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Identification and recombinant expression of glyceraldehyde-3-phosphate dehydrogenase of *Plasmodium falciparum* [☆]

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Abstract

The gene coding for the cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) was isolated from *Plasmodium falciparum*. The gene contains 1 intron and the A+T content is characteristic for the codon usage of *P. falciparum*. The predicted open reading frame codes for 337 amino acids (36 651 Da) and is 63.5% identical to the human erythrocytic GAPDH. GAPDH sequences from several field isolates of *P. falciparum* displayed 100% conservation. Phylogenetic analysis supports the hypothesis that dinoflagellates and *Plasmodium* are closely related. The protein encoded by the pfGAPDH was expressed recombinantly in *Escherichia coli* and exhibited enzymatic activity with NAD⁺ but not with NADP⁺ as cofactor. Antiserum raised against the recombinantly expressed enzyme detected specifically all developmental stages of cultured *P. falciparum* blood-stage parasites. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Glyceraldehyde-3-phosphate dehydrogenase; Localization; *Plasmodium falciparum*; Recombinant expression

1. Introduction

Malaria is the most important parasitic disease in humans, and one of the major health problems in developing countries. Worldwide, the number of cases is rising at a rate of 5% annually. Increasing mosquito resistance to insecticides is a contributor to this effect, as is parasite multi-drug resistance. It is estimated that 300–500 million people are infected every year, of whom 2.3 million die of malaria (Engers and Godal, 1998). Inhibition of biosynthetic pathways employed by patho-

gens has been a useful therapeutic strategy (Ridley, 1999). Glycolysis is an evolutionary conserved pathway in which one molecule of glucose is cleaved in multiple steps into two molecules of pyruvate. The human pathogen *Plasmodium falciparum* has no functional citric acid cycle, and ATP production therefore depends fully on the glycolytic pathway (Sherman, 1998). The level of glycolysis of parasite-infected red blood cells is about 100-times greater than that observed in uninfected cells. This increased activity is accompanied by the appearance of parasite-encoded glycolytic enzymes displaying physical and catalytic properties that are different compared to their host homolog (Roth et al., 1988). The obligate dependence of the parasite's bloodstream form on glycolysis for ATP production suggests that interference with the glycolytic pathway can be used for drug development (Döbeli et al., 1990; Wanachiwanawin et al., 1999). Several genes of the glycolytic pathway of *P. falciparum* have been cloned and fully sequenced. They include hexokinase, glucose-6-phosphate isomerase, phosphofructokinase, aldolase, triose-phosphate isomerase, phosphoglycerate kinase and lactate dehydrogenase (Sherman, 1998). Glyceraldehyde-3-phosphate

Abbreviations: aa, amino acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MSP 1, Merozoite Surface Protein 1; mAb, monoclonal antibody; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; ORF, open reading frame; PCR, polymerase chain reaction; pfGAPDH, GAPDH of *Plasmodium falciparum*; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

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dehydrogenase (GAPDH, EC 1.2.1.12) is a key enzyme in glycolysis and catalyzes the oxidative phosphorylation of glyceraldehyde-3-phosphate into 1,3-bisphosphoglycerate in the presence of NAD^+ and inorganic phosphate. GAPDH is a tetramer, composed of four identical polypeptide chains that contain sulfhydryl groups essential to their catalytic activity (Harris and Waters, 1976). The mechanism of the GAPDH-catalyzed reaction involves an initial formation of a covalent hemithioacetal intermediate between GAP and essential Cys. The hemithioacetal is then oxidized to a thioester, with concomitant reduction of NAD^+ to NADH. The product biphosphoglycerate is finally released by phosphorylytic attack on the thioester by an inorganic phosphate. We describe here the cloning and expression of a glyceraldehyde-3-phosphate dehydrogenase gene of *P. falciparum* (pfGAPDH). We expressed the protein recombinantly in *Escherichia coli* and demonstrate that it is enzymatically active. Furthermore, using immunofluorescence analysis, we demonstrate the presence of the protein in blood-stage parasites.

2. Materials and methods

2.1. Cloning of pfGAPDH

A cDNA library was constructed from total RNA isolated from *P. falciparum* strain K1 employing the SMART PCR cDNA Library Construction Kit (Clontech). Briefly, 2 μg of total RNA was reverse transcribed using a modified oligo (dT) primer. Additionally, the SMART oligonucleotide was added to the reaction to serve as a short, extended template at the 5' end of the RNA for reverse transcription. When the reverse transcriptase reaches the 5' end of the RNA, the enzyme switches templates and continues replicating to the end of the SMART oligonucleotide. The resulting single-stranded cDNA contains the SMART oligonucleotide sequence at the 5' end, which then serves as a universal PCR priming site in the subsequent PCR. This single-stranded cDNA can be used directly for PCR to amplify the library employing the SMART oligonucleotide and the oligo (dT) primer. To select for PCR products bigger than 0.7 kb, PCR products were run on a 1% agarose gel, selectively excised and purified. PCR products were ligated into pGem5 T vector (Promega). DH125[®] cells (BRL-Life Technologies) were transformed by electroporation with the *P. falciparum* cDNA library. Plasmid DNA of randomly picked clones was digested with enzymes *NotI*/*NcoI* and the insert size was analyzed on 1% agarose gels. Clones carrying inserts of more than 1 kb were chosen for further analysis. Linearized DNA was transcribed and translated in vitro as described (Ebel et al., 1997). ³⁵Met-labeled translation products were analyzed by SDS-PAGE and clones encoding ORFs

