APPENDIX

Materials

All the chemical reagents and kits used during this study were of molecular biology grade. These were procured from Sigma Chemical Company St Louis USA, Boehringer Mannheim; GmbH-Mannheim, Germany, Amersham Life Sciences, Illinois USA, New England Biolabs, MA. USA, Genei, Bangalore, India, BIO–RAD, California, USA, Promega, Pharmacia, Sweden, Qiagen, Gibco BRL and United States Biochemicals, USA.

Concentration of the protein

In all cases mentioned, protein concentration was carried out by ultrafiltration using YM 10 membrane (stirred Amicon cell, Millipore).

Media

LB (Luria-Bertani) medium. 20 g of LB powder (Sigma) /litre, sterilize by autoclaving.

LB-Agar. 30 g of LB-Agar powder (Sigma)/litre, sterilized by autoclaving.

Antibiotics

Ampicillin. Stock solution – 100 mg/ml salt of ampicillin in distilled water and sterilize by filtration through 0.22 μM syringe filter (sterile). Store at –20°C.

Kanamycin. Stock solution – 50 mg/ml of salt of kanamycin monosulfate in distilled water and sterilize by filtration through 0.22 μM syringe filter (sterile). Store at –20°C.
Stock solutions of commonly used reagents

1M Tris. Dissolve 121.1 g Tris base in 800 ml H₂O and adjust the desired pH (7.4 and 8.0) with concentrated HCl. Make up to 1 litre and autoclave.

0.5M EDTA. Add 186.1 g of disodium EDTA 2H₂O to 800 ml H₂O. Stir vigorously on a stirrer, adjust the pH to 8.0 with NaOH (~20 g of NaOH pellets), make up to 1 litre and autoclave.

5M NaCl. Dissolve 292.2 g NaCl to 800 ml H₂O, make up to 1 litre and autoclave.

3M Sodium acetate. Dissolve 408.1 g of sodium acetate 3H₂O in 800 ml H₂O. Adjust the pH to 5.2 with glacial acetic acid, make up to 1 litre and autoclave.

20% SDS. Dissolve 20 g of electrophoresis grade SDS in 70 ml of H₂O, heat to 68°C to dissolve and make up to 100 ml.

20 x SSC. Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml H₂O. Adjust the pH to 7.0 with NaOH. Make up to 1 litre with H₂O and autoclave.

Denhardt's solution (50X). Ficoll 5 g, polovinyl pyrrolidone 5 g, BSA (fraction V) 5 g, make up to 500 ml with H₂O. Filter through a 0.45 μM filter; dispense in 50 ml aliquot and store at -20°C.

Ethidium bromide. (10 mg/ml), stored in a dark bottle at 4°C.

10 x PBS. 53 g NaCl, 58 g Na₂HPO₄, 16.3 g KH₂PO₄, make up to 1000 ml with H₂O. Filter through 0.45 μM filter and autoclave.
30% Acrylamide – 0.85 Bis. 30 g of Acrylamide, 0.8 g of bis-acrylamide. Make upto 1 litre with H₂O, filter and degas.

X-gal (2 ml). 100 mg X-gal in 2 ml of N,N' dimethyl-formamide. Store in dark at -20°C.

IPTG (0.1M). 120 mg IPTG in 5 ml H₂O. Filter sterilize and store at -20°C.

**Buffers**

5 x TBE (Tris-borate-EDTA). 54 g Tris base, 27.5 g boric acid, 20 ml 0.5 M EDTA. Make upto 1 litre with H₂O and store at room temperature.

10 x SDS-PAGE running buffer. 30.3 g Tris base, 144.1 g glycine, 10 g SDS. Make upto 1 litre with H₂O.

10 x Protein transfer buffer. 60.55 g tris base, 71.37 g glycine, 1.0 g SDS. Make upto 1 litre with H₂O. For 1 x buffer add 100 ml of 10 x buffer to 200 ml of methanol and make up the volume 1 litre with H₂O.

2 x SDS PAGE sample buffer. 2 ml of 0.625 M Tris-Cl, pH 6.8, 1 ml of βMercaptoethanol, 2 ml of 20% SDS, 2 ml of Glycerol, 3 ml of H₂O. Add bromophenol blue to desired intensity.

DNA loading dye (6 x). 0.25% bromophenol blue, 0.25% xyline cynol, 40% (w/v) sucrose in H₂O. Store at room temperature.

