Review

Unique posttranslational modifications in eukaryotic translation factors and their roles in protozoan parasite viability and pathogenesis

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A B S T R A C T

Protozoan parasites are one of the major causes of diseases worldwide. The vector transmitted parasites exhibit complex life cycles involving interactions between humans, protozoa, and arthropods. In order to adapt themselves to the changing microenvironments, they have to undergo complex morphological and metabolic changes. These changes can be brought about by expressing a new pool of proteins in the cell or by modifying the existing repertoire of proteins via posttranslational modifications (PTMs). PTMs involve covalent modification and processing of proteins thereby modulating their functions. Some of these changes may involve PTMs of parasite proteins to help the parasite survive within the host and the vector. Out of many PTMs known, three are unique since they occur only on single proteins: ethanolamine phosphoglycerol (EPG) glutamate, hypusine and diphthamide. These modifications occur on eukaryotic elongation factor 1A (eEF1A), eukaryotic initiation factor 5A (eIF5A) and eukaryotic elongation factor 2 (eEF2), respectively. Interestingly, the proteins carrying these unique modifications are all involved in the elongation steps of translation. Here we review these unique PTMs, which are well conserved in protozoan parasites, and discuss their roles in viability and pathogenesis of parasites. Characterization of these modifications and studying their roles in physiology as well as pathogenesis will provide new insights in parasite biology, which may also help in developing new therapeutic interventions.

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Abbreviations: eEF1A, eukaryotic elongation factor 1A; eIF5A, eukaryotic initiation factor 5A; eEF2, eukaryotic elongation factor 2; EPG, ethanolamine phosphoglycerol; DHS, deoxyhypusine synthase; DOHH, deoxyhypusine hydroxylase.

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1. Introduction

The genomic content of every organism is limited, yet the coding capacity, i.e. the corresponding proteome, is very diverse. A cell has two basic ways to diversify its proteome: first, at the transcriptional level via alternate splicing and trans-splicing of mRNAs and second, through posttranslational modifications (PTMs) of proteins. The regulation of gene expression in kinetoplastids takes place at multiple levels, such as trans-splicing, polyadenylation, mRNA stability, transcription elongation, RNA translation and protein stability. Transcription in trypanosomatids generates large polycistronic transcripts, which are processed to monocistronic mRNAs by polyadenylation and trans-splicing of a mini-exon, or splice-leader, to primary transcripts. As a result, regulation of gene expression in kinetoplastids takes place mainly at the post-transcriptional level.

PTMs refer to a broad array of covalent modifications and processing of proteins. These modifications can be reversible or irreversible. Reversible modifications include the specific attachment of small molecules, such as phosphate, hydroxyl groups to specific amino acid residues of a given protein. These modifications may not only affect the biochemical properties of proteins but also modulate their functions, localization, turnover and interactions with other macromolecules. Thus, PTMs increase the functional diversity of a given protein, thereby adding a layer of complexity to the proteome in eukaryotes and to a limited extent in prokaryotes [1,2].

Among many protein modifications known till date, three of them are exceptional as they occur only on single proteins: ethanolamine phosphoglycerol (EPG) glutamate, hypusine and diphthamide modifications of eukaryotic elongation factor 1A (eEF1A), eukaryotic initiation factor 5A (eIF5A) and eukaryotic elongation factor 2 (eEF2), respectively. Interestingly, all three proteins are involved in the elongation step of translation in eukaryotes [3]. Translation is a basic event required by the cell to make proteins. Therefore, homologs of translation factors involved in this process can be found in eukaryotes, archaeb and bacteria. Translation involves three major steps: (i) initiation, (ii) elongation, and (iii) termination (Fig. 1). During initiation, a 43S pre-initiation complex is formed comprising of the 40S subunit, eIF2-GTP, Met-tRNAi, and eIF3. This is joined by cap binding complex of eIF4F and the factors eIF4A and eIF4B which further assist the complex in binding mRNA, thus forming the 48S complex. This complex associates with the 60S large ribosomal subunit to form the 80S ribosome [4], which has a mettRNA bound at its P site. The process starts with a cognate aminoacyl tRNA brought to the A site of the ribosome in form of a ternary complex with eEF1A and GTP. Following hydrolysis of the bound GTP to GDP, eEF1A is released, while the growing polypeptide is transferred from the P site tRNA to the aminoacyl tRNA at the A site. The peptidyl tRNA is then translocated from the A to the P site by GTP-bound eEF2, whereby eIF5A has been suggested to assist eEF2 in translocation. This cycle repeats until the ribosome reaches the stop codon where the process stalls, resulting in termination of elongation (Fig. 1).