yielding proteins of >20 kDa were selected. The corresponding cDNAs were characterized further by sequencing employing an ABI 310 automatic sequencer (Perkin Elmer). Genomic DNA was prepared from cultures of *P. falciparum* strains K1, MAD20 and different field isolates as described (Jiang et al., 1999). Primers to amplify pfGAPDH from genomic DNA were located at the untranslated 5' and 3' ends of the gene (5'-TTA-TATAAAAACATATATTTTTTCTTCGG-3'; 5'-AA-AATGCTTCATGTTTAAATAACTTAGTTG-3'). The amplification was achieved by incubating the reaction mixture (50 μl) for 4 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 45°C and 2 min at 72°C and a final extension for 10 min at 72°C. The PCR product was cloned into pGem5 T vector (Promega). The amplicons were sequenced as described (Jiang et al., 1999).

2.2. Expression of pfGAPDH in *E. coli*

ORF of pfGAPDH was amplified from plasmid A55 by PCR using primer GATGATCGCCCATATGGCAGTAACAAAACCTTGGAAATTAATGG and CATGTT TAC TCG AGT TAG TTG TTA GTA ATG TGT ACG GC containing *NdeI* and *XhoI* sites, respectively. The amplicon was digested with *NdeI* and *XhoI*, gel purified and cloned in frame into the bacterial expression vector pET28a⁺ (Novagen). The recombinant plasmid was sequenced to confirm that the pfGAPDH insert was in the proper reading frame and to exclude PCR errors. Competent *E. coli* BL21 (DE3) cells were transformed with the recombinant pET28a⁺ plasmid and expression of the fusion protein was induced by the addition of 1 mM isopropyl thiogalactoside (IPTG) (Calbiochem) after the A_{600} reached 0.6. The cells were induced at 37°C for 4 h and total cell lysates prepared and analyzed by 10% SDS-PAGE using 10% gels. *E. coli* cells expressing recombinant protein were harvested by centrifugation and lysed for 30 min on ice in lysis buffer [50 mM NaH_2PO_4 , 300 mM NaCl, 10 mM imidazole, 1 mg/ml lysozyme (Appligene Oncor)] and sonicated. After centrifugation at 10 000 $\times g$, the supernatant was loaded onto a NTA column (Qiagen) and purified according to the manufacturer's instructions. The recombinant protein was recovered using elution buffer (50 mM NaH_2PO_4 , pH 8.0, 300 mM NaCl, 500 mM imidazole). After analysis by SDS-PAGE and Western blotting, the purified protein was pooled, dialyzed against assay buffer and stored at +4°C. Protein concentration was determined according to Bradford (1976) using BSA as standard.

2.3. *Plasmodium falciparum* parasite cultures

Parasite strains K1, MAD20, IFA 4, IFA 7, IFA 16.9, IFA 16.3, IFA 11.2, IFA 11.5 and IFA 93 (Jiang et al., 1999) were grown in vitro essentially as described (Matile and Pink, 1990). They served as source for

