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Modification of eukaryotic initiation factor 5A from *Plasmodium vivax* by a truncated deoxyhypusine synthase from *Plasmodium falciparum*: An enzyme with dual enzymatic properties

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Abstract—The increasing resistance of the malaria parasites enforces alternative directions in finding new drug targets. Present findings from the malaria parasite *Plasmodium vivax*, causing tertiary malaria, suggest eukaryotic initiation factor 5A (eIF-5A) to be a promising target for the treatment of malaria. Previously we presented the 162 amino acid sequence of eukaryotic initiation factor 5A (eIF-5A) from *Plasmodium vivax*. In the present study, we have expressed and purified the 20 kDa protein performed by one-step Nickel chelate chromatography. In Western blot experiments eIF-5A from *P. vivax* crossreacts with a polyclonal anti-eIF-5A antiserum from the plant *Nicotiana plumbaginifolia* (*Solanaceae*). Transcription of eIF-5A can be observed in both different developmental stages of the parasite being prominent in trophozoites. We recently published the nucleic acid sequence from a genomic clone of *P. falciparum* strain NF54 encoding a putative deoxyhypusine synthase (DHS), an enzyme that catalyzes the post-translational modification of eIF-5A. After removal of 22 amino acids DHS was expressed as a Histidin fusion protein and purified by Nickel affinity chromatography. Truncated DHS from *P. falciparum* modifies eIF-5A from *P. vivax*. DHS from *P. falciparum* NF54 is a bi-functional protein with dual enzymatic specificities, that is, DHS activity and homospermidine synthase activity (HSS) (0.047 pkatal/mg protein) like in other eukaryotes. Inhibition of DHS from *P. falciparum* resulted in a K_i of 0.1 μM for the inhibitor GC7 being 2000-fold less than the nonguanylated derivative 1,7-diaminoheptane. *Dhs* transcription occurs in both develomental stages suggesting its necessity in cell proliferation.

1. Introduction

Despite three decades of research on eukaryotic initiation factor eIF-5A, the role of this ubiquitous protein remains mysterious. eIF-5A is unusually activated by the formation of the novel amino acid hypusine. This process occurs in a two-step mechanism by deoxyhypusine synthase (DHS)[1] [EC 1.1.1.249] transferring an aminobutyl moiety from the triamine spermidine to a specific lysine residue in the eIF-5A precursor protein to form deoxyhypusine and in a second step by deoxyhypusine hydroxylase (DOHH)[2] [EC 1.14.9929] which completes hypusine biosynthesis through hydroxylation.

Homospermidine synthase (HSS)[3,4] [EC 2.5.1.44], an enzyme involved in the biosynthesis of pyrrolizidine alkaloids, has shown to be recruited from the *dhs* gene by gene duplication. Although HSS resembles DHS in its reaction mechanism, that is, the transfer of the aminobutyl moiety with NAD as a cofactor, HSS does not modify eIF-5A.[5]

eIF-5A is a highly conserved protein encoded in the genomes of eukaryotes and archaebacteria.[6] Bacteria do not contain eIF-5A, but a distant homolog, the eukaryotic elongation factor P (EF-P).[7] Recently however, it turned

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out that bacteria possess genes with significant homology to the deoxyhypusine synthase (*dhs*) genes.[8] The authors propose that the putative *dhs* genes in bacteria might be acquired from archae by horizontal gene transfer (HGT). Hence, the function of these genes is enigmatic since there is no evidence for an interaction between these genes and the bacterial eIF-5A homolog EF-P.

The sub cellular localization and the function of eIF-5A have been controversially discussed during the last years. Initially eIF-5A was characterized as a translation initiation factor in yeast, but its depletion only resulted in a 30% decrease of protein biosynthesis[9] arguing against its role as a general translation initiation factor. The findings that eIF-5A functions as a nucleocytoplasmatic shuttle protein of a subset of mRNAs related to the G1/S cell cycle transition opened a new scenario.[10] These data suggested that eIF-5A may be operational in the post-transcriptional processing of a specific subset of mRNAs. These transcripts may encode factors that are required for cell viability and efficient proliferation.[11–14] This notion was supported even more by the discovery that eIF-5A acts as a cofactor of the human immunodeficiency virus type 1 (HIV-1) Rev mRNA transport factor.[15] However, recent results from yeast suggest a role for this factor in translation elongation rather than in translation initiation.[16]

In parasitic protozoa a small number of nucleic acid sequences which encode eIF-5A proteins from different parasites appeared in the databases, that is, from the malaria parasites *Plasmodium falciparum*,[17,18] *P. vivax*,[19,20] *Toxoplasma gondii*[21] and from *Leishmania major*.[22] Recently we published the modification of eIF-5A (EMBL Accession No. AJ422210) from *P. vivax Salvador* PEST strain by a novel DHS protein (EMBL Accession No. AJ549098) expressed from *P. vivax*.[23] Previous reports showed that expression of DHS from *P. falciparum* in contrast to *P. vivax* was not successful.[18] To address this question we present the first biochemical data of eIF-5A from *P. vivax* and its modification by DHS from *P. falciparum* showing that DHS from *P. falciparum* has low substrate specificity for eIF-5A from different malaria species. In contrast to *P. vivax* DHS,[23] expression of DHS from *P. falciparum* was only obtained by truncation of the protein.