The three translation factors eEF1A, eEF2 and eIF5A are phylogenetically well conserved [3]. The eukaryotic translation elongation factor complex consists of three or four subunits: eEF1A, eEF1B α, eEF1 β, eEF1B γ and eEF1B β. It is a GTP-binding complex, where GTP acts as a positive allosteric regulator of eEF1A [5]. The intrinsic GTPase activity of eEF1A is very low but is enhanced by

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** Translation elongation cycle. (1) eEF1A along with GTP escorts specific aminoacyl tRNA (aa-tRNA) to the ribosome bound with mRNA at the interface of the two subunits and a peptidyl tRNA at the P site. Following GTP hydrolysis, the aa-tRNA is transferred to the A site of the large subunit. (2) Peptidyl transferase reaction transfers the growing polypeptide from peptidyl tRNA to aa-tRNA. (3) eEF2A bound to GTP assists in translocation of the ribosome, followed by GTP hydrolysis. eIF5A is supposed to assist eEF2A in this process. The peptidyl tRNA moves from A to P site. (4) Uncharged tRNA exits the ribosome from the E site. (5) Ribosome ready to start another cycle. Three post-translational modifications of elongation factors are: (A) EPG modification of eEF1A. (B) Hypusine modification of eIF5A. (C) Diphthamide modification of eEF2. Diphthamide is involved in maintaining the reading frame during translation. The roles of hypusine and EPG in translation are not known.
a factor of 105 after binding to the ribosome [6]. eEF1A is modified at several amino acid side chains by phosphorylation [7], lysine methylation [8] and EPC addition, eEF2 is also a GTPase, which in its GTP bound form catalyses the co-ordinated movement of the two RNA molecules, the mRNA and conformational changes in the ribosome, following which its GTPase activity is activated. The diphthamide modification located at the tip of domain IV of eEF2 helps in the maintenance of the reading frame [9]. In addition, eEF2 is phosphorylated by calcium-dependent kinases, leading to down-regulation of the protein [10]. eIF5A, initially known as elf4D, is well conserved from yeasts to humans and contains a lysine residue that is modified to hypusine. In addition, another lysine residue can be acetylated, resulting in inactivation of eIF5A [11].

A diverse group of protozoan parasites represents a major cause of diseases in humans. Usually, these parasites have a digenetic life cycle, where one form of the parasite resides in an insect vector and the other infective form within the human host. Due to their complex life cycles, parasites have to undergo dramatic morphological and metabolic changes to adapt to the changing extracellular environments in the hosts and vectors. At least some of these changes may involve PTMs of parasitic proteins to help the parasite survive within the host and the vector [12]. The above-mentioned modifications of eEF1A, eEF2 and eIF5A are well conserved in protozoan parasites. This review describes the biosynthetic pathways and physiological significance of these unique modifications in protozoan parasites and discusses their possible roles in parasite biology.

2. Hypusine modification of eIF5A

eIF5A is a small, acidic protein that is highly conserved in archaea and eukaryotes [13]. It is the only protein carrying the specific polyamine-derived amino acid hypusine [Ne-(4-aminobutyl)lysine]. Hypusine was first identified in bovine brain extracts [14] and, subsequently, as modified amino acid in elf4D [15,16]. Hypusine is synthesized post-translationally in a two step process (Fig. 2a): first, deoxyhypusine synthase (DHS) catalyses the transfer of a 4-aminobutyl moiety from spermidine to the ε amino group of a specific lysine residue of eIF5A, in a NAD+ dependent reaction, thus forming the intermediate deoxyhypusine. In a second step, deoxyhypusine is hydroxylated to hypusine by deoxyhypusine hydroxylase (DOHH) [13].