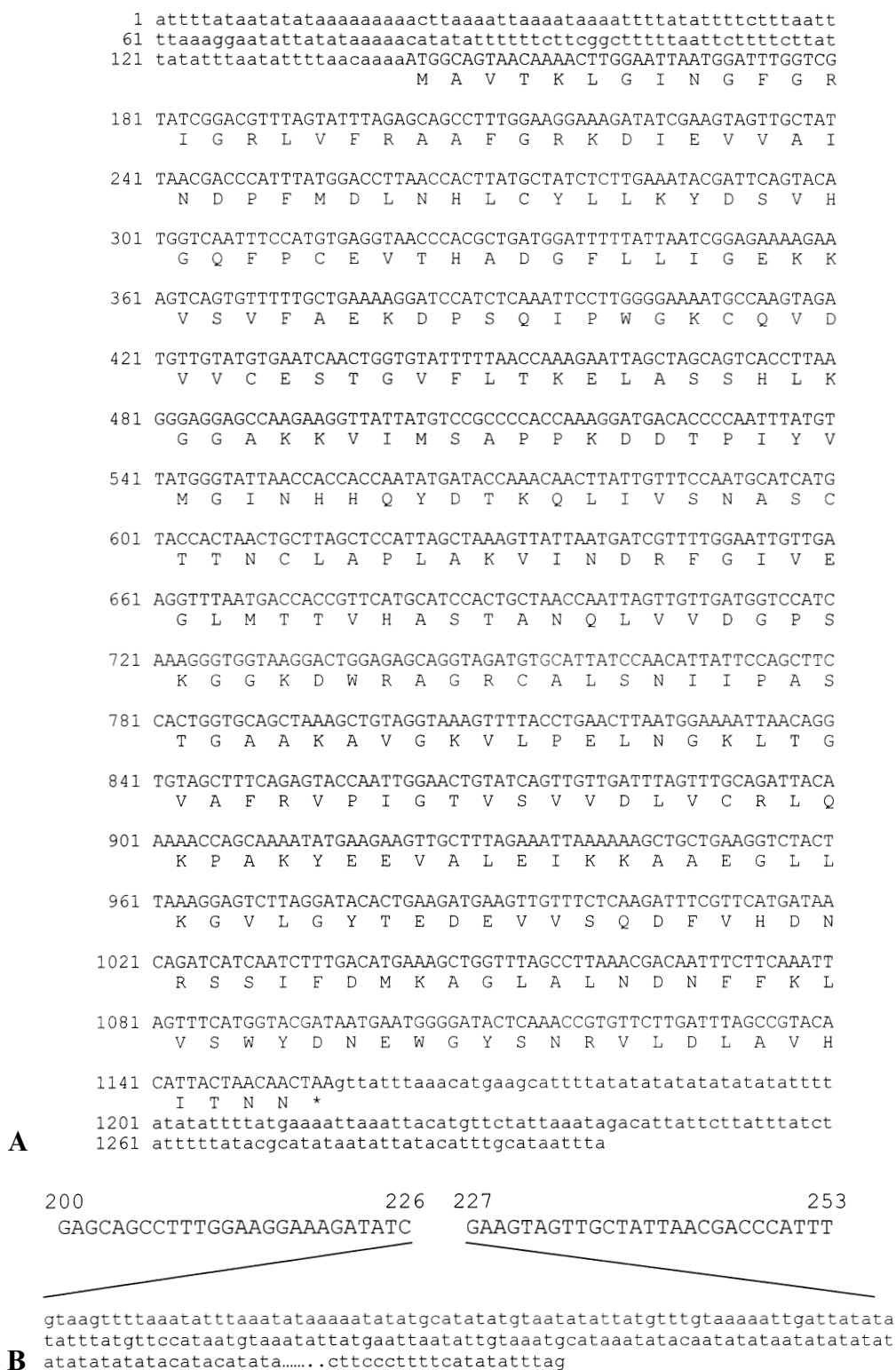


Fig. 1. (A) Complete cDNA sequence and deduced amino acid sequence of the pfGAPDH clone A55. The complete cDNA sequence was submitted to GenBank with the accession No. AF030440. The coding sequence is shown in uppercase, the non-coding sequence in lowercase. (B) Amplification of pfGAPDH gene from genomic DNA of *P. falciparum* strain K1. The position of the single intron sequence of about 300 bp is depicted. It was not possible to sequence the intron completely, due to its high AT-richness.