Modification of eIF-5A from *P. vivax* by DHS from *P. falciparum* was performed with respect to the following aspects: (i) for pharmacological evaluation of the expressed DHS protein from *P. falciparum* as a potential drug target for inhibitor development and (ii) comparative analysis between eIF-5A modification of DHS enzymes from different human malaria parasites.[17,19,23]

2. Materials and methods

2.1. Cloning and isolation of eukaryotic initiation factor 5A from *P. vivax*

Genomic DNA from *P. vivax salvador* PEST strain was kindly provided by John Barwell (University of Florida). The PCR with a total volume of 20 μl contained 200 pmol of each primer 1# forward 5′-ATG TCA GAC CAC GAA ACG T-3′ and primer 4 # reverse 5′-GGA GGA CAA CTC CTT CAC CG-3′, 89 ng genomic *P. vivax* DNA, dNTP-mix (10 mM each dNTP) 0.08 mM, 1× PCR buffer, Q solution 1-fold, H_2O, $MgCl_2$ (25 mM) 0.4 mM, and 1 μl Taq Polymerase (0.25 U/μl) (Genaxxon). PCR was performed at 60 °C for 30 cycles in a Thermocycler (Biometra®). Sequence identity of the 486 basepair fragment was confirmed by sequence analysis performed by MWG, Munich. After purification the amplified PCR fragment was purified and cloned into pSTBlue Acceptor vector (Novagen). The recombinant plasmid containing the eIF-5A gene served as a template for a next amplification step with primers containing an NdeI pex forward # 5′-TTA ATC ATA TGTCAG ACC AAA CG-3′ and BamHI restriction site pex reverse # 5′-TTA ATG GAT CCCTAG GAG GAC AAC TC-3′, respectively (Nde I and BamHI sites are underlined). PCR was performed as described previously. The obtained 486 bp fragment was ligated into the pet 15b vector (Novagen) with the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T7-RNA polymerase promoter.[24] The recombinant plasmid was resequenced by MWG, Munich.

2.2. Isolation and identification of the *dhs* gene from the chloroquine susceptible *P. falciparum* strain NF54

Genomic DNA from *P. falciparum* strain NF54 was isolated according to a protocol from Qiagen Blood amplification kit. The genomic DNA was used in a PCR protocol as a template for amplification with primer 7 # forward 5′-ATG GTG GAT CAC GTT TC-3′ and primer 8# reverse 5′-TCA CAT ATC TTT TTT CCT C-3′. Primers were used in a concentration of 100 pmol/μl each. The PCR had a total volume of 20 μl and the same composition as described for the amplification of eIF-5A. Annealing was performed at 50 °C for 30 cycles. The PCR product had a size of 1491 bp. The amplified PCR product was gel purified, cloned into pSTBlue Acceptor vector (Novagen), and sequenced by MWG, Munich. For protein expression the recombinant pSTBlue Acceptor vector containing the *dhs* gene was used as a template for subcloning the full length *dhs* sequence into the pet15b vector (Novagen) under the control of the T7 RNA polymerase promoter.[24] PCR amplification was performed as described for eIF-5A and primers with an NdeI restriction site (underlined) pex forward# 5′-GGT ATC ATA TGG ATC ACG TTT C-3′ and pex reverse # 5′-TTA ATG GAT CCT CAC ATA TCT TTT TTC CTC-3′ with a BamHI restriction site (underlined) were used for subcloning into pet15b (Novagen) vector.

Alternatively, because of a low expression of *P. falciparum* DHS, a fragment from the *dhs* gene which lacks the first 22 amino acids and has a synthetic translation initiation codon was amplified by PCR with primers forward # 5′-ATG AGT CAT AAT GAA GGA GAC-3′ and reverse 5′-TCA CAT ATC TTT TTT CCT C-3′ using the 1491 bp insertion as a template. The fragment was cloned into pGEM-T Easy Vector (Promega) and

cloning was verified by sequencing. Expression was performed by subcloning the fragment with expression primer #5′-TTT CAT ATG AGT CAT AAT GAA GGA GAC-3′ and pex reverse primer for subcloning into pet15b vector.

2.3. Isolation of cellular RNA from cell fractions containing trophozoites and schizonts of *P. falciparum* chloroquine resistant (QCR) R strain

Total cellular RNA from trophozoites and schizonts from *P. falciparum* infected human erythrocytes with a parasitemia of 8% (enriched in trophozoites and schizonts) was isolated with PAXgene blood RNA isolation kit (Preanalytix).

2.4. Transcriptional analysis of the eIF-5A and *dhs* genes by RT-PCR from different developmental stages

For RT-PCR a defined region of 588 bp of the *dhs* gene from *P. falciparum* was used. RNA fractions from trophozoites and schizonts were applied as a template in the Access RT-PCR system from Promega. The incubation mixture with a final volume of 50 μl contained: AMV/Tfl 5-fold reaction buffer 1-fold, dNTP-mix 0.2 mM, primer forward # -ATA GTG CCT AAT GAT AAT TA- 4 μmol, primer reverse# -AAC CTC CTC CGA GAA TAA TAA TAC CAG- 4 μmol, 25 mM $MgSO_4$ 1mM, AMV RV (0.1 U/μl), Tfl polymerase (0.1 U/μl), and RNA sample 250 ng. In case of eIF-5A we used the full length sequence of the gene for RT-PCR amplification with the primers defined under cloning. The control reaction compiled the RT-PCR control (2.5 atomol) with carrier, 15 μmol of upstream 5′-GCC ATT CTC ACC GGA TTC AGT CGT C-3′ and downstream primer 3′-GAC TGA ACT GCC CTG CCC TGC CA-5′. The following temperature profile was performed: 45 °C 45 min, 94 °C 2 min, 94 °C 30 s, 50 °C 1 min, 68 °C 2 min. (40 cycles). The RT-PCRs were analyzed on 1% agarose gels.