eIF5A as well as the two enzymes, DHS and DOHH, are well conserved among eukaryotes. In addition, a significant amino acid sequence identity is observed between eukaryotic eIF5A and its orthologs, elf5A and EF-P, in archaea bacteria and eubacteria, respectively [13]. Crystal structures have been determined and structural modeling has been done for yeast and human eIF5A (PDB 3er0, 3cpf), four elf5A proteins (PDB 1eif, 2eif, 1iz6, 1kkb), and two Leishmania proteins (PDB 1x6o, 1xtd), showing only minor differences between organisms [17]. eIF5A has two domains, a basic N-terminal and an acidic C-terminal domain. Hypusine modification takes place on a conserved lysine residue (Lys50 in humans) in the N-terminal domain. Structure of yeast eIF5A (PDB: 3ER0) with the conserved lysine residue (Lys51) involved in hypusination is shown in ball and stick model in Fig. 2b. Amino acid substitution and site directed mutagenesis studies have demonstrated that the lysine residue is a strict requirement for hypusination to take place [18]. Multiple alignments of eIF5A sequences from various organisms reveal that there is a high degree of amino acid sequence conservation, especially among the residues forming the hypusination loop [19]. The fact that hypusine is restricted to eIF5A and that the two enzymes required for hypusine modification are conserved, implies a vital role of eIF5A and hypusine in the cell [20].

Early studies on rabbit reticulocytes identified eIF5A as translation initiation factor [21,22]. More recently, the role of eIF5A has been implicated directly in translation elongation, eIF5A was shown to interact with structural components of 80S ribosomes and eEF2 [23]. Polysome profiles from temperature sensitive mutants of yeast for eIF5A revealed an increase in polysome/monosome ratio at the restrictive temperature, suggesting a block in translation elongation. The profile was similar to that in wild type cells treated with sordarin, an inhibitor of eEF2, suggesting that eIF5A might function together with eEF2 to promote ribosomal translational activity [24]. Moreover, a strain harboring both an eIF5A mutant and a translation initiation (elf4E) mutant showed an intermediate polysome profile phenotype, further supporting the role of eIF5A at the elongation step of translation [25]. Furthermore, independent evidence for a role of eIF5A in translation elongation was obtained by measuring the ribosome transit time (the time during which a growing nascent polypeptide chain remains attached to
the translating ribosome, i.e. the time of elongation plus the time of termination). The ribosomal transit was significantly delayed for different eIF5A temperature-sensitive mutants at the restrictive temperature [24], supporting the notion that eIF5A is involved in the elongation step of translation.

2.1. Functional significance of eIF5A and hypusine modification

In general, eukaryotes contain two or more isoforms of eIF5A [26]. S. cerevisiae encodes two isoforms, TIF51A and TIF51B, which share ~90% amino acid sequence identity. Both of them are of similar size, but are regulated by heme and oxygen in opposite manner, thus showing differential expression under aerobic and anaerobic conditions [27]. Contrary to this, in mammals eIF5A1 is constitutively expressed in all tissues, while the expression of eIF5A2 is limited to testis and some parts of brain [26].

Hypusination affects the subcellular localization of eIF5A. While non-hypusinated eIF5A is equally distributed between the nucleus and cytoplasm, the hypusinated form is exclusively present in the cytoplasm. Furthermore, mutant eIF5A lacking various amino acid substitutions at Lys50 shows a subcellular distribution profile similar to non-hypusinated form [28].

eIF5A and its hypusine modification are essential for growth of yeast. Null mutants of eIF5A in yeast were not able to survive [18]. Similarly, null mutants of eIF5A carrying a plasmid with a TIF51A gene having a Lys51Arg point mutation were not able to grow since they lacked hypusinated eIF5A [18]. In mammals, eIF5A2 is up-regulated in many cancers [29], hence considered as an oncogene whereas eIF5A1 is highly expressed in proliferating cells. Expression pattern of eIF5A1 shows that it is ubiquitously expressed in mice during all post-implantation days [30]. In addition, when CHO cells were treated with mono guanyldiamine GC7, a DHS inhibitor, it curtailed the growth of cells without depleting the spermidine or eIF5A precursor levels, demonstrating an essential role of hypusine in cellular proliferation [31]. Nishimura et al. [79] further investigated the role of eIF5A in mammalian development, where they showed its essentiality in embryogenesis. eIF5A has also been shown to have pro-apoptotic function. Over-expression of eIF5A1 induced apoptosis in colon carcinoma cells [32] via mitochondrial apoptotic pathway. Overproduction and accumulation of unmodified eIF5A precursor (but not the hypusinated form) upon transduction with eIF5A adeno-viral vectors has been implicated in apoptosis [33].