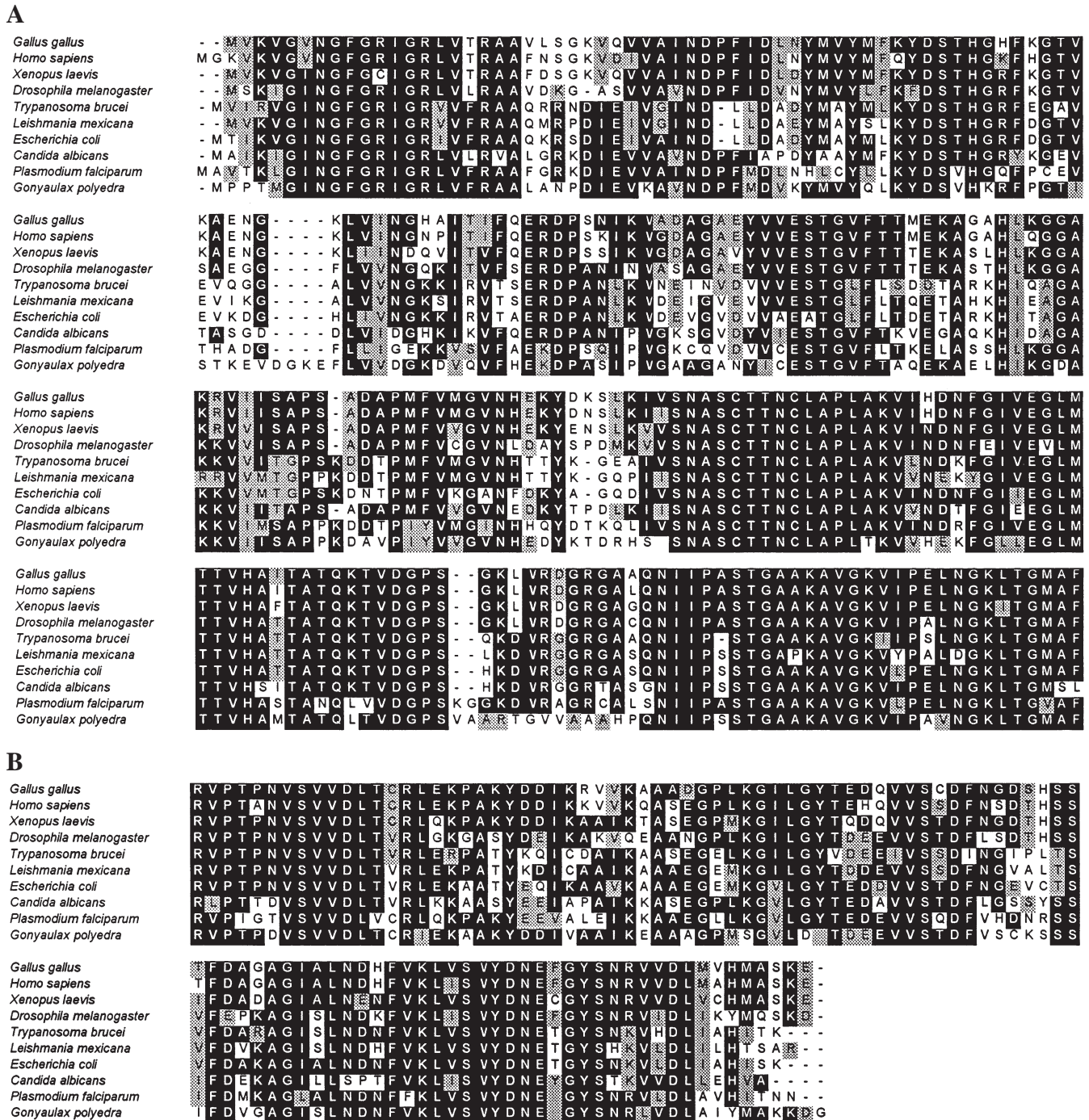


Fig. 2. Alignment of pfGAPDH amino acid sequences with representative GAPDH amino acid sequences of other organisms. The alignment was done with ClustalW and prepared for display using BOXSHADE (<http://bioweb.pasteur.fr>). For references to the source of sequences used, see legend to Fig. 3.

genomic DNA, cDNA and parasitized erythrocytes for immunofluorescence analysis.

2.4. Western blot analysis

SDS-PAGE was performed essentially as described (Gentz et al., 1988). Briefly, total cell lysates and purified

proteins were run on 10% gels. As molecular weight marker, RPN 800 (Amersham) was used. Separated proteins were transferred electrophoretically to nitrocellulose filter (Protean Nitrocellulose, BA 85, Schleicher & Schuell). Blots were blocked and then incubated with the Penta-His tag antibody (IgG₁; 1:1000) (Qiagen). After several washing steps, blots were incubated with

a 1:1000 dilution of a goat anti-mouse IgG alkaline phosphatase conjugated IgG (Sigma) for 2 h. Blots were developed using BCIP (5-bromo-chloro-3-indolyphosphate, Biorad) and NBT (nitrobluetetrazolium, Biorad) to visualize bands.

2.5. Production of antisera in mice and immunofluorescence analysis of blood-stage parasites

Four mice were immunized subcutaneously with 40 μ g of pfGAPDH formulated in RibiAdjuvant (Ribi-ImmunoChem, Old Corvallis, MT). After the third immunization, serum was used for immunofluorescence analysis of blood-stage parasites. Staining of in vitro cultured blood-stage parasites was conducted essentially as described (Pörtl-Frank et al., 1999). Briefly, multitest immunofluorescence microscopy slides (Flow Labs, Switzerland) were pre-treated with 0.01% (w/v) poly L-lysine (Sigma) for 30 min at room temperature and washed. Erythrocytes from an in vitro culture (Matile and Pink, 1990) of the *P. falciparum* clone K1 with about 10% parasitemia were washed and mixed with two volumes of a solution containing 4% paraformaldehyde and 0.1% Triton X-100. Droplets of 30 μ l cell suspension were added to each well and incubated for 30 min at room temperature. Cells were blocked with blocking solution containing 100 mg/ml fatty acid-free bovine serum albumin in PBS and then incubated with a 1:100 dilution of mouse sera for 1 h. The Merozoite Surface Protein 1 (MSP 1) specific mAb P.190-III:18 (Hugues Matile, personal communication) served as positive control and was used as undiluted hybridoma supernatant. After several washings, cells were incubated with 20 μ l of Cy3-conjugated affinity pure F(ab)₂ fragment goat anti-mouse IgG antibodies (Jackson ImmunoResearch Labs). For staining of parasite nuclei, Hoechst No. 33258 (Sigma) was added to the wells for 1 h at room temperature. After additional washing steps, the slides were mounted and assessed by fluorescence microscopy.

2.6. Enzyme assays

Glyceraldehyde-3-phosphate dehydrogenase activity for the forward reaction was assayed spectrophotometrically at 340 nm as described (Ferdinand, 1964). Briefly, the reaction was measured in triethanolamine (40 mM), Na₂HPO₄ (50 mM), EDTA (0.2 mM), 2 μ M D-glyceraldehyde-3-phosphate solution, 2 μ M NAD⁺ and recombinantly expressed pfGAPDH or commercially available porcine muscle GAPDH (Sigma). Reaction was initiated at 25°C by the addition of glyceraldehyde-3-phosphate. One unit is defined as the amount of enzyme that converts 1 μ mol of NAD⁺ in 1 min at 25°C.

