Phosphatidylethanolamine Is the Precursor of the Ethanolamine Phosphoglycerol Moiety Bound to Eukaryotic Elongation Factor 1A*5

Aita Signorell, Jennifer Jelk, Monika Rauch, and Peter Büttikofer
From the Institute of Biochemistry and Molecular Medicine, University of Bern, Bühlstrasse 28, Bern 3012, Switzerland

In addition to its conventional role during protein synthesis, eukaryotic elongation factor 1A is involved in other cellular processes. Several regions of interaction between eukaryotic elongation factor 1A and the translational apparatus or the cytoskeleton have been identified, yet the roles of the different post-translational modifications of eukaryotic elongation factor 1A are completely unknown. One amino acid modification, which so far has only been found in eukaryotic elongation factor 1A, consists of ethanolamine-phosphoglycerol attached to two glutamate residues that are conserved between mammals and plants. We now report that ethanolamine-phosphoglycerol is also present in eukaryotic elongation factor 1A of the protozoan parasite Trypanosoma brucei, indicating that this unique protein modification is of ancient origin. In addition, using RNA-mediated gene silencing against enzymes of the Kennedy pathway, we demonstrate that phosphatidylethanolamine is a direct precursor of the ethanolamine-phosphoglycerol moiety. Down-regulation of the expression of ethanolamine kinase and ethanolamine-phosphate cytidylyltransferase results in inhibition of phosphatidylethanolamine synthesis in T. brucei procyclic forms and, concomitantly, in a block in glycosylphosphatidylinositol attachment to procyclins and ethanolamine-phosphoglycerol modification of eukaryotic elongation factor 1A.

Eukaryotic elongation factor 1A (eEF1A)2 is a member of the G-protein family and represents an essential component during protein synthesis by binding aminoacyl-tRNAs in a GTP-dependent reaction to the acceptor site of ribosomes during peptide chain elongation (1–3). Crystal structures of eEF1A in complex with components of the nucleotide exchange factor eEF1B have recently been reported (4, 5). Besides its role during protein synthesis, eEF1A is involved in other cellular processes. It has long been proposed that eEF1A associates with and modulates microtubules and actin filaments in several cell types (1, 6). However, only recently could it be demonstrated that the conventional role of yeast eEF1A in protein synthesis and its non-canonical role in cytoskeleton organization clearly are separate functions (7). Furthermore, it has been reported that eEF1A, at least in the protozoan parasite Trypanosoma brucei, has yet another role in mediating the specificity of mitochondrial tRNA import (8).

Almost 20 years ago, two groups independently showed that eEF1A from a human erythroleukaemia cell line (9) and a murine lymphocyte cell line (10) is modified by ethanolamine-phosphoglycerol (EPG), which is covalently attached to two conserved glutamate residues in the polypeptide chain (Fig. 1). Subsequently, the same modification was found in plant (11), but not yeast (12), eEF1A. The discovery of the EPG modification was prompted by the observation that a 49-kDa cytosolic protein was labeled after incubation of mammalian cells in culture with tritiated ethanolamine (Etn) (9, 10), an approach that was originally aimed at identifying glycosylphosphatidylinositol (GPI)-anchored proteins. Etn is a component of the GPI core structure consisting of ethanolamine-phosphate-6-mannose-α1,2-mannose-α1,6-mannose-α1,4-glucosamine-α1,6-myo-inositol-1-phospholipid (13). The pathway of Etn incorporation into GPI-anchored proteins involves uptake into cells via choline/ethanolamine transporter, incorporation into phosphatidylethanolamine (PE) via common phospholipid biosynthetic pathways, transfer of the Etn moiety from PE onto a GPI precursor lipid, and attachment of the GPI to a polypeptide precursor in the endoplasmic reticulum (14–16).

Incorporation of tritiated ethanolamine into protein has also been observed for another lipid-modified protein, microtubule-associated protein 1 light chain 3 (17). This protein, together with its yeast homologue Atg8, is modified by PE, which renders these proteins membrane-bound (17, 18). The attachment of PE to a C-terminal glycine residue in Saccharomyces cerevisiae Atg8 occurs in a ubiquitin-like conjugation reaction (18). Recently, PE modification of Atg8 was shown to be linked to Atg8 function in membrane tethering and hemifusion during autophagosome formation (19).
The EPG modification of eEF1A, we studied if blocking PE biosynthesis in *T. brucei* procyclic (insect) forms using RNAi against enzymes of the Kennedy pathway affects attachment of EPG to eEF1A.