2.2. Hypusine modification of eIF5A in protozoan parasites

Like in all other eukaryotes, the hypusine pathway is well conserved in kinetoplastids and apicomplexans. Synthesis of hypusine is one way by which polyamines are covalently incorporated in proteins. Polyamines (spermidine, putrescine and spermine) are present at high concentrations in many protozoan parasites, where they are required for essential functions and viability. There are several reports highlighting the importance of polyamines in parasites and their potential roles as drug targets (reviewed in [34]). These studies have been done by manipulating polyamine levels using various inhibitors of the enzymes involved in the polyamine pathway. In addition, treatment of neoplastic cells in culture with α-difluoromethylornithine, an inhibitor of ornithine decarboxylase (ODC), the first enzyme of this pathway, leads to cessation of cell growth due to depletion of putrescine and spermidine [35]. This cessation was due to depletion of hypusine synthesis resulting in accumulation of immature eIF5A [36]. Together, these studies highlight the essential roles of polyamines and hypusine in parasite survival, development and pathogenesis.

Kinetoplastids are flagellated protozoans and include the genus Trypanosoma and Leishmania. Leishmania has two genes coding for DHS, one on chromosome 34 (LinJ.34.0350) and one on chromosome 20 (LinJ.20.0270) ([http://tritrypdb.org/tritrypdb/]), with both of them showing little sequence homology to human DHS (P49366). Pfam domain assignments of DHSL34 and DHSL20 show the presence of the deoxyhypusine synthase domain ([http://pfam.sanger.ac.uk] (Table 1). Interestingly, only DHSL34 exhibits deoxyhypusine synthase activity [37,38]. In Leishmania, chromosomal null mutants of DHSL34 could only be obtained in

<table>
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<tr>
<th>Table 1</th>
<th>Candidate proteins involved in hypusine biosynthesis in L. infantum, T. brucei gambiense and P. falciparum 3D7.</th>
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<tbody>
<tr>
<td><strong>Gene name</strong></td>
<td><strong>Gene ID</strong></td>
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<tr>
<td>DHS20</td>
<td>LinJ.20.0270</td>
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<tr>
<td></td>
<td>Tbg972.1.280</td>
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<tr>
<td>DHS34*</td>
<td>LinJ.34.0350</td>
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<td>PF14_0125</td>
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<td>DOHH</td>
<td>LinJ.26.1920</td>
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<td>Tbg972.9.1120</td>
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<td>PF13_0013</td>
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* Active enzyme with the conserved active site Lysine; * Domain organization based on PFAM domain assignments
the presence of a rescuing episome that contained the DHSL34 gene, suggesting that DHSL34 is essential for \( \text{L. donovani} \). These results are in line with a previous study showing that the hypusination pathway plays a vital role in \( \text{L. donovani} \) survival and proliferation [37].

The second enzyme of this pathway, DOHH, has been characterized in \( \text{L. donovani} \) (Lin.J.26.1920) [38]. Pfam domain assignments of DOHH show two HEAT repeat domains (http://pfam.sanger.ac.uk) (Table 1). The DOHH inhibitor, ciclopixor, inhibited cell proliferation of AG83 promastigotes as well as intracellular amastigotes, indicating that DOHH inhibition might lead to decreased levels of active eIF5A in the cell, thus compromising growth of parasites.

An ODC null mutant of \( \text{L. donovani} \) lost the ability to sustain infection. Parasitemia load in murine macrophages was reduced by 80% compared to wild-type cells [39]. This reduced infectivity was restored either by epibosomal complementation of the chromosomal lesion in ODC [39] or by external administration of putrescine [40]. Similarly, null mutants of spermidine synthase made the parasite auxotrophic for polyamines. There was a substantial reduction in parasite burden in mice compared to the wild type, i.e. a reduction by 3 orders of magnitude in liver and 2 orders of magnitude in the spleen [41]. These experiments show that a reduction in polyamine biosynthetic capacity of the parasite results in decreased ability of the parasite to infect the mammalian host. The reduced virulence of the parasite can be correlated to reduced levels of putrescine and spermidine, ultimately affecting the levels of hypusinated eIF5A.

At present, little is known about eIF5A hypusination in trypanosomes. Candidate DHS genes have been annotated in the genomes of \( \text{Trypanosoma brucei} \) (Tb927.1.870) and \( \text{Trypanosoma cruzi} \) (Tc00.1047053511421.60) (http://tritrypdb.org/tritrypdb) [42]. In addition, two putative DHS-like sequences (Tb927.1.870 and Tb927.10.2750) have been identified in the genome of \( \text{T. brucei} \) 927 strain. Multiple amino acid sequence alignments of the putative protein sequences reveal that they share 38% and 37% sequence identity to human DHS. At present we are characterizing the proteins in trypanosomes.