2.7. Phylogenetic analysis

Phylogenetic analysis was performed by the neighbor-joining method (Saitou and Nei, 1987) for distances estimated with the Dayhoff matrix using the PROTDIST, SEQBOOT and NEIGHBOUR programs of the PHYLIP package, Version 3.572, available under <http://bioweb.pasteur.fr> (Felsensten, 1999).

3. Results

3.1. Cloning and characterization of pfGAPDH

During the characterization of a number of cDNA clones from a *P. falciparum* cDNA library which had yielded in vitro translated products, we identified a cDNA clone carrying the complete pfGAPDH gene coding sequence. This nucleotide sequence (Fig. 1A) predicts an open reading frame of 337 amino acids, with a predicted molecular mass of 36 651 Da and a theoretical *pI* of 7.59. The base composition is characteristic of *P. falciparum* DNA (Saul and Battistutta, 1988), being 62% A-T rich in the coding region and 87% A-T rich in both the 5' and 3' non-coding regions. The initiation codon was assigned by analogy to known GAPDH sequences and is strongly supported by the nucleotide context of the ATG start codon, AAAATGG, which is described in other genes of *P. falciparum* (Saul and Battistutta, 1990). An eukaryotic TATA-box is located about 20 nucleotides upstream of the initiation codon (Fig. 1A).

The pfGAPDH gene was amplified from genomic DNA of *P. falciparum* clones K1, MAD 20 and different field isolates from Tanzania (Jiang et al., 1999). The sequences obtained revealed that the gene contains a single intron of about 300 bp length which is flanked by the typical splice consensus sequences found in other *P. falciparum* genes (Saul and Battistutta, 1988) (Fig. 1B). No differences in the nucleotide sequences of pfGAPDH derived from different isolates were identified.

A comparison of deduced amino acid sequences of GAPDH from selected organisms are shown in Fig. 2. Comparison of the amino acid sequences of pfGAPDH and human erythrocyte GAPDH revealed 63.5% identity. Despite considerable divergence from other characterized GAPDH molecules, the sequence of pfGAPDH retains most of the residues considered to be important for binding both substrate and co-factor as identified by X-ray crystallography of human GAPDH (Hannaert et al., 1998). The residues involved in catalysis (C165, H193), in substrate-phosphate binding (T196, A197, T198, R248) and in inorganic phosphate binding (S164, T166, T167, T225, G226, A227) are fully conserved in all organisms, except for position T198 which is changed to N198 in *P. falciparum* (Fig. 2). Furthermore, at

position 206, a unique insert of a K and G residue is present in *P. falciparum*. At the same position, the dinoflagellate *Gonyaulax polyedra* has an insert composed of V and A (Fig. 2).

Phylogenetic analyses were carried out on the protein sequences of selected organisms using the PHYLIP program. Distance matrices of proteins (PROTDIST) were calculated and the result of the neighbor-joining method is shown in Fig. 3. The pfGAPDH is localized on the same branch as *Gonyaulax polyedra*, belonging to the class of dinoflagellates.

3.2. Expression and enzymatic activity of pfGAPDH

In order to characterize the enzymatic activity of the protein encoded by the cDNA clone A55, pfGAPDH

was expressed in *E. coli*. For affinity purification, we attached a hexa-histidine tail at the N-terminus, which allows binding to Ni²⁺-chelate resins. This reengineering resulted in the addition of 18 amino acid residues not present in the native enzyme, namely MGSSHHHHHSSGLVPRGSH. After affinity purification, the expressed protein was analyzed by SDS-PAGE and Western blotting for purity and homogeneity. As demonstrated in Fig. 4, a single band of 36 kDa was detected after both protein staining with Coomassie Blue (Fig. 4A) and immunostaining using mouse antiserum raised against the recombinant pfGAPDH (Fig. 4B). The recombinant pfGAPDH runs slightly higher as native GAPDH, which could be explained by the additional 18 amino acid residues not present in the native sequence (Fig. 4B). Bands of different sizes pre-

