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Single or multiple localization of ADP/ATP transporter in human malarial *Plasmodium falciparum*

Malarial parasites develop and propagate asexually inside the erythrocytes of their vertebrate host. Although the parasite is considered to be a homolactate fermentor [1] its acristate mitochondrion [2] is capable of electron transport [3] and maintains a membrane potential that responds classically to various inhibitors [4]. The same cytosol inhibitors also compromise parasite growth and reduce the ATP levels in the parasite's cytosol, thus, assigning a role for the mitochondrion in ATP production [5]. Quite unexpectedly, it was discovered recently that this membrane contains an ADP/ATP translocator whose biochemical characteristics are identical to the transporter that is usually located at the inner membrane of mitochondria [6–8]. Since host cell ATP production is compromised, because glycolytic activity is presumably very much reduced due to the acidification of its cytosol [9], the parasite membrane ADP/ATP translocator has the crucial role of supplying ATP produced by the parasite to its host cell [10].

Studies on the biogenesis of mitochondria indicate that the ADP/ATP transporter (adenylate translocase, AdT*) is coded by the nuclear genome and is synthesized in the cytosol of eukaryotic cells. Unlike other nucleus-coded mitochondrial proteins, its molecular weight is identical to that of its precursor, implying that it does not contain a cleavable leader sequence [11]. In the presence of an adequate concentration of nucleotide triphosphates, the precursor assumes a conformation that allows it to bind to a specific receptor on the mitochondrial surface and is then driven electrophoretically by the membrane potential to its final destination at the inner membrane of the organelle [12, 13]. Cloning and sequencing of the AdT gene from various types of cells revealed that the polypeptide consists of three repeats of about 100 amino acid residues displaying a rather impressive homology [14]. The C-terminal end of each repeat contains a stretch rich in residues of basic amino acids that confers the necessary charge for the electrophoretic translocation [15]. While the transmembrane sequences are highly conserved throughout evolution, the hydrophylic sequences that contain the charged residues display a substantial variability both in composition and in net charge.

From the above description, it is clear that the presence of AdT at the parasite's cell membrane poses some intriguing questions concerning its targeting and translocation. The requisite for a specific receptor at the cell membrane is probably not absolute, inasmuch as substantial translocation into mitochondria can occur even in its absence. However, the polarity of the cell membrane potential required for the electrophoretic translocation, is opposite that of the mitochondrial membrane [16], and the question then arises as to the identity of the peptide sequence(s) that would

* Abbreviations: AdT, the ADP/ATP transporter adenylate translocase; bp, base pair; anti-AdT, rabbit polyclonal antibody directed against mitochondrial AdT; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