**EXPERIMENTAL PROCEDURES**

Unless otherwise specified, all reagents were of analytical grade and were from Merck (Darmstadt, Germany), Sigma-Aldrich (Buchs, Switzerland) or MP Biomedicals (Tägerig, Switzerland). [1-3H]Ethan-1-ol-2-amine hydrochloride ([3H]Etn, 60 Ci mmol⁻¹) and [9,10(n-3)H]myristic acid ([3H]myristate, 60 Ci mmol⁻¹) were purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). L-[3H(G)]Serine ([3H]serine, 29.5 Ci mmol⁻¹) was from PerkinElmer Life Sciences. BioMax MS films were from GE Healthcare (Buckinghamshire, UK), and Kodak MBX films from Kodak SA (Lausanne, Switzerland).

**Trypanosomes and Culture Conditions.—** The *T. brucei* EP/GPEET procyclin null mutant Δprocyclin#1 (30), and the derived procyclic cell line expressing tagged eEF1A, were cultured at 27 °C in DTM supplemented with 15% heat-inactivated fetal bovine serum (Invitrogen), *T. brucei* 29-13 procyclic forms (31) (obtained from Paul Englund, John Hopkins University School of Medicine) were cultured at 27 °C in SDM-79 containing 15% heat-inactivated fetal bovine serum, 25 μg/ml hygromycin, and 15 μg/ml G418 to maintain constitutive expression of the T7 RNA polymerase and the tetracycline repressor. Derived RNAi strains, including the RNAi strain against *T. brucei* eEF1A (8) (a kind gift of André Schneider, University of Fribourg), were cultured in the presence of an additional 2 μg/ml puromycin. The expression of double-stranded RNA was induced by the addition of 1 μg/ml tetracycline. *T. brucei* 427 bloodstream forms were cultured at 37 °C and 5% CO₂ in HMI-9 containing 10% heat-inactivated fetal bovine serum. Differentiation of bloodstream forms to procyclic forms was induced by the addition of 6 mM cis-aconitate and transferring the cells to 27 °C in SDM-79 containing 15% heat-inactivated fetal bovine serum (32).

Protein synthesis was blocked in Δprocyclin#1 parasites by the addition of cycloheximide (50 μg/ml, final concentration) to the culture medium 30 min before the start of the experiment. Protein synthesis was measured by incorporation of [3H]serine into protein.

**Construction of Epitope-tagged *T. brucei* eEF1A.—** To express a hemagglutinin-tagged variant of eEF1A (HA-eEF1A), the annotated *T. brucei* eEF1A (TEF1) gene (GenDB accession number Tb10.70.5670) was amplified by PCR with flanking HindIII and BamHI restriction sites (primers TbEF1A, supplemental Table S1), cloned into the TOP10F vector with a TOPO TA Cloning Kit (Invitrogen), and subsequently subcloned between HindIII and BamHI restriction sites into the pCorleone vector (a kind gift of Isabel Roditi, University of Bern) (33). The vector was linearized with HindIII, and an oligonucleotide coding for the HA tag and flanked by two HindIII sites (primers HA, supplemental Table S1) was cloned into the same vector at the N terminus of the *T. brucei* eEF1A gene. Before transfection into Δprocyclin#1, the vector (pASHaEF) was linearized with NotI and Sall.

---

**PE Synthesis Is Required for EPG Attachment to eEF1A**

FIGURE 1. Chemical structure of the EPG modification linked to the side chains of Glu301 and Glu374 of murine eEF1A (10).
RNAi-mediated Gene Silencing—Putative T. brucei ethanolamine kinase (GeneDB accession number Tb11.18.0017) and putative T. brucei ethanolamine-phosphate cytidylyltransferase (Tb11.01.5730) were down-regulated by RNAi-mediated gene silencing using stem loop constructs containing a puromycin resistance gene. Cloning the gene fragments into the vector pALC14 (a kind gift of André Schneider, University of Fribourg) was performed as described previously (34), using PCR products obtained with primers Tb0017 (spanning nucleotides 261–810 of Tb11.18.0017) and Tb5730 (spanning nucleotides 51–585 of Tb11.01.5730) (supplemental Table S1), resulting in plasmids pA0017 and pAS5730, respectively. For transfection of T. brucei procyclic forms, the vectors were linearized with NotI.