2.5. Expression and analysis of eIF-5A protein from *P. vivax* and truncated DHS protein from *P. falciparum* strain NF54 in *Escherichia coli* BL21pLysS cells

Escherichia coli cell cultures harboring the expression plasmids were grown in 200 ml LB medium with the appropriate antibiotic for 15.5 h overnight at 37 °C until an OD_{600} of 1.5–1.6 was reached. One hour after addition of 50 ml LB medium cells was induced with 0.4 mM IPTG. From the beginning of induction, samples of 1 ml were taken at intervals of 1 h and analyzed on 10% sodiumdodecylsulfate (SDS) polyacrylamide gels.

1 ml samples from the eIF-5A- and DHS-expressing strains were taken and centrifuged at 13000 rpm for 2 min. Cells were lysed with 400 μl lysis buffer (50 mM Tris–HCl, pH 8.0, 2 mM EDTA), centrifuged and again resuspended in lysis buffer and sonicated (tip 1 at 50% two times 30 s at 4 °C). After centrifugation for 10 min at 16000 rpm at 4 °C, samples were diluted 1:1 in loading buffer (20 mM Tris, pH 6.8; 2% (w/v) SDS, 2 mM EDTA, 20% (V/V) glycerin, 0.3% and bromo phenol blue) heated at 100 °C, and run on a 10% SDS–polyacrylamide gel at 100 V.

2.6. Purification of *P. vivax* eIF-5A and *P. falciparum* DHS protein on Nickel–Nitrilotriacetic acid spin columns under native conditions

Escherichia coli BL21 (DE3) *pLysS* cells (Invitrogene, Germany) expressing either eIF-5A or truncated DHS from a 50 ml LB medium culture were thawed for 15 min and dissolved in 1 ml lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazol, pH 8.0). Lysozyme was added at a concentration of 1 mg/ml and cells were incubated on ice for 30 min and sonicated for 10 s each time with 5 s pauses in between. The lysate was centrifuged for 10.000 rpm for 20–30 min at 4 °C. Lysate (600 μl) was centrifuged for 2 min at 2000 rpm on a Nickel–Nitriloacetic acid (Ni–NTA) spin column which was preequilibrated with lysis buffer. The Ni–NTA spin column was washed twice with 600 μl wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazol, pH 8.0) for 2 min at 2000 rpm. The protein was eluted with 200 μl elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazol, pH 8.0) for 2 min at 2000 rpm.

2.7. Enzymatic activity assays of expressed DHS and HSS activities from *P. falciparum* strain NF54

DHS activity was measured by the incorporation of 40 μM [^{14}C] spermidine into eIF-5A precursor protein. A total volume of 50 μl compiled 83.3 μM Nickel-affinity purified eIF-5A, NAD^+ 1 mM, 0.1 M glycine–NaOH buffer, pH 9.5, 1 mM dithiothreitol (DTT), and Nickel-affinity purified DHS enzyme (3–6 μg). DHS enzymatic activity was determined after a second step of buffer exchange on a NAP column (Amersham). To ensure linearity of the reaction, samples were taken at certain time intervals between 1 min and 60 min at 37 °C. The reaction was stopped by adding 10 μl of 1 M potassium phosphate, pH 6.3, with 60 mM spermidine. The incubation mixture was absorbed to a Whatman No. 3 mM paper disk and developed according to a method by Sasaki et al.[25] Filters were washed until the difference in radioactivity incorporation did not significantly differ more than 1000 cpm.

Homospermidine synthase activity was determined according to the established assay from *Senecio* species[4] with 40 μM [^{14}C] putrescine instead of eIF-5A precursor protein. For identification of homospermidine an aliquot of the reaction was derivatized with methyl chloroformate and analyzed by GC–MS.[4]

2.8. Western blot analysis of eIF-5A from *P. vivax*

Western blots were performed according to the sandwich method by electroblotting in a Novex Western Transfer Apparatus for 2 h at 25 V. Protein extracts from *E. coli* cells expressing eIF-5A after zero and 3 h (protein concentration of the extracts 50 μg/μl) were transferred to a nitrocellulose membrane in transfer buffer (12 mM Tris, 96 mM glycin, and 20% methanol). The primary antibody, a polyclonal antibody raised against the eIF-5A homolog from *Nicotiana plumbagin-*

ifolia, diluted 1:5000 in TBS (10 mM Tris, 15 mM NaCl) buffer was incubated for 1 h at room temperature. The primary antibody was detected with an anti-IgG biotin conjugate at a dilution of 1:10,000. The membrane was washed and incubated with streptavidin alkaline phosphatase diluted 1:10,000. Detection was performed with nitrobluetetrazolium phosphate (NBT).

2.9. Determination of K_i values

Enzyme velocity at a variety of substrate concentrations in the presence and absence of the inhibitors GC7 and 1,7-diaminoheptane was determined and plotted graphically to determine K_m and (observed) $K_{m,obs}$. The determined K_m and $K_{m,obs}$ plus and minus a single concentration of inhibitor were used to rearrange the equation to determine the K_i.[26]

$$K_i = \frac{[\text{inhibitor}]}{\frac{K_{m,obs}}{K_m} - 1.0}$$

3. Results

3.1. eIF-5A and *dhs* are transcribed equally in different developmental stages of the parasite's life cycle

Total cellular RNA from *P. vivax* from different developmental stages, that is, trophozoites and schizonts was used in reverse transcription (RT) experiments. The eIF-5A transcript (486 bp) which had the same size as the band of the control (cloned eIF-5A gene) (Fig. 1, lane 5) was most prominent in the trophozoite fraction (Fig. 1a, lane 1) with a second weaker transcript band of approximately 900 bp. A faint transcript band appeared from the RNA enriched fraction of schizonts (Fig. 1a, lane 2) although the PCR compiled the same amount of total cellular RNA (approximately 250 ng). These results suggest that eIF-5A is present in the two different developmental stages of the parasite (Fig. 1a, lanes 1 and 2). RT-PCR was controlled by an RT-PCR control (Fig. 1, lane 3). A quantification of the transcripts in different developmental stages is currently being performed by RT-PCR.

