng Biotechnol* 2003; **84**: 143–182.
12 Lobo CA, Kar SK, Ravindran B, Kabilan L & Sharma S. Novel proteins of *Plasmodium falciparum* identified by differential immunoscreening using immune and patient sera. *Infec Immun* 1994; **62**: 651–656.
13 Uchiumi T, Wahba AJ & Traut RR. Topography and stoichiometry of acidic proteins in large ribosomal subunits from *Artemia salina* as determined by crosslinking. *Proc Natl Acad Sci USA* 1987; **84**: 5580–5584.
14 Singh S, Sehgal A, Waghmare S, Chakraborty T, Goswami A & Sharma S. Surface expression of the conserved ribosomal protein P0 on parasite and other cells. *Mol Biochem Parasitol* 2002; **119**: 121–124.
15 Rexach M & Blobel G. Protein import into nuclei; association and dissociation reactions involving transport substrate transport factors, and nucleoporins. *Cell* 1995; **83**: 683–692.
16 Gorlich D, Henklein P, Laskey PRA & Hartman E. A 41 amino acid motif in importin alpha confers binding to importin beta and hence transit in to the nucleus. *EMBO J* 1996; **15**: 1810–1817.
17 Goel VK, Li X, Chen H, Liu SC, Chishti AH & Oh SS. Band 3 is a host receptor binding merozoite surface protein 1 during the *Plasmodium falciparum* invasion of erythrocytes. *Proc Natl Acad Sci USA* 2003; **100**: 5164–5169.
18 Howard RJ, Uni S, Aikawa M, *et al.* Secretion of a malarial histidine-rich protein (Pf HRP II) from *Plasmodium falciparum*-infected erythrocytes. *J Cell Biol* 1986; **103**: 1269–1277.
19 Akompong T, Kadekoppala M, Harrison T, *et al.* Trans expression of a *Plasmodium falciparum* histidine-rich protein II (HRPII) reveals sorting of soluble proteins in the periphery of the host erythrocyte and disrupts transport to the malarial food vacuole. *J Biol Chem* 2002; **277**: 28923–28933.
20 Futagami S, Takahashi H, Norose Y, Nagata K, Kobayashi M & Nomura T. Analysis of immune response to *Helicobacter pylori* urease in patients with chronic gastroduodenal diseases. *Jap J Gastroenterol* 1994; **91**: 2202–2213.
21 Phadnis SH, Parlow MH, Levy M, *et al.* Surface localization of *Helicobacter pylori* urease and a heat shock protein homolog requires bacterial autolysis. *Infect Immun* 1996; **64**: 905–912.
22 Ferrero RL, Jean-Michel T, Kansau I, Wuscher N, Huerre M & Labigne A. The GroES homologue of *Helicobacter pylori* confers protective immunity against mucosal infection in mice. *Proc Natl Acad Sci* 1995; **92**: 6499–6503.
23 Michetti P, Corthesy-Theulaz I, Davin C, *et al.* Immunization of BALB/c mice against *Helicobacter felis* infection with *Helicobacter pylori* urease. *Gastroenterology* 1994; **107**: 1002–1011.
24 Myers GA, Ermark TH, Georgakopoulos K, *et al.* Oral immunization with recombinant *Helicobacter pylori* urease confers long lasting immunity against *Helicobacter felis* infection. *Vaccine* 1999; **17**: 1394–1403.
25 Kennedy MC, Wang J, Zhang Y, *et al. In vitro* studies with recombinant *Plasmodium falciparum* apical membrane antigen 1 (AMA1): Production and activity of an AMA1 vaccine and generation of a multiallelic response. *Infect Immun* 2002; **70**: 6948–6960.
26 Lambros C & Vanderberg JP. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J Parasitol* 1979; **65**: 418–420.
27 Anders RF. Multiple cross-reactivities amongst antigens of *Plasmodium falciparum* impair the development of protective

immunity against malaria. *Parasite Immunol* 1986; **8**: 529–539.
28 Schofield L. On the function of repetitive domains in protein antigens of *Plasmodium* and other eukaryotic parasites. *Parasitol Today* 1991; **7**: 99–105.
29 Danier-Ribeiro CT. Is there a role of autoimmunity in immune protection against malaria? *Mem Inst Oswaldo Cruz* 2000; **95**: 199–207.
30 Shaper AG, Kaplan MH, Mody NJ & McIntyre PA. Malarial antibodies and autoantibodies to heart and other tissues in the immigrant and indigenous peoples of Uganda. *Lancet* 1968; **1**: 1342–1347.
31 Kreier JP & Dilley DA. *Plasmodium berghei*; nucleic acid agglutinating antibodies in rats. *Exp Parasitol* 1969; **26**: 175–180.
32 Danier-Ribeiro CT & Zanini G. Autoimmunity and malaria: what are they doing together? *Acta Tropica* 2000; **76**: 205–221.