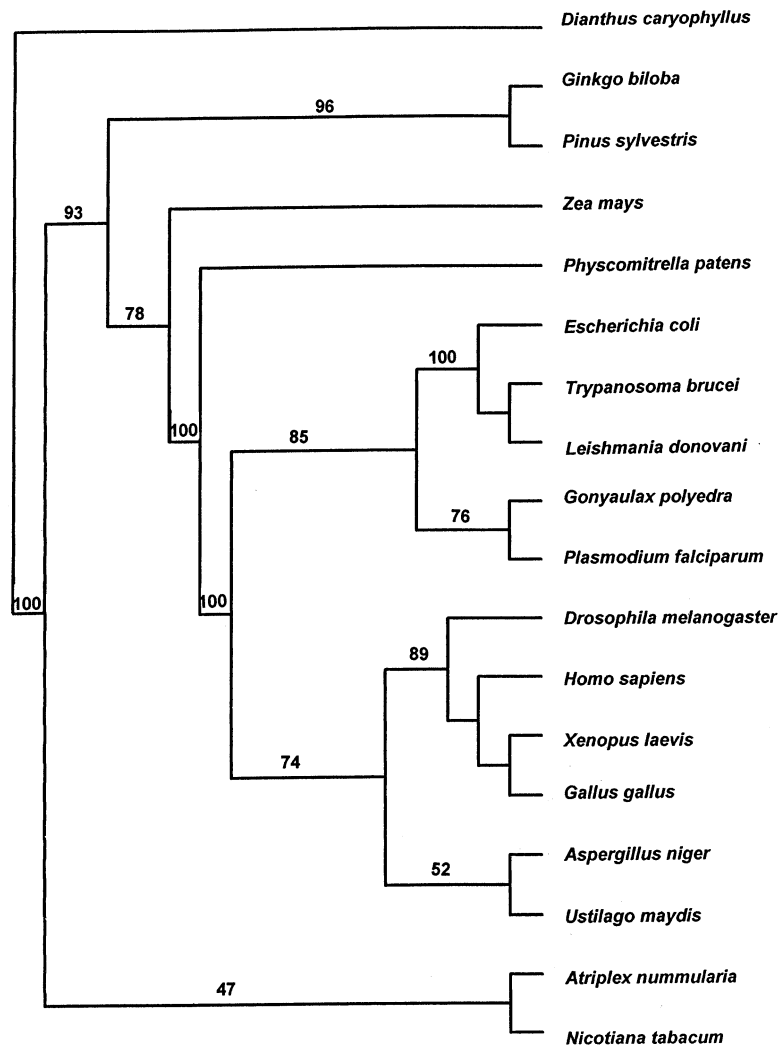


Fig. 3. Phylogeny of 18 species inferred from the protein sequences of GAPDH. The numbers on the branches are bootstrap values obtained with 1000 replications and indicate the percentage of times all species to the right appear as a monophyletic cluster. The sequences are from *Ginkgo biloba* (Q39769), *Physcomitrella patens* (P34923), *Dianthus caryophyllus* (P34921), *Nicotiana tabacum* (CAB 39974), *Xenopus laevis* (P51469), *Gallus gallus* (P00356), *Atriplex nummularia* (P34783), *Pinus sylvestris* (P34924), *Escherichia coli* (P06977), *Aspergillus niger* (Q12553), *Homo sapiens* (P0034), *Ustilago maydis* (P09317), *Drosophila melanogaster* (Q01597), *Trypanosoma brucei* (P00097), *Plasmodium falciparum* (AF030440), *Gonyaulax polyedra* (AF028562), *Leishmania mexicana* (Q01558) and *Candida albicans* (Q92211).

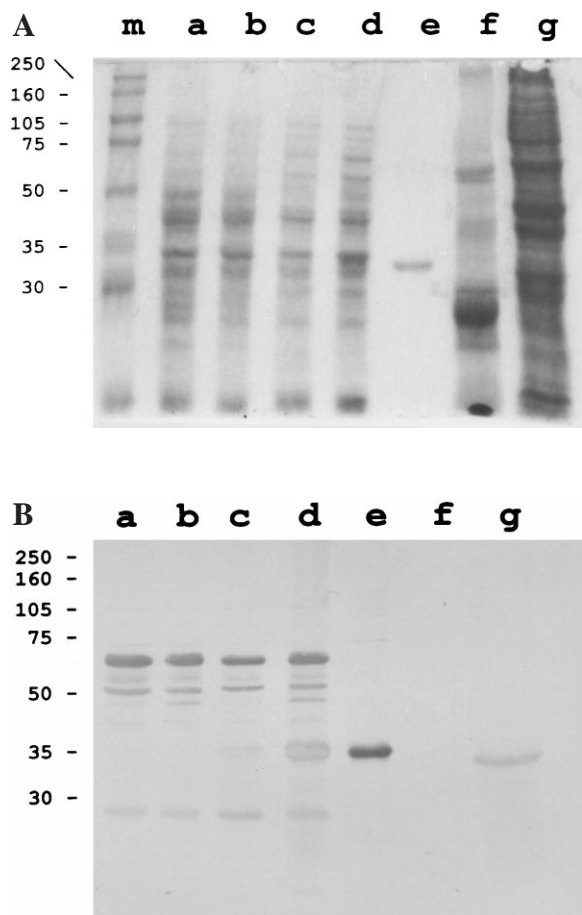


Fig. 4. (A) Recombinant expression and purification of pfGAPDH. Induction and purification of pfGAPDH was monitored. A 10% SDS-PAGE was run and stained with Coomassie Blue. Lanes a and b: total cell lysate of BL21 (DE3) *E. coli* cells transformed with empty pET28a⁺ vector before and after induction with IPTG for 4 h, respectively. Lanes c and d: BL21 (DE3) *E. coli* cells transformed with pET28a⁺ carrying ORF of pfGAPDH before and after induction with IPTG for 4 h, respectively. Lane e: the purified pfGAPDH protein. Lanes f and g: lysates from uninfected erythrocytes and from erythrocytes infected with *P. falciparum* asexual blood stages, respectively. Sizes of molecular weight standards are given in lane m. (B) Specificity of mouse antiserum raised against recombinant pfGAPDH. The same samples as in (A) are run in parallel and analyzed by Western blotting using a 1:1000 diluted antiserum from a mouse immunized with purified pfGAPDH. Lanes a and b: total cell lysate of BL21 (DE3) *E. coli* cells transformed with empty pET28a⁺ vector before and after induction with IPTG for 4 h, respectively. Lanes c and d: BL21 (DE3) *E. coli* cells transformed with pET28a⁺ carrying ORF of pfGAPDH before and after induction with IPTG for 4 h, respectively. Lane e: the purified pfGAPDH protein. Lanes f and g: lysates from uninfected erythrocytes and from erythrocytes infected with *P. falciparum* asexual blood stages, respectively. Sizes of molecular weight standards are given on the left.