In the case of transcription of the *dhs* gene a band of 588 bp (as expected from the control Fig. 1b, lane 5) encoding the amino acid region between amino acid residues 208–404 of the *dhs* gene could be observed in the enriched trophozoite (Fig. 1b, lane 2) and schizont enriched RNA (Fig. 1b, lane 3) fractions. Hence, the detected transcript band in the schizont fraction was weaker than in the trophozoite fraction although similar RNA concentrations were used. These results indicate that transcription of *dhs* and *eIF-5A* genes is important in both developmental stages.

3.2. Expression of eIF-5A from *P. vivax* and DHS from *P. falciparum* strain NF54 in *Escherichia coli* cells and their one-step purification by Nickel-chelate affinity chromatography

Expression of the *eIF-5A* gene and *dhs* gene in *E. coli* cells was performed under the control of a T7 promoter

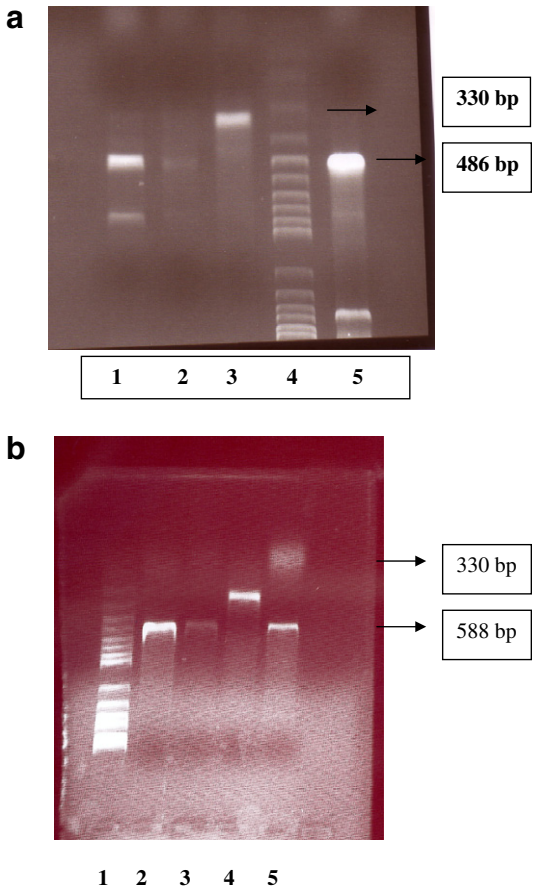

Figure 1. RT-PCR of the *eIF-5A* and *dhs* genes, respectively, with subcellular total RNA fractions as template which are enriched in trophozoites and schizonts: (a) RT-PCR of the *eIF-5A* gene lane 1 cDNA fragment of 486 bp obtained after RT-PCR with primers encoding the full length sequence of eIF-5A from trophozoite enriched subcellular RNA; lane 2 cDNA fragment of 486 bp obtained after RT-PCR with primers encoding the full length sequence of eIF-5A from schizont enriched subcellular RNA; lane 3 330 bp RT-PCR control; lane 4 100 bp ladder extended (Roth); lane 5 recombinant full length *eIF-5A* plasmid. (b) RT-PCR of the *dhs* gene lane 1 100 bp ladder extended (Roth); lane 2 cDNA fragment of 588 bp obtained after RT-PCR with primers encoding the amino acid region between amino acid residues 208 and 404 of the *dhs* gene from the trophozoite enriched subcellular total RNA fraction; lane 3 cDNA fragment of 588 bp obtained after RT-PCR with primers encoding the amino acid region between amino acid residues 208 and 404 of the *dhs* gene from the schizont enriched subcellular total RNA fraction; lane 4 330 bp RT-PCR control; lane 5 recombinant *dhs* plasmid control harboring amino acid residues 208 and 404.

by a method according to Studier et al.[24] For expression and subsequent ligation of the *eIF-5A* coding DNA sequence into the pet 15b expression vector (Novagene®) after PCR amplification, we used primers containing an NdeI restriction site 5-upstream of the translation initiation codon and a BamHI site 3-downstream of the translation termination codon. After confirmation of sequence identity, the recombinant *eIF-5A* and *dhs* expression plasmids were transformed into *E. coli* BL21 (DE3) *plysS* cells, and after induction with isopropylthiogalactoside (IPTG) expression for 3 h was monitored on 10% SDS gels. The strongest expression of eIF-5A was observed after 2 h of induction and remained

constant for up to 3 h (Fig. 2, lanes 2 and 3). Even without IPTG induction at time point zero eIF-5A was expressed because of a basal level of T7 promoter transcription (Fig. 2, lane 1). The expressed eIF-5A protein had an estimated size of 20 kDa in contrast to the predicted size of 17. 49 kDa calculated from the nucleic acid sequence and eluted as a single band in the eluate fractions (Fig. 2, lanes 6 and 7).

In contrast, expression of DHS was only possible after the removal of a signal peptide similar structure. The expressed DHS protein was monitored as a protein of 55 kDa (predicted molecular size: 57.19 kDa) (Fig. 3).

Purification of the eIF-5A and the DHS proteins was performed by a Nickel–Nitriloacetic acid spin column (Figs. 2 and 3) according to a protocol from Qiagen. The N-terminal histidine-tagged eIF-5A protein could be detected in both eluate fractions (Fig. 2, lanes 6 and 7) as analyzed on a 10% SDS protein polyacrylamide gel. Nickel-purified DHS protein showed a constant increase after 1–3 h of expression (Fig. 3).