In contrast, the entire hypusine synthetic pathway has been characterized in \( \text{Plasmodium} \). eIF5A (Q8M7X6) (http://www.uniprot.org/) was cloned by screening a cDNA library of \( \text{P. falciparum} \) DH2 strain. The CDNA open reading frame (ORF) of 486 bp encodes a predicted protein of 161 amino acids [43]. Unlike other eukaryotes, \( \text{Plasmodium} \) has only one gene for eIF5A, which is expressed at all erythrocytic phases of the parasite [43]. Quantitative time course analysis of relative protein abundance for schizont-stage parasites (34–46 h) after invasion exhibited a 15-fold increase [44]. Two isoforms of eIF5A were identified, showing a modest decrease in expression during schizont development and correlating well with the mRNA levels with a delay. In addition, DHS (Q0KH1M1) (http://www.uniprot.org/) from \( \text{P. vivax} \) was cloned and functionally characterized. Amino acid sequence alignment of \( \text{Plasmodium} \) DHS with homologous sequence from human DHS showed 44% identity [45]. The active site and the residue responsible for binding spermidine and mono guanyl diamine GC7 are highly conserved between human and all \( \text{Plasmodium} \) DHS sequences. The DOHH (C0QNK6) (http://www.uniprot.org/) cDNA sequence revealed an ORF of 1236 bp encoding a protein of 412 amino acids. DOHH proteins from \( \text{Plasmodium} \) and humans share only 27% sequence identity [46].

Polyamines, the precursors of hypusine, are also important for growth of malarial parasites. There are two key enzymes in the polyamine pathway, ODC, which forms putrescine, and S-adenosylmethionine (SAM) decarboxylase, which decarboxylates SAM to form the intermediate for subsequent production of spermidine. This spermidine acts as the source of the 4-amino butyl moiety for hypusine formation. Parasites treated with \( \alpha \)-difluoromethylornithine had their development completely blocked, i.e. they were unable to transform from trophozoite to schizont stage [47]. \( \text{P. falciparum} \) treated with the SAM decarboxylase inhibitor, SAM486A, had reduced hypusine formation due to suppression of spermidine levels. The drug also protected infected mice from cerebral malaria and prolonged the survival time in comparison to untreated mice. The parasitemia load was also reduced to 10% after 8–10 days of treatment with the inhibitor [48]. These findings indicate that reduced hypusination of eIF5A affects the ability of the parasite to cause the disease. Similar results were obtained when chloroquine-susceptible strain NF54 and chloroquine-resistant R strain of \( \text{P. falciparum} \) were treated with 1,7-diaminoheptane, a competitive inhibitor of DHS. The inhibitory effect of the compound was most prominent after 48 h of treatment, which coincides with the observation that the activity of DHS is maximal in rapidly proliferating cells due to onset of nucleic acid synthesis, indicating that the effect of the inhibitor was due to the inhibition of DHS activity [49].

Further characterization of the hypusine biosynthetic pathway in kinetoplastids and \( \text{Plasmodium} \) would help in evaluating the role of this pathway in virulence and pathogenesis.

3. Diphthamide modification of eEF2

Diphthamide (2-[3-carboxyamido-3-(trimethylammonio)-propyl] histidine) is a unique amino acid formed by posttranslational modification of a strictly conserved histidine residue on eEF2 or its archaeal ortholog, eEF2. Diphtheria toxin from Corynebacterium diphtheriae and Pseudomonas exotoxin A target eEF2 by transfer of ADP-ribose from NAD+ to the diphthamide residue of eEF2 [50]. This modification is well conserved in archaea and eukaryotes, but absent from the eubacterial ortholog. eEF-G, suggesting that eubacterial toxins have evolved a mechanism of specifically targeting diphthamide without inactivating endogenous EF-G.