Stable Transfection of Trypanosomes—T. brucei procyclic forms were transfected with pA0017, pAS5730, or pASHaEF and selected for antibiotic resistance by addition of 10 μg/ml blasticidin S HCl (Invitrogen) for Δprocyclin#1 cells or 2 μg/ml puromycin for 29–13 RNAi cells to the culture medium. Clones were obtained by limiting dilution.

RNA Isolation and Northern Blot Analysis—Total RNA for Northern blotting was prepared by the standard acidic guanidium isothiocyanate method (35). Total RNA (10 μg) was separated on formaldehyde agarose gels and transferred to GeneScreen Plus nylon membranes (PerkinElmer Life Sciences). The 32P-labeled probes were made by random priming of the same PCR products used as inserts in the stem-loop vector (Prime-a-Gene Labeling System, Promega, Madison, WI). Hybridization was performed overnight at 60 °C, and the membrane was analyzed by autoradiography using BioMax MS film (Kodak). Ribosomal RNA was visualized on the same gel by ethidium bromide staining to control for equal loading.

Metabolic Labeling and Examinations—Trypanosomes were labeled with [3H]Etn or [3H]myristate for 2–20 h (36) and sequentially extracted with 2 × 10 ml of chloroform:methanol (CM, 2:1, by volume) to extract bulk phospholipids, followed by 3 × 5 ml chloroform:methanol:water (CMW, 10:10:3, by volume) to solubilize GPI precursors and free GPIs (30, 37). The resulting pellet was solubilized in 1% SDS. CMW fractions were pooled, dried under nitrogen, and partitioned between butanol-1-ol (CMWbut) and water (CMWaq) (37). In some experiments, CMWbut extracts were treated with purified GPI-specific phospholipase D (GPI-phospholipase D) from bovine serum as described before (38). [3H]Etn-labeled acid-soluble metabolites were extracted from trypanosomes as described elsewhere (39).

TLC—One-dimensional TLC was performed on Silica Gel 60 plates. To separate phospholipids, CM extracts were run in solvent system 1, composed of chloroform:methanol:acetic acid:water (25:15:4:2, by volume). For separation of GPI precursors, CMWbut phases were run in solvent system 2, composed of chloroform:methanol:water (4:4:1, by volume). Ethanolamine metabolites were separated in solvent system 3, composed of 25% ammonium hydroxide:methanol:0.6% NaCl in water (1:10:10, by volume). Radioactivity was detected by scanning the air-dried plate with a radioisotope detector (Berthold Technologies, Regensdorf, Switzerland) and quantified using the Rita Star® software provided by the manufacturer. Alternatively, the plate was sprayed with Enˈhance (PerkinElmer Life Sciences) and exposed to MXB film at −70 °C. On all TLC plates, appropriate lipid standards were run alongside the samples.

Lipid Phosphorous Determination—Phospholipid fractions were scraped from TLC plates and digested by boiling in perchloric acid, and the released inorganic phosphate was reacted with ammonium molybdate and quantified photometrically (40). Each determination was accompanied by a series of inorganic phosphate standards. The assay was linear between 0 and 200 nmol of phosphate per tube.

SDS-PAGE and Immunoblotting—Extracted proteins were separated on 12% polyacrylamide gels under reducing conditions (41). For detection of [3H]-labeled proteins, gels were fixed, stained with Coomassie Brilliant Blue, soaked in Amplify (GE Healthcare), dried, and exposed to MXB films at −70 °C. For immunoblotting, proteins were transferred onto Immobilon P polyvinylidene difluoride membranes (Millipore, Bedford, MA) by semi-dry blotting. Mouse monoclonal antibody against eEF1A (α-EF, Upstate, Lake Placid, NY) was used at a dilution of 1:5000. Mouse monoclonal antibody against HA (α-HA, Covance, Berkeley, CA) was used at a dilution of 1:3000. Mouse monoclonal antibody against EP procyclin (α-EP), generously provided by Terry W. Pearson (University of Victoria), was used at a dilution of 1:2500. Primary antibodies were detected with secondary rabbit anti-mouse IgG conjugated to horseradish peroxidase (Dako, Baar, Switzerland) at a dilution of 1:5000 and using an enhanced chemiluminescence detection kit (Pierce).