sent in lanes a–d loaded with lysates of *E. coli* are presumably derived from antibodies elicited by contaminating *E. coli* antigens co-purified with recombinant pfGAPDH. These bands do not appear in lanes loaded with lysates of parasitized or unparasitized erythrocytes (Fig. 4B).

The purified protein was tested for its enzymatic activity using a standard protocol for the GAPDH forward reaction (Ferdinand, 1964). Specific activity of a typical preparation of pfGAPDH was 126 U/mg protein, which is comparable with the activity of other GAPDH enzymes. Thus, introduction of the affinity tag did not inhibit the enzymatic activity. No activity was detectable when NADP⁺ was used instead of NAD⁺ as co-factor (data not shown).

3.3. Localization of pfGAPDH by immunofluorescence analysis

A mouse antiserum raised against purified, recombinant pfGAPDH was used to stain *P. falciparum* blood-stage parasites grown in vitro. As shown in Fig. 5, the immune serum stained every developing merozoite of a schizont. Ring and trophozoite stages were also stained intensively (not shown). No significant cross-reactivity with erythrocytic GAPDH was observed. Pre-immune serum yielded no staining and immune serum did not recognize uninfected erythrocytes (not shown). Comparison of staining patterns for pfGAPDH and the MSP 1 indicates that pfGAPDH is localized in the cytosol (Fig. 5)

4. Discussion

Expression screening of a *P. falciparum* cDNA library for in vitro translated products yielded several expressed proteins. One cDNA clone isolated encoded pfGAPDH not previously described in *P. falciparum*. The gene was 100% conserved at the nucleotide level in all nine *P. falciparum* isolates analyzed. The protein exhibited a molecular mass of 36 kDa when expressed in *E. coli*, which is in accordance to the predicted molecular mass and similar to GAPDH of other species. The lack of natural polymorphism of housekeeping genes like pfGAPDH supports the view that there might be a recent population bottleneck in the evolutionary history of this parasite (Rich et al., 1998). The phylogenetic analysis supports other studies suggesting that *P. falciparum* is closely related to dinoflagellates (Roos et al., 1999). The presence of *E. coli* in the eukaryotic cluster of the phylogenetic tree based on GAPDH sequences has been found also in another analysis (Hannaert et al., 1992). This has been explained by the acquirement of the gene (*gap A*) by horizontal transfer from a eukaryotic source (Doolittle et al., 1990).

Despite the tremendous impact of malaria on the human population, only a few effective anti-malarial drugs have been developed, several of which are becoming more and more ineffective because of the continuing emergence and spread of drug resistance in the malarial parasite (Newton and White, 1998). Pharmacological