 Biosynthesis of diphthamide involves a complex pathway, which includes six different enzymes acting in three steps (Fig. 3a). The first step involves the transfer of a 3-amino-3-carboxypropyl group from SAM to the C-2 of the imidazole of a specific histidine residue (His609 in \( \text{S. cerevisiae} \)). This step is catalyzed by the iron-sulfur cluster enzyme Dph2 in archaea while yeast and other eukaryotes require four enzymes (Dph1–4). The second step, involving trimethylation of the amino group resulting in a diphthine intermediate, is catalyzed by diphthine synthase (Dph5) using SAM as methyl group donor. The last step involves the ATP-dependent amidation of the carboxylate group resulting in the formation of diphthamide and is catalyzed by diphthine-ammonia ligase [51]. Out of the six enzymes involved, five have been identified by screening of recessive yeast mutants and CHO cells which were resistant to DT [52]. These mutants are sorted into five complementation groups (Dph1, Dph2, Dph3, Dph4 and Dph5) in yeast [51] and three groups (CG–1, CG–2 and CG–3) in CHO cells. The last enzyme involved in amidation was only recently identified in yeast (YBR246W or Dph7) by showing that a mutant of this ORF accumulated diphthine, suggesting that the product of this ORF may be involved in the last step of diphthamide synthesis [52]. However, this enzyme is not conserved in archaea and it contains WD domains. Using integrated data-mining, de Crécy-Lagard et al. [53] have demonstrated that the DUF71/COG2102 family contains at least two subfamilies, one of which was predicted to be the missing diphthine-ammonia ligase (Dph6), the last enzyme in the diphthamide biosynthesis pathway. Dph6 orthologs are present in all archaea and eukaryotes.

 eEF2 belongs to the family of GTP-binding proteins. It catalyzes the coordinated movement of the ribosome, mRNA and bound tRNA during translation, thus maintaining the reading frame. Structure
of yeast eEF2 (PDB:1NOU) with the conserved histidine involved in diphthamide biosynthesis (His699) is shown in Fig. 3b. The molecule has five structural domains with the diphthamide modification being present at a histidine residue at the tip of domain IV [54]. Three dimensional cryo electron microscopy studies of yeast eEF2 revealed that the tip of domain IV lies close to the cognate tRNA. Domain IV reaches deeper into the ribosome, where it interacts with both the 40S and 60S subunit in the region of the decoding center [55]. Within the decoding center there are two important adenine residues, A1492 and A1493, which are highly conserved in helix 44 of both 16S and 18S rRNA. These two adenines interact with the first two base pairs of the codon anticodon helix, thereby allowing the ribosome to closely monitor the match and the mismatch between the anticodon of the incoming tRNA and the codon of the mRNA [56]. This interaction involves a conformational cycle of stacked adenines in the absence of tRNA and flipped bases in the presence of tRNA. Diphthamide is thought to stabilize these adenines in their stacked position, thereby stabilizing codon anticodon pairing, hence maintaining the reading frame [57] and preventing -1 frame shifting during translation [58]. As a consequence diphthamide modification promotes the progression of the translation process.

3.1. Functional significance of eEF2 and diphthamide modification

The exact role of diphthamide in cellular physiology is still an enigma, but the conservation of all six enzymes, Dph1-Dph6, in all eukaryotes suggests an important function for diphthamide. Yeast mutants of Dph1, Dph2, Dph4 and Dph5 show only subtle changes in phenotypes when compared to wild type cells. Only Dph3 mutant of yeast showed varied growth defects indicating that Dph3 might also have other roles in cellular physiology [59]. In mammals, Dph1 was identified as OVCA1 [59,60], which is a candidate tumor suppressor gene whose deletion leads to ovarian malignancies. In addition, it is required for embryonic and postnatal viability [61]. It shares ~53% amino acid sequence identity with yeast Dph1. Mouse mutants of OVCA1 have a high risk of developing tumors [61], suggesting a role for dph1 in tumorigenesis. Results from studies using Dph3 knock-out mice revealed that Dph3 may
be involved in development. Dph3 heterozygous mice were pheno-
typically normal but the null mutant showed embryonic lethality. In
addition, Dph3 null mutants showed abnormalities in placenta
development, wherein there is a lack of fusion of allantois with the
chorion [62].