3.3. Truncated *P. falciparum* deoxyhypusine synthase modifies eIF-5A precursor protein from *P. vivax*

As a substrate for the DHS assay, the purified eIF-5A precursor protein from *P. vivax* and the purified DHS enzyme from *P. falciparum* were applied after Nickel-chelate chromatography. The incorporation of radioactively labeled spermidine into the substrate precursor protein was assayed using a filter paper assay.[25] The purified samples were immediately used for the determination of specific enzymatic DHS activity, that is, DHS from *P. falciparum* strain NF54 with eIF-5A precursor protein from *P. vivax*. The average specific enzymatic activity for three experiments was 337 pkatal/mg

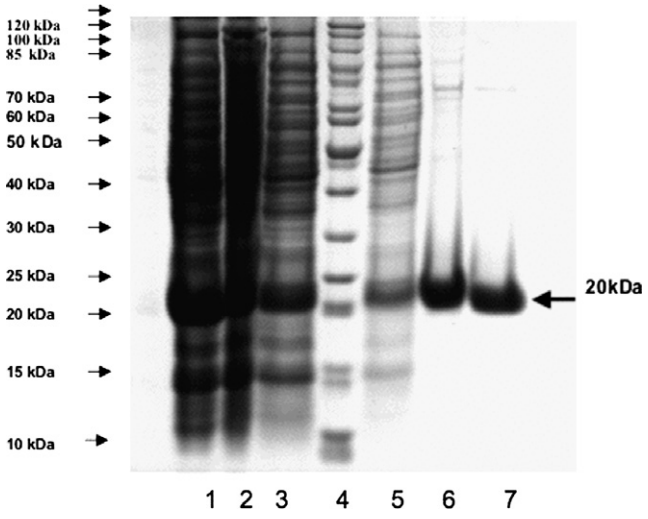

Figure 2. Purification of histidine-tagged eIF-5A by Nickel-chelate affinity chromatography under native conditions: lane 1 basal level of expression of eIF-5A at time point 0; lane 2 crude, lysed extract after expression of eIF-5A after 2 h of induction; lane 3 crude, lysed extract after expression of eIF-5A after 3 h of induction; lane 4 protein marker 10–200 kDa (MBI fermentas); lane 5 wash fraction after Nickel-chelate affinity chromatography; lanes 6 and 7 eluate fractions.

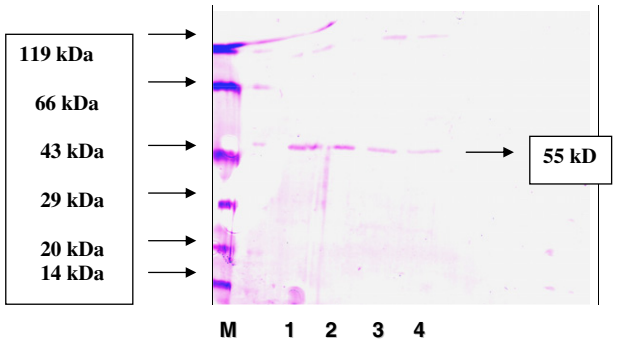

Figure 3. Purified, histidine-tagged DHS obtained by Nickel-chelate affinity chromatography under denaturing conditions after different time points of expression: lane M protein marker (Roth); purified DHS after lane 1 3 h of induction; lane 2 2 h of induction and lane 3 after 1 h of induction.

(pktal/mg) DHS protein suggesting that the truncated DHS protein from *P. falciparum* is able to modify its homolog from *P. vivax* (Table 1, row 1). In control experiments we detected no specific enzymatic activity when DHS enzyme was either denatured (Table 1, row 4) or the cofactor NAD^+ was left out of the incubation mixture (Table 1, row 3).

eIF-5A from *P. vivax* can also be modified by human DHS obtained from crude extract with a specific activity of 200 pktal/mg protein (Table 1, row 5). In the next set of experiments we employed the competitive inhibitor *N*-guanyl-1,7-diaminoheptane which is known to inhibit DHS at its active site. The recombinant, truncated DHS from *P. falciparum* strain NF54 had a K_i value of 0.1 μM for GC7 and less inhibitory properties for 1,7-diaminoheptane with a determined K_i of 50 μM.

3.4. Deoxyhypusine synthase from *P. falciparum* is a bi-functional enzyme with homospermidine synthase activity

With regard to its reaction mechanism, DHS shows striking similarities to homospermidine synthase, a key enzyme in the production of pyrrolizidine alkaloids[3,4] which are plant secondary metabolites. The fact that both enzymes are involved in the transfer of an aminobutyl moiety from spermidine to different acceptors in a NAD^+ dependent reaction mechanism prompted us to test whether DHS from *P. falciparum* strain NF54 also shares homospermidine synthase activity like DHS enzymes from other eukaryotes.[5] The purified DHS enzyme from *P. falciparum* was incubated in the presence of ^{14}C-labeled spermidine and putrescine (Table 1). We determined a specific HSS enzymatic activity of 0.047 pktal/mg protein (Table 1, row 2). These data suggests that DHS from *P. falciparum* is a bi-functional enzyme which is capable of transferring the aminobutyl moiety from spermidine to putrescine.

3.5. eIF-5A from *P. vivax* shows crossreactivity to its homolog from the plant *Nicotiana plumbaginifolia* (Solanaceae)

A comparison on the amino acid level between eIF-5A from *P. vivax* and its homolog from *P. falciparum* shows

Table 1. Determination of specific activities of truncated recombinant deoxyhypusine from synthase from *P. falciparum* strain NF54

Assay	[^{14}C] Spermidine (μmol)	eIF-5A lys (μmol)	Putrescine (μmol)	NAD^+ (μmol)	DHS enzyme from	Specific enzymatic activity (pktal/mg)
1	40	83.3	—	1	*P. falciparum*	337
2	40	—	625	1	*P. falciparum*	0.047
3	40	83.3	—	—	*P. falciparum*	not detected
4	40	83.3	—	1	*P. falciparum* denatured	not detected
5	40	83.3	—	1	Human (not purified)	200

Rows:

(1) Determination of DHS activity from *P. falciparum* strain NF54 representing the standard assay using spermidine as the donor of the aminobutyl moiety for the modification of the eIF-5A precursor protein.