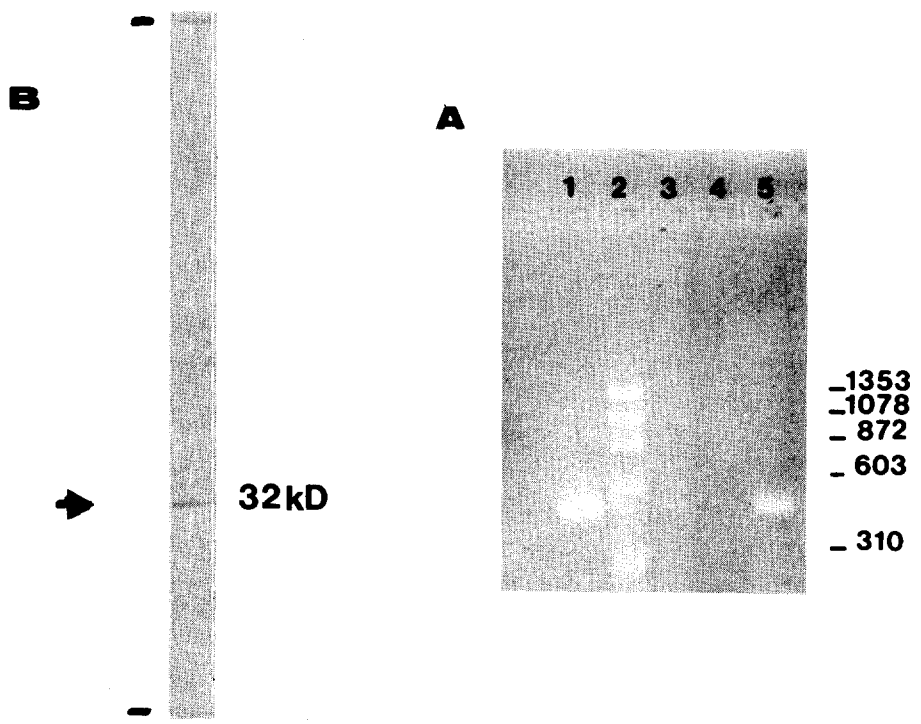

Fig. 1. (A) Ethidium bromide stained 2% agarose gel. 1 and 5: Amplified *P. falciparum* DNA; 2: 0.25 μg ΦX174 cut by *Hin*dIII as marker size in bp; 3: 2 μL of reaction mixture alone; 4: oligoprimer alone. (B) Western blotting of *P. falciparum*-infected cells electrophoretically separated by SDS–PAGE with rabbit polyclonal anti-Adt.

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Sc  F P T Q A L M F A F K D K I K S L L S Y D R E R
Nc  * * * * * * * * * * R * * F * K M F G Y K K D V
Bb  * * * * * * * * * * * * * Y * Q I F L G G V D *
Pf  F P T Q A L M F * * * * Y F * M K F P R Y D Q M

Sc  D G Y - A K W F A G N L F S G G A A G G L S L L
Nc  * * * - W * * M * * * * A * * * * * * A T * * *
Bb  H K Q F W R Y * * * * * A * * * * * * A T * * C
Pf  T D F - S * F * C V * I L * * A T * * A I * * *

Sc  F V Y S L D Y A R T R L A A D A R G S K S T S Q
Nc  * * * * * * * * * * * * * N * * K S A * K G G E
Bb  * * * P * * F * * * * * * * * V - - G * G A A *
Pf  I * * P * * F * * * * * * S * I - * - * G K D -

Sc  R Q F N G L L D V Y K K T L K T D G L L G L Y R
Nc  * * * * * * V * * * R * * I A S * * I A * * * *
Bb  * E * T * * G N C I T * I F * S * * * R * * * Q
Pf  * * * T * * F * C L A * I Y * Q T * * * S * * S

Sc  G F V P S V L G I I V Y R G L Y F G L Y D S F K
Nc  * * G * * * A * * V * * * * * * * * * * * I *
Bb  * * N V * * Q * * * I * * A A * * * V * * T A *
Pf  * * G V * * T * * * * * * * S * * * * * * * A *

Sc  P V L L T G A L E G S F V A S F L L G W V I T M
Nc  * * * * V * D * K N N * L * * * A * * * C V * T
Bb  G M * P D P Q N V H I - I V * W M I A Q T V * A
Pf  A L I F * N D K N T N I * L K W A V A Q S L * I

Sc  G A S T A S Y P L D T V R R R M M M
Nc  A * G I * * * * * * * * * * * * *
Bb  V * G L V * * * F * * * * * * * * *
Pf  L * G L I * * * F * T V R R R M M M
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Fig. 2. Comparison between primary sequence of ADP/ATP carrier from *S. cerevisiae* (Sc), *N. crassa* (Nc), *B. bovine* (Bb) and *P. falciparum* (Pf). * Amino acid idem. to Sc.