**Gen Bank Accession Number of PcTRAP nucleotide sequence:** Y12541
Short communication

Cloning and sequence analysis of the thrombospondin-related adhesive protein (TRAP) gene of *Plasmodium cynomolgi bastianelli*

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Received 18 July 1997; received in revised form 2 September 1997; accepted 15 September 1997

**Keywords:** Thrombospondin-related adhesive protein (TRAP); *Plasmodium cynomolgi*; Sporozoite surface protein 2 (SSP2); Sulphatide binding domain

Thrombospondin-related adhesive protein (TRAP) is one of the two major proteins identified on the sporozoite surface of *Plasmodium falciparum* that are involved in hepatocyte or HepG2 cell line recognition or invasion [1–3]. A number of studies using recombinant or synthetic constructs have shown that the *P. falciparum* TRAP (PfTRAP) binds specifically to hepatocytes as well as heparin sulphate proteoglycans, facilitating the parasite-host cell interaction [4,5]. Furthermore, experimentally raised antibodies to certain PfTRAP fragments have been shown to inhibit the sporozoite invasion of hepatocytes [4]. In humans living in malaria endemic areas, Scarselli et al. have shown close correlation between levels of TRAP antibodies and clinical protection against malaria [6]. Others have demonstrated that vaccination of mice with the TRAP homologue of *P. yoelii*, termed as the sporozoite surface protein-2 (SSP2), along the circumsporozoite protein (CSP) of *P. yoelii*, confers complete protection against challenge

Abbreviations: aa, Amino acid; AMA-1, apical membrane antigen-1; CSP, circumsporozoite protein; gDNA, genomic DNA; PbTRAP, *P. berghei* TRAP; PchTRAP, *P. cynomolgi* TRAP; PfTRAP, *P. falciparum* TRAP; PgTRAP, *P. gallinaeum* TRAP; PkTRAP, *P. knowlesi* TRAP; PvTRAP, *P. vivax* TRAP; PyTRAP, *P. yoelii* TRAP; SSP-2, sporozoite surface protein 2; SSUrRNA, small-subunit ribosomal RNA; TRAP, thrombospondin-related adhesive protein.

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† Note: Nucleotide sequence data reported in this paper is available in the EMBL, GenBank™ and DDJB databases under the accession No Y12541
with a lethal inoculation of *P. yoelii* sporozoites [7]. However, the role of TRAP and indeed, its very expression in the erythrocyte stages of *P. falciparum*, is not yet clearly established. Nevertheless, antisera to the CSP-like Region II of TRAP inhibits the in vitro invasion of erythrocytes by the asexual blood stage merozoites and also recognizes a TRAP-like protein in the blood stage lysate of *P. falciparum* [8]. Because of its possible role in two most important events viz. the sporozoite invasion of hepatocytes and merozoite invasion of erythrocytes, TRAP represents a putative vaccine molecule, potentially effective against two different stages of the parasite [7,8].

Recently TRAP genes from a number of *Plasmodium* species, like *P. vivax* (PvTRAP), *P. knowlesi* (PkTRAP), *P. gallinacium* (PgTRAP) [9] and *P. berghei* (PbTRAP) [10] have been cloned and sequenced. The overall organisation of TRAP seems to be conserved in all species; specifically an N-terminal signal sequence, an integrin A domain, a sulfatide-binding motif similar to one seen in Region II of Plasmodium CS proteins, followed by an acidic, proline rich region, a transmembrane domain and a short cytoplasmic tail [9,10].

We present here, the nucleotide sequence analysis of the TRAP gene from a simian malaria parasite, *P. cynomolgi*; the PcTRAP maintains the overall structural organisation of Plasmodial TRAP. *P. cynomolgi* is a natural parasite of Asian macaques and is considered a useful model for studying *P. vivax* malaria, which is the second most important malaria parasite infecting humans. The study of the PcTRAP and its expression will help us to elucidate the potential role of TRAP as a vaccine candidate in rhesus monkeys, which represents a close approximation to the human immune system and also immunological studies with the expression products will allow us to ascertain the possible presence of PcTRAP in different stages of the life cycle of this parasite.