(2) HSS activity assay of *P. falciparum* strain NF 54 with putrescine substituting the eIF- 5A precursor protein.

(3) Control reaction: Determination of DHS activity from *P. falciparum* strain NF54 representing the standard assay using spermidine as the donor of the aminobutyl moiety for the modification of eIF-5A precursor protein without the cofactor NAD^+.

(4) Control reaction: Determination of DHS activity from *P. falciparum* strain NF54 representing the standard assay using spermidine as the donor of the aminobutyl moiety for the modification of eIF-5A precursor protein with denatured DHS from *P. falciparum*.

(5) Determination of human deoxyhypusine synthase activity with eIF-5A precursor protein from *P. vivax*.

97% amino acid identity. Hence, it is remarkable that there is 61% identity on the amino acid level of *P. vivax* eIF-5A to a putative eIF-5A protein from the plant *Arabidopsis thaliana* and 59% identity to two isoforms from the plant *N. plumbaginifolia* (Solanaceae).[27] This data prompted us to test in a Western blot experiment whether a polyclonal antibody raised against one of the eIF-5A isoforms crossreacts with the eIF-5A protein from *P. vivax*. The results in Figure 4, lanes 1 and 2 show that crude extracts of the expressed plasmodial eIF-5A protein harvested at time points zero and 3 h after IPTG induction crossreact with the polyclonal antibody against its homolog from the plant. In a control experiment we used protein extracts from *E. coli* which is known to lack the eIF-5A protein (Fig. 4, lanes 3 and 4).

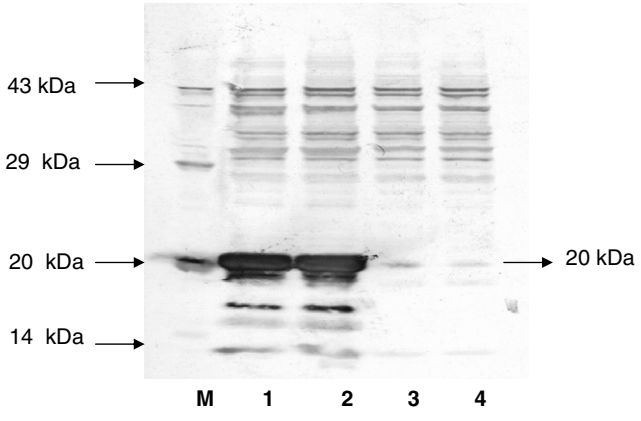

Figure 4. Western blot experiment with crude protein extracts expressing eIF-5A protein from *P. vivax* with a polyclonal antibody raised against NIF-5A protein from *N. plumbaginifolia* (Solanaceae). Lane 1: protein marker 10–200 kDa (MBI Fermentas) Lanes 2 and 3: Crude bacterial extracts expressing eIF5A protein from *P. vivax* in two different concentrations. The crossreacting protein band of 20 kDa is marked by an arrow. Lanes 4 and 5: Crude bacterial extracts in different concentrations from *E. coli* BL21 cells used as a negative control.

4. Discussion

In this paper, we report about target evaluation of eukaryotic initiation factor 5A (eIF-5A)[19] from *P. vivax* by modification of DHS from *P. falciparum* strain NF54. eIF-5A has shown to be an important target in cancer therapy to potentiate apoptosis and cell death[28] since transglutaminases induce apoptosis and decrease hypusine levels in eukaryotic cells. The latter effect is probably due to γ-glutamyl-conjugate formation by *t*-TGAses or by the inhibition of polyamine uptake. DHS enzyme inhibition with *N*-guanyl-1,7-diaminoheptane (GC7) impairs melanoma growth[29] by a decrease of eIF-5A hypusination. Recently the guanylhydrazone CNI-1493 turned out to be an efficient inhibitor of human DHS enzyme.[30] The compound suppresses activation of eIF-5A which is a cellular cofactor of HIV-1 regulatory protein (Rev) and thus virus replication. The same effect was demonstrated by RNA interference for the human *dhs* gene which efficiently suppressed the retroviral replication cycle of HIV1-virus in cell culture.

eIF-5A is a well conserved protein with 97% amino acid identity in human malaria parasites and known to be involved in cell proliferation. Our strategy for an antimalarial chemotherapy in the future will be directed towards inhibitor development against its modifying enzymes, that is, DHS and DOHH catalyzing the sequential steps of eIF-5A hypusination. DHS from *P. falciparum* 3D7[17] and *P. vivax*[23] share only 68% amino acid identity.

Previous reports[18] have demonstrated that recombinant expression of full length DHS from *P. falciparum* was not successful. In this study, we show that DHS activity of *P. falciparum* was expressed from a genomic clone of strain NF54 when the first 22 amino acids were omitted. A similar strategy was applied to spermidine synthase from *P. falciparum* where the enzyme was expressed abundantly when the first 29 amino acids were truncated.[31] The determined average specific activity of recombinant DHS from *P. falciparum* is 337 pkat/mg

protein at 37 °C (Table 1, row 1) and comparable to that of DHS of the plant *Nicotiana tabacum* (Solanaceae) that is, 350 pktal/mg protein.[32] In control experiments incorporation of 1.8 [^{1}4C] labeled spermidine into eIF-5A from *P. vivax* was impossible either with denatured DHS enzyme (Table 1, row 4) or by omission of the NAD^+ cofactor (Table 1, row 3). In the case of human DHS enzyme (crude bacterial extract), a specific activity of 200 pktal/mg DHS protein at 37 °C was determined suggesting that eIF-5A from *P. vivax* is modified by human DHS but to a lower extent due to its low substrate affinity.