Immunoprecipitation—HA-eEF1A from trypanosomes lysed in 0.1% Nonidet P-40 was immunoprecipitated with Anti-HA Affinity Matrix (Roche Applied Science) according to the manufacturer’s instructions and boiled in sample loading buffer for SDS-PAGE.

Mass Spectrometry—Immunoprecipitated HA-eEF1A was subjected to SDS-PAGE, followed by in-gel reductive alkylation and trypsin digestion. Identification of peptide masses was done at the FingerPrints Proteomics Facility, Wellcome Trust Biocenter, University of Dundee (Dundee, Scotland), using an ABI 4700 matrix-assisted laser desorption ionization-time-of-flight/time-of-flight (MALDI-Tof/Tof) mass spectrometer. The tryptic peptides were further analyzed by liquid chromatography tandem mass spectrometry (nano-LC-MS/MS) using a Dionex Ultimate LC, equipped with a Pepmap C18 column (75 μm × 15 cm), coupled to an ABI Q-Trap 4000 mass spectrometer. The peptide-resolving part of the nano-LC gradient was from 1% to 40% acetonitrile in 0.1% formic acid over 20 min at 300 nl/min.

Chemical Treatment of eEF1A—Immunoprecipitated [3H]Etn-labeled HA-eEF1A was incubated in 50 µl of 70% formic acid at 37 °C for 4 or 22 h. The reaction was stopped by diluting the sample with 150 µl of water. After drying in a SpeedVac, peptides were resuspended in sample loading buffer and analyzed by SDS-PAGE, followed by immunoblotting or fluorography. Alternatively, SpeedVac-dried SDS fractions of [3H]Etn-labeled Δprocyclin#1 parasites were incubated with 50 µl of 70% formic acid at 37 °C for 4 or 22 h, diluted with 150 µl of water, and analyzed as above.
RESULTS

*T. brucei* eEF1A is Labeled with [3H]Etn—Alignment of the deduced protein sequence of *T. brucei* eEF1A with carrot and mouse eEF1A shows 76% identity with both sequences (Fig. 2). In particular, the two glutamate residues shown to be modified with EPG in plant, Glu289 and Glu362 (11), and mammalian eEF1A, Glu301 and Glu374 (9, 10), are conserved in *T. brucei* eEF1A (Glu289 and Glu362). The original identification of EPG bound to eEF1A in mammalian cells was prompted by the observation that eEF1A was labeled with [3H]Etn (9, 10). We used the same approach in *T. brucei* and found that incubation of procyclic forms lacking the genes for procyclins (*H9004* procyclin#1) with [3H]Etn resulted in labeling of a single strong band at 49 kDa (Fig. 3A, left panel). The reason to use this particular mutant cell line was to avoid possible misidentification of labeled bands with the multiple forms of procyclins, which readily incorporate large amounts of [3H]Etn into their GPI anchors (36, 42). In addition, we found that a band of similar molecular mass is also recognized by a monoclonal antibody against eEF1A (Fig. 3A, right panel). The band at 37 kDa likely represents a degradation product of eEF1A. Further evidence that the 49-kDa labeled protein is eEF1A was obtained from experiments using a *T. brucei* 29-13 procyclic cell line expressing an RNAi construct designed to knock down the expression of eEF1A (8). The results show that, after induction of RNAi by the addition of tetracycline to the culture medium, the 49-kDa band is no longer labeled with [3H]Etn; in contrast, incorporation of label into GPI-anchored EP procyclin, which migrates as a broad band at ~42 kDa, continued (Fig. 3B, left panel). Immunoblotting revealed that induction of RNAi against eEF1A resulted in complete disappearance of the 49- and 37-kDa bands that are recognized by eEF1A antibody in control cells (Fig. 3B, right panel). In addition, we found that when protein synthesis, as measured by incorporation of [3H]serine into total protein, is inhibited by the addition of cycloheximide to the culture medium (Fig. 3C, left panel), incorporation of label into eEF1A is blocked, indicating that the attachment of Etn to eEF1A occurs during, or shortly after, protein synthesis (Fig. 3C). Furthermore, the 49-kDa [3H]Etn-labeled band was also seen in *T. brucei* 427 bloodstream forms and during differentiation of bloodstream to procyclic forms in culture (Fig. 3D). The additional [3H]-labeled band at 55 kDa in *T. brucei* 427 bloodstream forms represents the variant surface glycoprotein, which is replaced during differentiation to procyclic forms by
**PE Synthesis Is Required for EPG Attachment to eEF1A**