3.2. Diphthamide modification of eEF2 in protozoan parasites

eEF2 has been reported as a T-helper cell Th1-stimulatory pro-
tein of Leishmania donovani, generating strong IFN-γ and IL-12
responses in cured Leishmania-infected patients and protecting
hamsters against Leishmania challenge [63]. At present, there are
no reports on diphthamide modification of eEF2 in protozoan
parasites. The histidine residue of eEF2 (His715 in humans and
His699 in yeast) carrying the diphthamide modification is con-
served in eEF2 of kinetoplastids and Plasmodium, indicating that
protozoan parasites may also make this modification. Our bio-
informatic survey using Hidden Markov Models [64] (HMMs) against
the L. infantum genome database shows that the genome contains
candidate ORFs for all six enzymes involved in the diphthamide
biosynthetic pathway, Dph1 (LinJ.29.1900), Dph2 (LinJ.33.2540),
Dph3 (LinJ.26.2660), Dph4 (LinJ.18.1470), Dph5 (LinJ.31.1640) and
Dph6 (LinJ.25.0300) (Table 2). While LinJ.29.1900, LinJ.31.1640 and
LinJ.18.1470 have already been annotated as diphthamide
synthetase, diphthine synthase and chaperone DnaJ-like protein,
respectively, in the Trityp database, the hypothetical pro-
teins LinJ.33.2540, LinJ.26.2660 and LinJ.25.0300 have now been
annotated based on our bioinformatics analysis. While several
proteins encoding DnaJ domains are present in L. infantum, PFAM
HMM-based domain assignments revealed the presence of a DnaJ
domain and a ZF-CSL zinc finger domain in LinJ.18.1470, suggesting
that it may represent Dph4 (Table 2). Furthermore, the last enzyme
in the diphthamide biosynthetic pathway in yeast, encoded by
YBR246W (Dph7), contains WD repeats. However, a recent report
suggests the presence of Dph6 which contains an ATP binding
domain which is often accompanied by an Endoribonuclease L-PSP
domain at the C-terminus. Unlike yeasts, kinetoplastids (Leishmania
and Trypanosoma) encode Dph6 (LinJ.25.0300 and Tbg972.11.240,
respectively) containing an ATP binding domain. Similar can-
didate genes are also present in the genome of P. falciparum
(Table 2).

Multiple amino acid sequence alignments of Dph1–6 from L.
infantum with T. brucei, T. cruzi, human and S. cerevisiae using
CLUSTALW [80] are shown as Suppl. Figs. 1–6. Multiple sequence
alignment of Dph3 shows the four strictly conserved cysteines as
well as conservation of the “CSL” motif, confirming it being a CSL
type Zinc finger. Furthermore, all Dph4 homologs have a CSL-type
Zinc finger domain tethered C-terminally to a DnaJ domain, which
contains the conserved tripeptide “HPD” motif found in all func-
tional DnaJ proteins. The four cysteines essential for zinc binding
and the “CSL” motif are conserved in the Zinc finger domain. Mul-
tiple sequence alignment of Dph6 shows the conservation of the
“SGGKDS” motif found in all PP-loop ATPases essential for ATP
binding. While the human homolog contains only the ATP binding
domain, Leishmania and Trypanosoma Dph6 have an unassigned C-
terminal domain. In contrast, the yeast homolog has an N-terminal
ATP binding domain and a C-terminal ribonuclease L-PSP (endori-
bonuclease L-PSP) domain. In silico identification of the enzymes
involved in the diphthamide modification of eEF2 may open up an
totally new area of research in kinetoplastids and apicomplexans.

Table 2


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<th>Gene name</th>
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<td>Dph1</td>
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<td>diphthamide synthetase, diphthine synthase, chaperone DnaJ-like protein</td>
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<td>247</td>
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* Hypothetical proteins with HMM-based functional annotation;
@ Proteins annotated as Chaperone protein DnaJ putative;
# Domain organization based on PFAM domain assignments.
4. **EPG modification of eEF1A**

*Fig. 4.* Post-translational modification of eEF1A showing EPG formation (a) theoretical structural model of human EF1A (PDB:1SYW). The three domains GTP_EFTU, GTP_EFTU_D2 and GTP_EFTU_D3 are shown in pink, orange and violet colors, respectively. Modified Glu residues (301 and 374) are shown in white color. GTP binding residues are shown as green ball and stick models. (b) Proposed pathway of EPG attachment involving modification of eEF1A by phosphatidylethanolamine and subsequent deacylation to EPG.

**eEF1A** is an essential protein involved in the elongation step of peptide synthesis in all eukaryotes. It mediates GTP-dependent binding of aminoacylated tRNA to the acceptor site of a ribosome. Following GTP hydrolysis, eEF1A dissociates from the ribosome and is reactivated by nucleotide exchange factor eEF1B [65]. The activity of eEF1A is modulated by protein modifications, including phosphorylation [7], lysine methylation [8] and C-terminal methyl esterification [66]. In addition, numerous reports indicate...
that eEF1A may fulfill various other functions in the cell, such as organization of the cytoskeleton, regulation of protein degradation, and viral propagation [67].