In a second set of experiments we investigated the inhibitory effect of *N*-guanyl-1,7-diaminoheptane (GC7) and 1,7-diaminoheptane on truncated DHS from *P. falciparum* strain NF54. The determined K_i value shows that GC7 with a K_i of 0.1 μM (being 50% higher than the K_i of the human enzyme) is a competitive inhibitor of DHS from the parasite and significantly better in inhibition than 1,7-diaminoheptane with a K_i of 50 μM.[34] In the human enzyme Asp^{316} and Glu^{323} are at the bottom of the tunnel and have close contacts with the GC7 guanidinium group.[1] Near the entrance of the tunnel is Asp^{243} which forms a salt bridge to the GC7 terminal amino group. This model is comparable to Plasmodium since these amino acids are highly conserved.[23]

Truncated DHS expressed from the *P. falciparum* genomic clone NF54 turned out to be a bi-functional enzyme with dual specific activities, that is, DHS and HSS activities (0.047 pktal/mg protein). This observation has been made for a variety of deoxyhypusine synthases, first for human DHS,[3,33]and later for DHS from tobacco[32] and the plant *Senecio vernalis* (Asteraceae).[4]

A recently performed phylogenetic analysis with eIF-5A from *P. vivax*[19] and from *P. falciparum*[18,19] with that of other eukaryotic sequences shows that both apicomplexan eIF-5A proteins are to a higher degree more similar to their homologs from plants, that is, *A. thaliana* (61%) and *N. plumbaginifolia* (59%). These findings are strongly supported by the crossreactivity of the eIF-5A protein from *P. vivax* with an antibody against its homolog from the plant *N. plumbaginifolia* (Fig. 4). Although a background between the polyclonal *E. coli* antiserum and different *E. coli* proteins is observed, a strong prominent band of 20 kDa for eIF-5A from *P. vivax* was detectable.

While genomic Southern blot analysis suggests that only one locus for eIF-5A exists in *P. falciparum*,[18] different loci, presumably different isoforms are present for *dhs* genes in Plasmodium 3D7 strain on chromosomes 9, 13, and 14.

RT-PCRs performed with cellular RNA obtained from schizonts and trophozoites showed that *eIF-5A* and *dhs* genes from *P. vivax* are present in both developmental stages. However, it seems likely that the occurrence of both transcripts varies in the different developmental stages being prominent in the trophozoite stage where protein synthesis is enhanced. Molitor et al.[18] who used RT-PCR for a quantitative determination of eIF-5A from *P. falciparum* Dd2 ring, trophozoite and schizont stages showed no significant variation in expression patterns at different developmental stages. However, it is possible that expression patterns differ between the two Plasmodium species. A second *eIF-5A* transcript of 900 bp appeared in the trophozoite fraction suggesting a putative second gene locus for *eIF-5A* in *P. vivax*.

It has been known for more than a decade that eIF-5A is an essential protein for cell survival and proliferation.[25] The occurrence of transcripts of *eIF-5A* and *dhs* genes in different developmental stages of the parasite confirms these findings. However, the band intensity of both *eIF-5A* and *dhs* transcripts was much weaker in schizont enriched RNA fractions (Fig. 1a, lane 2 and b, lane 3). The fact that eIF-5A is not just a bona fide translation factor, but has role in translation of certain mRNA molecules in a hypusine dependent manner, has been recently demonstrated by the presence of two binding motifs, that is, UAACCA and AAAUGU.[35] Previous results confirm its role in translation elongation[16] by the interaction with structural components of the 80S ribosome and translation elongation factor 2 (eEF2). Since the amino acid identity between DHS proteins from both human malaria parasites is only 44%, now target evaluation of DHS can be performed to exploit the essential modification of eIF-5A. This strategy will be pursued either by RNA interference or by pharmacological active compounds after a more detailed biochemical analysis of both parasitic enzymes.

A second alternative to interfere with modification of eIF-5A is the cloning and expression of DOHH from Plasmodium. *Dohh* genes have now been cloned from yeast and human.[35] Sequence and structural analysis reveal that DOHH belongs to a family of HEAT-repeat-containing proteins, consisting of eight tandem repeats of an α-helical pair (HEAT motif) organized in a symmetrical dyad. DOHH contains two potential iron coordination sites (one on each dyad) composed of two strictly conserved His-Glu motifs.[36] Recent data showed that a 2,6-di-2-pyridine substituted 4-oxo-piperidine saturated monocarboxylate deriving from mimosine as a lead structure has antiplasmodial activity in vitro and in vivo[37] presumably by chelating the iron coordination sites of DOHH.

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References and notes

1. Umland, T. C.; Wolff, E. C.; Park, M. H.; Davies, D. R. *J. Biol. Chem.* **2004**, *27*, 28697–28705.
2. Abbruzzese, A.; Park, M. H.; Folk, J. E. *J. Biol. Chem.* **1986**, *261*, 3085–3089.