To confirm that peptide FAIESK was modified and to localize the modification, the same tryptic peptide samples were analyzed by nano-LC-MS/MS. In both samples, a peptide eluting at 13.3 min produced an [M+2H]⁺ ion at m/z 510.8 that gave a product ion spectrum that clearly localized an EPG-modified glutamic acid residue (E*) in the sequence FAE*IESK. The spectrum is consistent with a facile elimination of glycerol phosphate, leaving an aminoethene modified Glu⁶⁶² residue, as indicated in Fig. 5.

To further demonstrate that Glu⁶⁶² is the only site modified with EPG, [³H]Etn-labeled eEF1A was treated with mild acid. Formic acid has been shown to selectively and efficiently cleave Asp–Pro bonds in proteins (44); in addition, it also cleaves Asp–Gly bonds, albeit at much lower efficiency (45). Because the EP/GPEET procyclins (43). Together, these results strongly indicate that the 49-kDa [³H]Etn-labeled band in *T. brucei* procyclic and bloodstream forms represents eEF1A.

**FAIESK (823.420 Da, amino acids 360–366 of the eEF1A sequence, see Fig. 2) plus a predicted mass increase of 197.045 Da resulting from the attachment of EPG to a glutamate residue. In contrast, the second tryptic peptide predicted to possibly contain an EPG modification at Glu⁶⁶², SIEMHHEQLAEATPGDNVGEFEK (amino acids 279–301 of the eEF1A sequence, see Fig. 2), was only found in unmodified form, i.e. with [M+H]⁺ 2523.193 Da. At present, we don’t know why HA-eEF1A runs as a doublet on SDS-PAGE.**

**Peptide synthesis is required for EPG attachment to eEF1A.**

A. After incubation of the procyclic *T. brucei* strain ∆procyclin1 with 2.5 μCi/ml [³H]Etn for 18 h, trypanosomes were washed to remove unincorporated label, delipidated with organic solvents, and sequentially extracted as described under “Experimental Procedures.” Proteins in the SDS extract were analyzed by SDS-PAGE and fluorography (left panel, 1 × 10⁶ cell equivalents) or immunoblotting using α-EF antibody (right panel, 2 × 10⁶ cell equivalents). B. Endogenous eEF1A in *T. brucei* 29-13 procyclic forms was down-regulated using RNAi by incubating the cells in the absence (−) or presence (+) of tetracycline (Tet). After 8 h of induction, 1.5 μCi/ml [³H]Etn was added, and incubation was continued for an additional 16 h. SDS extracts were prepared and analyzed as in A. The lanes contain 2 × 10⁶ and 5 × 10⁵ cell equivalents for fluorography (left panel) and immunoblotting (right panel), respectively. C. *T. brucei* Δprocyclin1 cells were incubated in the absence (−) or presence (+) of cycloheximide (CHX, 50 μg/ml final concentration) for 30 min to inhibit protein synthesis. Subsequently, trypanosomes were incubated with 2.5 μCi/ml [³H]Ser or 1.5 μCi/ml [³H]Etn for 8 h. Labeled proteins were extracted and analyzed as in A. The lanes contain 3 × 10⁷ [³H]Ser-labeled or 1 × 10⁷ [³H]Etn-labeled cell equivalents for fluorography, and 5 × 10⁶ cell equivalents for immunoblot analyses. D. *T. brucei* bloodstream forms were incubated at 37 °C with 0.3 μCi/ml [³H]Etn for 9 h. Labeled proteins were extracted and analyzed as in A (left lane). Differentiation of bloodstream forms to procyclic forms was triggered by the addition of 6 mM cis-aconitate to the culture medium and a temperature shift to 27 °C. After 0, 9, and 22 h of differentiation, trypanosomes were incubated an additional 16 h. SDS extracts were prepared and analyzed as in A (right lanes). The lanes contain 9 × 10⁸ cell equivalents.
FIGURE 4. Characterization and isolation of HA-tagged eEF1A in T. brucei. T. brucei Δprocyclin#1 trypanosomes expressing an ectopic copy of HA-tagged T. brucei eEF1A were labeled with 1.5 μCi/ml [3H]Etn for 18 h. Subsequently, HA-eEF1A was isolated by immunoprecipitation and analyzed by SDS-PAGE and fluorography (A, top panel), or immunoblotting using α-EF or α-HA antibodies (A, bottom panel). The left lane of the fluorograph shows total 3H-labeled eEF1A in Δprocyclin#1 parasites (endogenous eEF1A plus HA-eEF1A), the right lane represents immunoprecipitated HA-eEF1A. The lanes contain 3.5 × 10^6 cell equivalents for fluorography and 5 × 10^6 cell equivalents for immunoblotting. In a separate experiment, HA-eEF1A was isolated from 8.5 × 10^6 non-labeled parasites by immunoprecipitation and analyzed by SDS-PAGE and Coomassie staining (B). The two bands at 50 kDa were subsequently excised and used for mass spectrometry analysis.