*P. cynomolgi* infection is routinely maintained at the Central Drug Research Institute (Lucknow), India. Blood from *P. cynomolgi* infected monkeys was obtained by venipuncture and passed through CF-11 column (Whatman) to remove leukocytes. The parasitized erythrocytes were purified by centrifugation on a ficoll-hy-
sequence and transmembrane domain, respectively) are underlined. Amino acid sequence shown in bold italics (I) corresponds to aa position 98 and charged residues remain conserved across the sequences are not strictly conserved. The sulfatide Region II is indicated by dashed line. Hydrophobic regions at N-terminus and C-terminus (corresponding to predicted signal that derived from the degenerate primers. The N-terminal

Fig. 1. Alignment of the amino acid sequences of the TRAP from P. cayen­molgi (1), P. vivax (2), P. knowlesi (3), P. falciparum (4), P. berghei (5), P. yoelii (6) and P. gallinaceum (7). Boxed regions represent consensus sequences in six out of seven TRAP molecules.

Dash (-) represents absence of that residue/or region at that position. (*) Indicates conserved cysteine residue across the all species. Region II is indicated by dashed line. Hydrophobic regions at N-terminus and C-terminus (corresponding to predicted signal sequence and transmembrane domain, respectively) are underlined. Amino acid sequence shown in bold italics (1) corresponds to that derived from the degenerate primers. The N-terminal sequence for 1–3 is unknown.

acid (aa) position 98 and a TDGVP sequence at aa position 108. The DGSGS sequence is conserved in all the TRAP proteins with the exception of PfTRAP, where the sequence changes to DCUAGS [12], whereas TNMS and TDGVP sequences are not strictly conserved. The sulfatide binding motif also exhibits variation in sequences among different TRAP molecules, though the charged residues remain conserved across the species. However, this domain (P/DNT­PCSVTGC/NGHTRSSR) is virtually identical in PcTRAP, PtvTRAP and PfTRAP. The basic sequence, length and number of repeat units vary considerably among different TRAP molecules [9]. The consensus repeat unit in PcTRAP is PEN, which is the same as PkTRAP, although the number of such repeats in PcTRAP is five, as compared to seven in PkTRAP. Like all other TRAP molecules, PcTRAP also lacks the RGD motif, seen in PfTRAP.

The number and positions of cysteine residues, which may be crucial in determining the overall structure of the protein, are rigorously conserved across the TRAP molecules, though the charged residues remain conserved across the species.
TRAP is present in the N-terminal signal sequence. The acidic, proline rich region of PcTRAP shows more homology with PfTRAP and PkTRAP, as compared to the other four TRAP molecules. However, its length varies among all the TRAP molecules. At the carboxy terminus, PcTRAP, like other TRAP species, shows strict conservation of a sequence (N(G/K)YKGAGG) within the exodomain, immediately adjacent to the transmembrane region and a cysteine residue within the transmembrane domain. The cytoplasmic tail in PcTRAP shares similar features with other TRAP proteins; it has a very low PI together with conserved tryptophan and proline residues [9].

Southern blot analysis of P. cynomolgi genomic DNA, digested with EcoRI and EcoRI-PstI, was carried out using radiolabelled-PcTRAP gene under stringent hybridisation and washing conditions [13]. The pattern of southern blot suggested that PcTRAP is present as a single copy gene (data not shown).

A comparison of the PcTRAP aa sequence with other TRAP sequences available so far, reveals that PcTRAP is more closely related to PvTRAP (~73.9% homology) and to PkTRAP (~71.9% homology) than to other TRAP molecules. Sequence similarity among these three TRAP molecules exists throughout the gene to some extent, even in the highly variable repeat region. The greater homology of PcTRAP with that of PvTRAP and PkTRAP is in agreement with our earlier study of the apical merozoite surface antigen (AMA-1); PcAMA-1 was found to be more closely related with PvAMA-1 and PkAMA-1, than with AMA-1 molecules of other Plasmodium species [14]. Escalante et al. [15] have also shown, based on DNA sequences of small subunit rRNA genes, that P. cynomolgi is more closely related in evolution to P. vivax and P. knowlesi than to P. falciparum, and other malaria parasites. Based on the closer similarity of the amino acid sequences, and the fact that PcTRAP and PvTRAP are biologically closely related (e.g. both cause relapse), it seems that among these three closely related parasite species, P. cynomolgi and P. vivax are closer to each other. This divergence may have probably occurred after the separation in evolution of P. knowlesi from P. vivax and P. cynomolgi. For this reason P. cynomolgi may serve as a good model to develop malaria vaccine against P. vivax infection.

Acknowledgements

The authors thank Dr Pawan Sharma for critical suggestions and helpful discussions. We are grateful to the director, CDRI Lucknow and Dr G.P. Datta for providing P. cynomolgi material. Financial assistance given to the first author by the CSIR, India, is duly acknowledged.

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