EPG is a unique protein modification attached to eEF1A. It was discovered when mammalian cells were labeled with [3H]ethanolamine, a precursor often used to identify glycosylphosphatidylinositol–anchored proteins. Subsequent isolation and chemical and mass spectrometric analyses of a [3H]ethanolamine-labeled 49 kDa protein revealed that the radioactivity was not associated with a glycosylphosphatidylinositol structure but with a previously unknown EPG moiety [68]. The sites of EPG attachment were identified as two glutamate residues located in domains II and III of eEF1A from murine and human cells [68,69] (Fig. 4a). Subsequent studies in plants confirmed the presence of EPG in eEF1A and demonstrated that the modification sites were conserved between mammals and plants [70]. In contrast, no evidence for EPG modification was found in eEF1A from S. cerevisiae [8]. In addition, the EPG modification is absent in archaeal or bacterial eEF1A orthologs (reviewed in [3]).

The pathways for EPG synthesis and attachment to eEF1A, as well as the enzymes involved in these events, have remained elusive. An early study using a cell-free cytosolic extract from a murine lymphocyte cell line showed that free [3H] ethanolamine was incorporated into eEF1A, suggesting that EPG may be assembled stepwise [71]. However, since the modification lacked the other two components of the EPG moiety, glycerol and phosphate, no reaction sequence was proposed [71]. More recently, studies in T. brucei procyclic forms demonstrated that the cellular pool of phosphatidylethanolamine, a major phospholipid class in most eukaryotic cells, represents the donor of the ethanolamine residue for eEF1A [72]. Based on this finding, a reaction sequence involving attachment of phosphatidylethanolamine to eEF1A with subsequent cleavage of the two glycerol-bound acyl chains has been suggested (Fig. 4b).

4.1. Functional significance of eEF1A and EPG modification

Besides its canonical role during protein synthesis, eEF1A is also involved in many other cellular processes, such as binding to and mediating interactions of cytoskeletal proteins [73], nuclear export of proteins [74] and mitochondrial tRNA import [75]. Because of its high amino acid sequence conservation among eukaryotes, it is likely that non-canonical roles of eEF1A are also conserved in other eukaryotes. Remarkably, although attachment of EPG to eEF1A has been described for the first time more than 20 years ago, the biological function of this unique modification has not been addressed in mammalian cells and plants.

4.2. EPG modification of eEF1A in protozoan parasites

So far, EPG attachment to eEF1A in parasites has only been demonstrated in T. brucei procyclic and bloodstream forms [72]. Interestingly, and in contrast to mammalian and plant eEF1A, T. brucei eEF1A was found to contain a single EPG attached to a conserved glutamate residue, Glu362, in domain III [72]. More recently, trypansomes have been used as model eukaryote to investigate the role of EPG in eEF1A function. Amino acid point mutations of the EPG attachment site, Glu362, of T. brucei eEF1A, were shown to prevent EPG linkage, even when Glu was replaced by Asp, demonstrating that EPG attachment is strictly dependent on the presence of Glu [76]. In addition, conditional expression in T. brucei procyclic forms of an EPG-deficient form of eEF1A, after down-regulation of endogenous eEF1A, demonstrated that attachment of EPG to eEF1A is not essential for growth of parasites in culture [77]. However, the fact that the EPG glutamate modification has been preserved during eukaryotic evolution, occurring in mammals, plants and protozoa, suggests that it may provide a clear advantage, or represent an essential function, for the organisms under certain conditions. Interestingly, in a recent report, attachment of EPG was shown to be evolutionary conserved, i.e. human eEF1A expressed in T. brucei procyclic forms was modified with EPG [78].

5. Concluding remarks and future prospects

We have discussed the unique modifications of eEF5A, eEF2 and eEF1A and their roles in viability and pathogenesis of protozoan parasites. All three proteins are important factors required for the basic cell survival process of translation. Hence, they are well conserved among eukaryotes, including parasitic protozoa. The hypusine modification of eEF5A and its synthesis have been well characterized in kinetoplastids and Plasmodium. Various knock out studies have shown that the protein as well as its hypusine modification are essential for parasite survival. The physiological functions of the other two modifications, diphthamide on eEF2 and EPG glutamate on eEF1A, have not been characterized in kinetoplastids and apicomplexans. Further studies on the roles of these unique modifications in physiology as well as pathogenesis of protozoan parasites will provide new insights in parasitic biology, which in turn may facilitate identification of new drug targets and development of novel therapeutic interventions.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molbiopara.2012.11.001.

References


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