FIGURE 5. Product ion spectrum of the EPG-modified peptide from T. brucei eEF1A. A tryptic digest of T. brucei eEF1A was subjected to nano-LC-MS/MS and an ion at m/z 510.8 was subjected to collision-induced dissociation to produce the spectrum in panel A. The spectrum can be assigned to conventional y- and b-type ions assuming that the EPG-modified glutamic acid residue (Ex) undergoes rapid elimination of glycerol phosphate to generate an aminocetene-modified glutamic acid residue (Ex), as indicated in panel B, where the modified glutamic acid residue is indicated in italics. Other diagnostic ions are the y6 ion of the precursor peptide (FAE*YESK) at m/z 873.72 and the ethanolamine-phosphoglycerol ion at m/z 216.24.

Down-regulation of PE Biosynthesis—To test our working hypothesis that PE is the precursor of the EPG modification of eEF1A, we studied the effects of down-regulating possible PE biosynthetic pathways by RNAi on EPG attachment in T. brucei procyclic forms. Searching the T. brucei genome for homologues encoding enzymes involved in PE synthesis revealed candidate sequences for all three enzymes of the Kennedy pathway and, in addition, candidate sequences for PE/PS head group exchange and PS decarboxylation (supplemental Fig. S1). Subsequently, we targeted the first two putative enzymes of the Kennedy pathway, ethanolamine kinase (Tb11.18.0017) and ethanolamine-phosphate cytidylyltransferase (Tb11.01.5730), for RNAi using tetracycline-inducible expression of double-stranded RNA. The nucleotide sequences of the constructs used showed no significant homology to any other sequences in the trypanosome genome. Transfection of T. brucei procyclic forms with these constructs and selection by resistance to puromycin resulted in several mutant clones, two each of which were selected for all subsequent experiments.