3. Kaiser, A. *Plant J.* **1999**, *19*, 195–201.
4. Ober, D.; Hartmann, T. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 14777–14782.
5. Ober, D.; Harms, R.; Witte, L.; Hartmann, T. *J. Biol. Chem.* **2003**, *278*, 12805–12812.
6. Kim, K. K.; Hung, L. W.; Yokata, H.; Kim, R.; Kim, S. H. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 10419–10424.
7. Kyrpides, N. C.; Woese, C. R. *Proc. Nat. Acad. Sci. U.S.A.* **1998**, *95*, 224–228.
8. Brochier, C.; Lopez-Garcia, P.; Moreira, D. *Gene* **2004**, *330*, 169–176.
9. Kang, H. A.; Hershey, J. W. *J. Biol. Chem.* **1994**, *269*, 3934–3940.
10. Rosorius, O.; Reichert, B.; Kratzer, F.; Heger, P.; Dabau-Valle, M. C.; Hauber, J. *J. Cell Sci.* **1999**, *112*, 2369–2380.
11. Park, M. H.; Wolff, E. C.; Lee, Y. P.; Folk, J. E. *J. Biol. Chem.* **1994**, *269*, 27827–27832.
12. Caraglia, M.; Marra, M.; Giuberti, G.; D'Alessandro, A. M.; Baldi, A.; Tassone, P.; Venuta, S.; Tagliaferri, P.; Abbruzzesse, A. *J. Biochem.* **2003**, *133*, 757–765.
13. Lee, Y.; Kim, H. K.; Park, H. E.; Park, M. H.; Joe, Y. A. *Mol. Cell Biochem.* **2003**, *237*, 69–76.
14. Shi, X. P.; Yin, K. C.; Ahem, J.; Davis, L. J.; Stern, A. M.; Warman, L. *Biochim. Biophys. Acta* **1996**, *1310*, 119–126.
15. Ruhl, M.; Himmelspach, M.; Bahr, G. M.; Hammerschmid, F.; Jaksche, H.; Wolff, B.; Aschauer, H.; Farrington, G. K.; Probst, H.; Bevec, D.; Hauber, J. *J. Cell Biol.* **1993**, *123*, 1309–1320.
16. Zanelli, C. F.; Maragno, A. L.; Gregio, A. P.; Komili, S.; Pandolfi, J. R.; Mestriner, C. A.; Lustri, W. R.; Valentini, S. R. *Biochem. Biophys. Res. Commun.* **2006**, *348*, 1358–1366.
17. Gardner, M.; Hall, N.; Fung, E.; White, O., et al. *Nature* **2002**, *419*, 498–511.
18. Molitor, I. M.; Knobel, S.; Dang, C.; Spielmann; Allera, A.; König, G. M. *Mol. Biochem. Parasitol.* **2004**, *137*, 65–74.
19. Kaiser, A.; Gottwald, A.; Maier, W.; Seitz, H. M. *Parasitol. Res.* **2003**, *91*, 508–516.
20. Carlton, J.; Dame, J. *Parasitol. Today* **2000**, *16*, 409.
21. Kissinger, J. C.; Gajria, B.; Li, L.; Roos, D. S. *Nucleic Acids Res.* **2003**, *31*, 234–236.
22. Drummelsmith, J.; Brochu, V.; Girard, I.; Messier, N.; Ouelette, M. *Mol. Cell Proteomics* **2003**, *2*, 146–155.
23. Njuguna, J. T.; Nassar, M.; Hoerauf, A.; Kaiser, A. E. *BMC Microbiol.* **2006**, *16*, 6–91.
24. Studier, F. W.; Rosenberg, A. H.; Dunn, J. J.; Dubendorff, J. W. Use of T_7 RNA polymerase to direct expression of cloned genes. In *Methods in Enzymology*; Goeddel, D. V., Ed.; Academic Press: San Diego, 1990; vol. 185, pp 60–89.
25. Sasaki, K.; Abid Md, R.; Miyazaki, M. *FEBS Lett.* **1996**, *384*, 151–154.
26. http://www.graphpad.com/curvefit/inhibitors. Enzyme kinetics in the presence of an inhibitor.
27. Chamot, D.; Kuhlemeier, C. *Nucleic Acids Res.* **1992**, *20*, 665–666.
28. Caraglia, M.; Marra, M.; Guberti, G.; D'Allessandro, A. M.; Buddilton, S.; del, Prete; Lentini, A.; Benninati, S.; Abbruzzese, A. *Amino Acids* **2000**, *20*, 91–104.
29. Jasiulionis, M. G.; Luchessi, A. D.; Moreira, A. G.; Souza, P. P.; Suenaga, A. P.; Correa, M.; Costa, C. A.; Curi, R.; Costa-Neto, C. M. *Cell Biochem. Funct.* **2007**, *25*, 109–114.
30. Hauber, I.; Bevec, D.; Heukeshoven, J.; Kraetzer, F.; Choidas, A.; Harrer, T.; Hauber, J. *J. Clin. Invest.* **2005**, *115*, 76–85.
31. Haider, N.; Eschbach, M. L.; De Souza Dias, S.; Gilberger, T. W.; Walter, R. D.; Lüersen, K. *Mol. Biochem. Parasit.* **2005**, *142*, 224–236.
32. Ober, D.; Hartmann, T. *J. Biol. Chem.* **1999**, *274*, 32040–32047.
33. Lee, Y. B.; Folk, J. E. *Bioorg. Med. Chem.* **1998**, *6*, 253–270.
34. Park, J. H.; Wolff, E. C.; Folk, J. E.; Park, M. H. *J. Biol. Chem.* **2003**, *278*, 32683–32691.
35. Xu, A.; Chen, K. Y. *J. Biol. Chem.* **2001**, *276*, 2555–2561.
36. Park, J. H.; Aravind, L.; Wolff, E. C.; Kaevel, J.; Kim, Y. S.; Park, M. H. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 51–56.
37. Saeftel, M.; Sarite, R. S.; Njuguna, T.; Holzgrabe, U.; Ulmer, D.; Hoerauf, A.; Kaiser, A. *Parasitol. Res.* **2006**, *99*, 281–286.