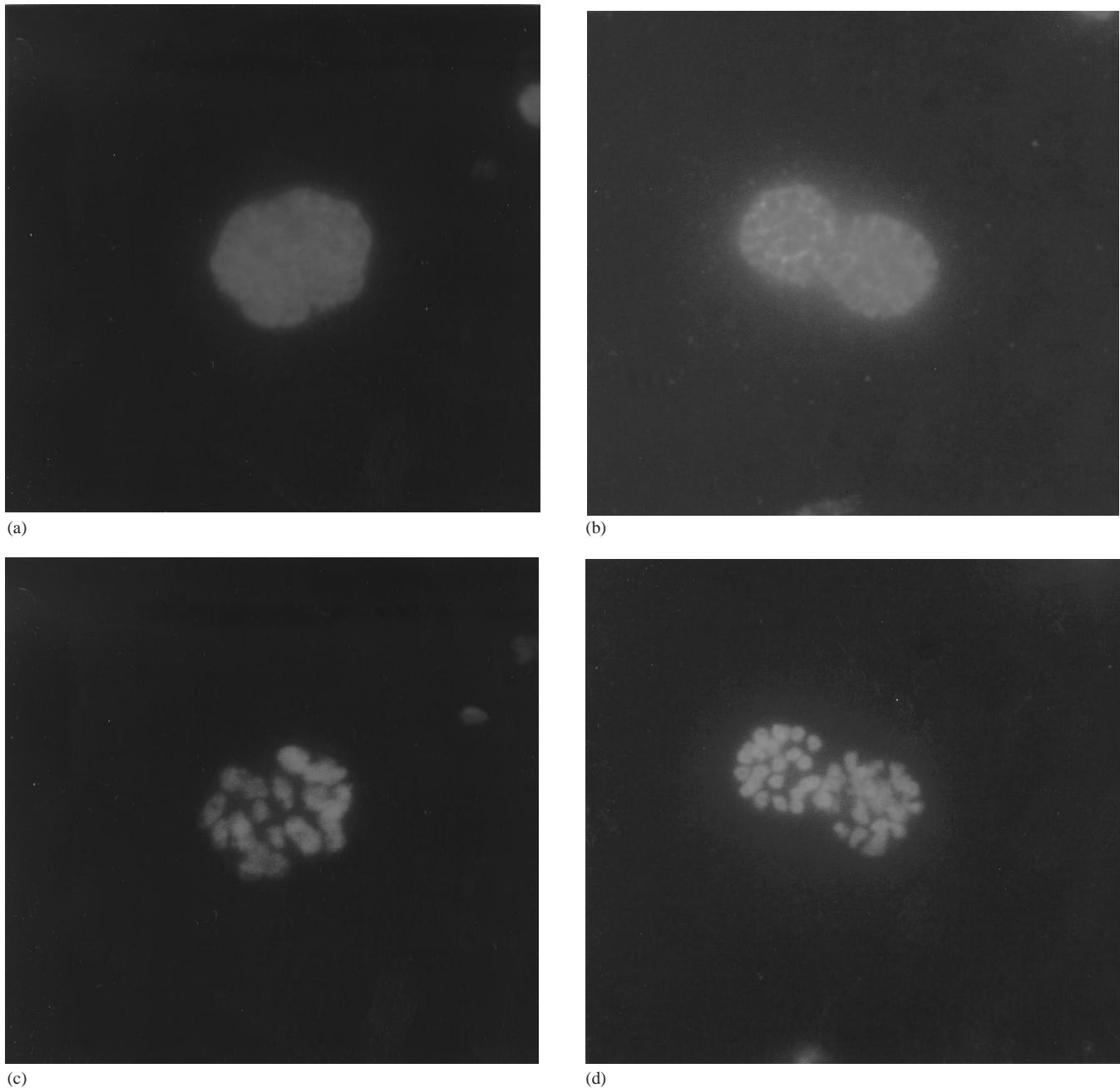


Fig. 5. Immunofluorescence staining of *P. falciparum* blood-stage parasites with mouse antiserum raised by immunization with recombinant pfGAPDH (A). Parasitized erythrocytes were fixed and permeabilized and sequentially incubated with the serum and CY3-conjugated goat anti-mouse IgG. In parallel, staining with the MSP 1 specific mAb P.190-III:18 was performed (B). DNA of the parasite was made visible by staining with Hoechst 33285 (C, D).

compounds designed to act as inhibitors at different points along the pathway of malarial glycolysis may offer variable efficacy based on the relative importance of the various reactions in the pathway. Assuming that the glycolytic reactions in *P. falciparum* follow the same scheme as in yeast and other eukaryotes, pfGAPDH catalyzes the oxidative reduction of glyceraldehyde-3-phosphate into 1,3-bisphosphoglycerate in the presence of NAD^+ and inorganic phosphate. If the glycolysis

could be interrupted at this point, the truncation of glycolysis will not generate ATP. This would waste energy, because the generation of the substrate will require the consumption of ATP. Cloning of glucose phosphate isomerase, phosphoglycerate kinase and aldolase genes of *P. falciparum* revealed that the homology of these proteins to the human equivalents is surprisingly low (Certa et al., 1988; Hicks et al., 1991; Kaslow and Hill, 1990). The pfGAPDH described in this study

displays a sequence similarity to human erythrocytic equivalent to 63.5%. However, to evaluate the capacity of pfGAPDH to serve as promising drug target, the three-dimensional structure would be more informative. The recombinantly expressed pfGAPDH might be suitable for future determination of the three-dimensional structure of this enzyme. With this information, it could be investigated whether a structure-based drug design for *P. falciparum* malaria using the evolutionary conserved pfGAPDH might become feasible. Structure-based design of inhibitors of trypanosomatid glyceraldehyde-3-phosphate dehydrogenase led recently to selective compounds active at submicromolar concentrations (Aronov et al., 1999; Callens and Hannaert, 1995).

A number of studies with mammalian GAPDH proteins have identified diverse biological functions in addition to the GAPDH enzymatic activity. These include a role for GAPDH in membrane transport and membrane fusion, microtubule assembly, nuclear RNA transport, protein phosphotransferase/kinase reactions, translational control of gene expression, DNA replication and DNA repair (Sirover, 1999). Interestingly, there are indications that mammalian GAPDH is involved in the biological effects of nitric oxide (NO). NO induces oxidatively the covalent binding of NAD⁺ to GAPDH and, by S-nitrosylation, inhibits its dehydrogenase activity (Dimmeler and Brune, 1992; Molina y Vedia et al., 1992). During *P. falciparum* infection in humans and mice, NO inactivates liver-invading sporozoites and blood-stage gametocytes (Good and Doolan, 1994). Furthermore, the mosquito *Anopheles stephensi*, a natural vector of human malaria, limits parasite development with the inducible synthesis of NO (Luckhart et al., 1998). With the availability of pfGAPDH preparations and specific immunological reagents, the physiological effects of NO on pfGAPDH and on *P. falciparum* can now be investigated in detail.

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