Down-regulation of ethanolamine kinase or ethanolamine-phosphate cytidylyltransferase by RNAi resulted in a complete disappearance of the respective RNA (Fig. 7, top). After 3 days of culture in the presence of tetracycline, growth of the individual clones slowed down and, in the case of ethanolamine-phosphate cytidylyltransferase, ceased after 7 days of induction (Fig. 7, bottom). To study the effects of RNAi on PE biosynthesis, mutant clones were incubated in the presence of [3H]Etn and the incorporation of radioactivity into PE, or other lipids, was determined by TLC analysis. The results show that essentially all radioactivity in the lipid fraction co-migrated with a PE standard (Fig. 8a, left panels). After down-regulation of ethanolamine kinase or ethanolamine-phosphate cytidylyltransferase by RNAi for 3 days, the amount of labeled PE decreased to <30% of control levels (Fig. 8a, right panels). A block in PE synthesis at ethanolamine-phosphate cytidylyltransferase is expected to result in accumulation of the immediate biosynthetic precursor of the reaction, Etn-P. Thus, we analyzed the 3H-labeled acid-soluble metabolites and found that control trypanosomes show small amounts of radioactivity co-migrating with Etn-P and CDP-Etn standards, whereas RNAi cells after down-regulation of ethanolamine-phosphate cytidylyltransferase show an accumulation of [3H]Etn-P, and a lack of CDP-[3H]Etn (Fig. 8b).
Although a decrease in [3H]Etn incorporation into PE is consistent with a decreased rate of PE synthesis by the Kennedy pathway, it may not reflect a decrease in cellular PE content, because, at least in mammalian cells and yeast, substantial amounts of PE are generated by decarboxylation of PS (22, 23). Thus, we determined the total PE content in T. brucei procyclic forms after down-regulation of ethanolamine kinase or ethanolamine-phosphate cytidylyltransferase RNAi. The results show that knocking down the two enzymes has a dramatic effect on cellular PE levels. After 5 days of incubation in the presence of tetracycline, ethanolamine kinase knockdowns showed a 38% reduction in total PE, whereas ethanolamine-phosphate cytidylyltransferase knockdowns had <20% of PE compared with cells incubated in the absence of tetracycline (Fig. 8C). In contrast, the PC content was unaffected during down-regulation of PE biosynthesis (results not shown), indicating that the two enzymes that were targeted by RNAi are only involved in PE synthesis and show no significant overlapping substrate specificity with the corresponding enzymes of the Kennedy pathway for PC biosynthesis. Furthermore, these results demonstrate that PC in T. brucei is not synthesized by methylation of PE, which is in agreement with the apparent lack of genes for the corresponding methyltransferases in the T. brucei genome. Furthermore, the dramatic reduction of cellular PE after down-regulation of ethanolamine-phosphate cytidylyltransferase demonstrates that the Kennedy pathway provides the majority of PE in T. brucei procyclic forms.

Down-regulation of PE Synthesis Blocks EPG Attachment to eEF1A—Because PE has been shown to be the donor of the Etn residue for GPI anchor assembly in trypanosomes (46), incorporation of [3H]Etn into GPI-anchored EP procyclin was expected to be severely affected after down-regulation of PE synthesis, and, thus, was used as positive control for a limited availability of PE for protein modification. The results show that, during labeling of procyclic T. brucei RNAi cells with [3H]Etn in the absence of tetracycline, significant amounts of radioactivity were incorporated into eEF1A and EP procyclin (Fig. 9A). In contrast, after RNAi-mediated down-regulation of PE synthesis, labeling of eEF1A and EP procyclin was partly (RNAi against ethanolamine kinase) or completely (RNAi against ethanolamine-phosphate cytidylyltransferase) inhibited (Fig. 9A). Immunoblotting of the same extracts shows that RNAi against the two enzymes did not reduce the amounts of eEF1A and EP procyclin—Because PE has been shown to be the donor of the Etn residue for GPI anchor assembly in trypanosomes (46), incorporation of [3H]Etn into GPI-anchored EP procyclin was expected to be severely affected after down-regulation of PE synthesis, and, thus, was used as positive control for a limited availability of PE for protein modification. The results show that, during labeling of procyclic T. brucei RNAi cells with [3H]Etn in the absence of tetracycline, significant amounts of radioactivity were incorporated into eEF1A and EP procyclin (Fig. 9A). In contrast, after RNAi-mediated down-regulation of PE synthesis, labeling of eEF1A and EP procyclin was partly (RNAi against ethanolamine kinase) or completely (RNAi against ethanolamine-phosphate cytidylyltransferase) inhibited (Fig. 9A). Immunoblotting of the same extracts shows that RNAi against the two enzymes did not reduce the amounts of eEF1A (Fig. 9B). In contrast, protein levels of EP were decreased in the induced cells compared with control uninduced trypanosomes (Fig. 9C). In addition, two bands with lower molecular masses were detected in cells after down-regulation of ethanolamine-phosphate cytidylyltransferase (Fig. 9C); these likely represent precursor forms of EP procyclin.
To further demonstrate that the reduction of PE synthesis had a major effect on GPI anchor biosynthesis and attachment to protein, *T. brucei* procyclic forms after RNAi were labeled with [3H]Etn or [3H]myristate, and the GPI precursors and free GPI anchors were analyzed by TLC and SDS-PAGE followed by fluorography. The results show that the major GPI precursor lipid, named PP1 (42), was labeled with [3H]Etn in control cells but not in cells after induction of RNAi against ethanolamine-phosphate cytidylyltransferase (supplemental Fig. S2A). In contrast, when trypanosomes were labeled with [3H]myristate, the amount of 3H-labeled GPI precursor lipids increased considerably (supplemental Fig. S2B). Similarly, induced cells showed a depletion of [3H]Etn-labeled and an accumulation of [3H]myristate-labeled free GPI anchors (supplemental Fig. S2C). Together, these results are consistent with a depletion of Etn-containing GPI lipids and free GPs and a concomitant accumulation of GPI lipids and free GPs lacking the terminal Etn group in cells after RNAi against ethanolamine-phosphate cytidylyltransferase.