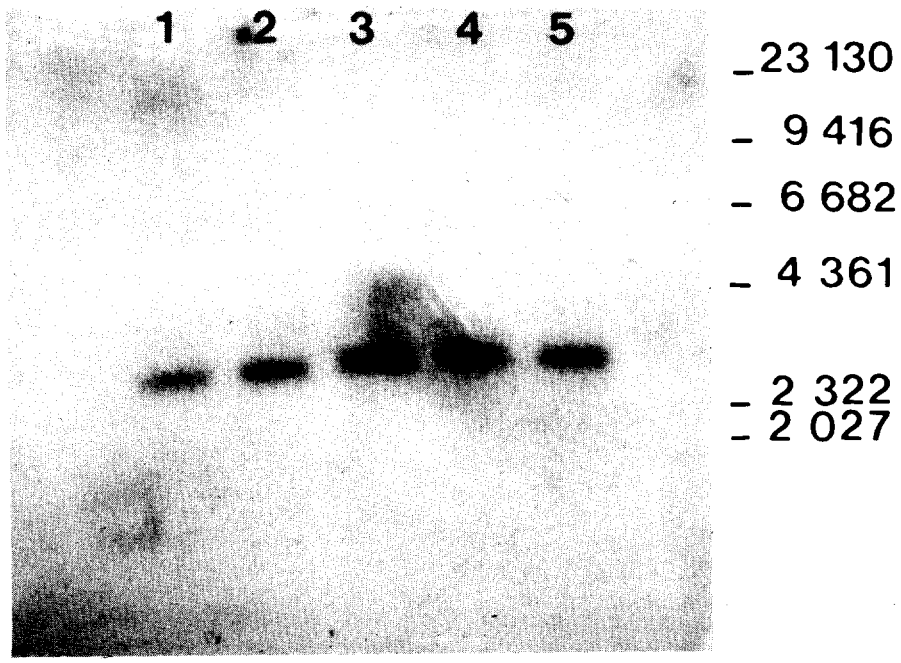

Fig. 3. Autoradiography of Southern blot of *P. falciparum* DNA. 1: DNA (1 mg) cut by *Hin*dIII; 2: cut by *Hin*dIII+*Bam*HI; 3, 4 and 5: cut by the three enzymes and hybridized with the transmembrane region fragment of AdT labelled with α ^{32}PdATP.

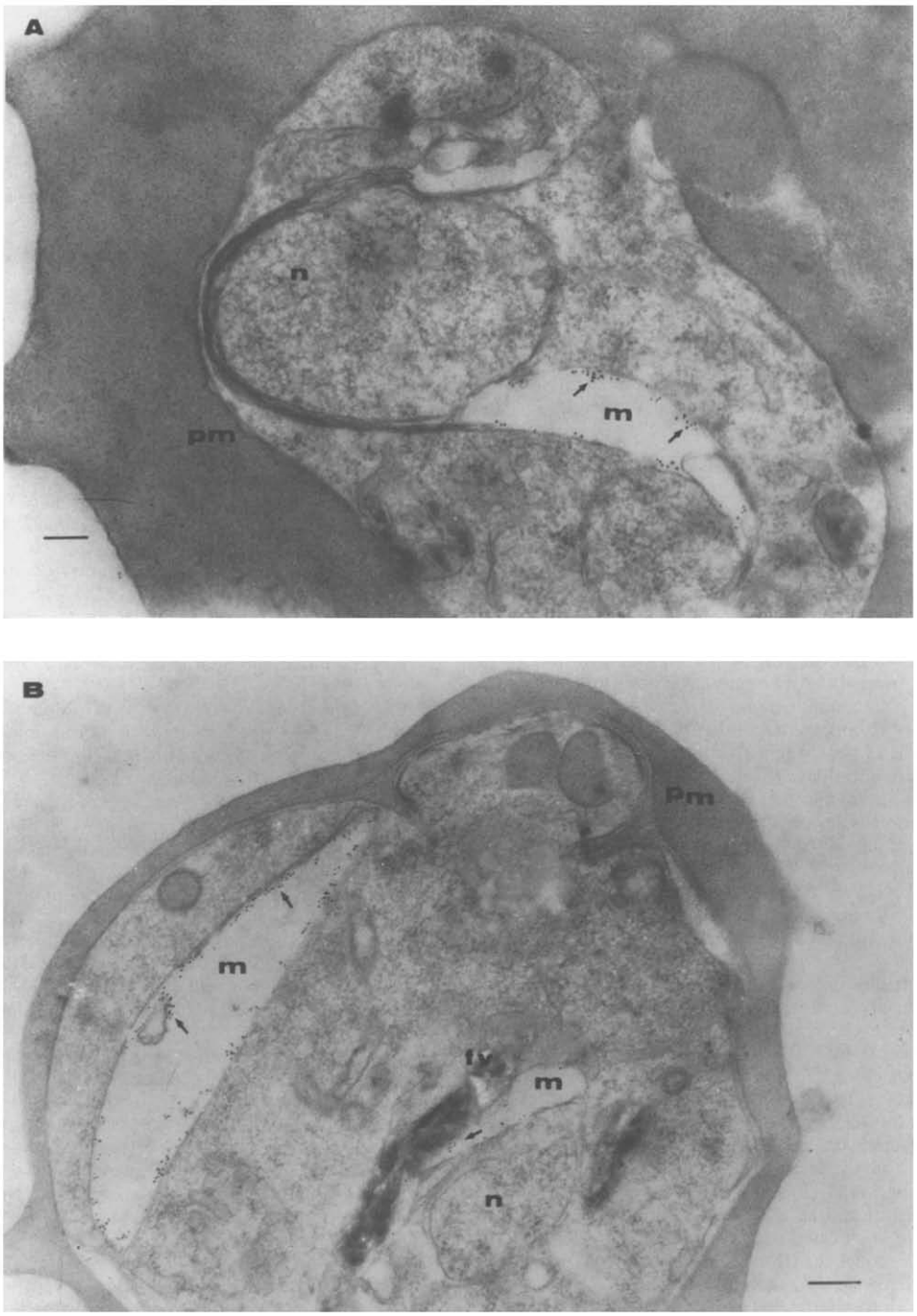

Fig. 4. (A and B) Immunogold labelling of infected red blood cells by *P. falciparum* with a rabbit polyclonal antibody directed against a mitochondrial ADP/ATP carrier from bovine heart cells. m, Mitochondria; fv, food vacuole; n, nucleus; pm, parasitophorus membrane. Arrows indicate gold labelling. Bar = 500 nm.

mediate this translocation. This enigma could probably be solved by the presence of two different genes in *Plasmodium*, one coding for the mitochondrial AdT and the other for that of the cell membrane. Indeed, three AdT genes have been identified in *Saccharomyces cerevisiae* [17] and two genes were found in human cells [18]. The expression of the yeast genes depends on growth conditions while that of the human genes is tissue specific. Therefore, one could envisage *a priori* two plasmodial genes that could code for the membrane specific AdTs.

To test this hypothesis, the AdT was cloned and sequenced from human malarial parasite *Plasmodium falciparum*. The subcellular localization of malarial ATP/ATP transporter was studied by immunoelectromicroscopy.

PCR was used with oligonucleotide primers constructed to match the conserved regions of *Neurospora crassa*

protein (essentially the transmembrane region) and in accordance with the codon usage of the parasite genome [19]. The 5′ primer was directed against the conserved transmembrane segment:

CCCAAGCTTTCCAACACAAGCATTAAATTTT

and the 3′ primer was directed against the ATP binding site:

CCCATGCATCATCATTCTTCTTCTTACAGT.

Amplification was performed according to Saïki *et al.* [20] in a Perkin–Elmer thermal cycler. The size of the resulting product was estimated by electrophoresis on agarose gels and staining with ethidium bromide (Fig. 1). The amplified fragment was cloned in the plasmic pUC 18 and sequenced. The DNA was annealed with either a universal straight or reversed primer of M13 phage, or with one of two primers complementary to a region already sequenced and having the following sequences:

5′GGTAAAGATAGACAAGTTTACAGA3′

and

5′ GATATATGTTATTATGGTCATTGAC3′

(Fig. 2). This sequence resides in open reading frame and contains 487 bp with 61 bp corresponding to the sequence of the primer used for PCR amplification. Therefore, 426 bp correspond to the transmembrane region of the AdT. The size of the fragment conforms with the length expected from the AdT gene structure and the primers chosen for the amplification.

The very high percentage of A + T (71%) found in the sequence is in concordance with base composition of the *P. falciparum* genome (82% A + T, 18% G + C) [19]. The deduced amino acid sequence (Fig. 2) is highly homologous to the corresponding zone of the hydrophobic and charged part of the other known AdT. The hybridization of this fragment with *Hin*dIII, *Bam*HI and *Eco*RI digests of parasite DNA, revealed one single band of 3100 bp. This DNA fragment is lined with *Hin*dIII sites and does not contain either *Bam*HI or *Eco*RI restriction sites (Fig. 3).

Western blotting of *P. falciparum*-infected cells electrophoretically separated by SDS–PAGE (7.5% acrylamide slab gel) with rabbit polyclonal antidody directed against mitochondrial AdT (anti-AdT) from bovine heart cell (generously given by Dr Brandolin) revealed a single band of 31–32 kDa (Fig. 1A). The immunogold labelling revealed a protein localized at the inner mitochondrial membrane. No label could be detected at the parasite cytoplasmic membrane in the immunoelectromicroscopy experiments with this anti-AdT (Fig. 4A, B).

In conclusion, the gene coding for the AdT of *P. falciparum* is located on a fragment of 3100 bp. The nucleotide sequence of the AdT has been partially elucidated and this sequence corresponds to its transmembrane region. Hybridization experiments with this fragment which always result in a single band imply the presence of one single AdT gene at the inner mitochondrial membrane and thus further reinforce the notion that the parasite's mitochondrion is engaged in ATP generation. This is in apparent contradiction to the expected presence of two genes coding for the mitochondrial and the plasma membrane AdT. However, an alternative interpretation consistent with the data is that the two putative genes are highly homologous and yield identical restriction fragments that hybridize successfully with the highly conserved transmembrane region. The lack of immunogold labelling of the cytoplasmic membrane may be explained by the method used for the extraction of the mammalian AdT used for raising the antibodies. Different methods can result in different conformations that have no common antigenic determinants. Further experiments are underway to resolve these apparent inconsistencies.

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