**DISCUSSION**

Our results demonstrate that eEF1A in the parasitic protozoa, *T. brucei*, is modified with EPG. Labeling experiments, in
**PE Synthesis Is Required for EPG Attachment to eEF1A**

Combination with chemical and mass spectrometric analyses, show that, in analogy to mammalian (9–11) and plants (11), endogenous and HA-tagged eEF1A in T. brucei procyclic and bloodstream forms can be labeled with [3H]Etn at an EPG moiety attached to the side chain of a glutamate residue. This amino acid, Glu362 in the T. brucei eEF1A primary sequence, is strictly conserved between the predicted amino acid sequences of mammalian, plant, and yeast cells. In contrast, in the yeast S. cerevisiae, the corresponding Glu372 has been reported not to be modified with EPG (12). The reason for this lack of EPG addition to eEF1A in S. cerevisiae is unknown. Mammalian cells (9, 10) and plants (11) contain a second EPG ethanolamine kinase), or complete (RNAi against ethanolamine-phosphate cytidylyltransferase completely prevents [3H]incorporation into eEF1A, demonstrating that the Kennedy pathway is the major route for PE synthesis in T. brucei eEF1A and bloodstream forms to label eEF1A with [3H]myristate or [3H]palmitate, or by incubating the cells with [3H]pyruvate, which is incorporated into the fatty acid pool in T. brucei procyclic forms (49), showed no incorporation of radioactivity into eEF1A (results not shown). However, this is not surprising, because we were unable to detect a PE-modified form of eEF1A by mass spectrometry, and, thus, this form may represent a minor fraction of total eEF1A only. After addition of PE to eEF1A, the acyl and/or alkyl (or alkenyl) chains of PE will be removed by the concerted action of esterases of the phospholipase A-type and/or glycerol ether-cleaving enzymes, respectively, releasing eEF1A to the cytosol. Such a pathway offers interesting implications for eEF1A function, because a small fraction of eEF1A may associate with membranes and could be involved in processes other than its canonical role in protein synthesis at the ribosome, such as tRNA import into mitochondria (8), actin-binding (7), association with sphingosine kinases (50) or other signaling molecules (51), or binding to peroxisomes (52).

Acknowledgments—We thank I. Roditi and A. Schneider for plasmids and cell lines and M. A. J. Ferguson for help in performing and interpreting the mass spectrometry experiments.

**REFERENCES**

1. Negrutskii, B. S., and El’skaya, A. V. (1998) Prog. Nucleic Acids Res. Mol. Biol. **60**, 47–78
2. Merrick, W. C., and Nyborg, J. (2000) in *Translational Control of Gene Expression* (Sonnenberg, N., Hershey, J. W. B., and Methews, M. B., eds) pp. 89–125, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
3. Browne, G. J., and Proud, C. G. (2002) *Eur. J. Biochem.* **269**, 5360–5368
4. Andersen, G. R., Valente, L., Pedersen, L., Kinzy, T. G., and Nyborg, J. (2001) *Nat. Struct. Biol.* **8**, 531–534
5. Andersen, G. R., Nissen, P., and Nyborg, J. (2003) *Trends Biochem. Sci.* **28**, 434–441
PE Synthesis Is Required for EPG Attachment to eEF1A

6. Condeelis, J. (1995) Trends Biochem. Sci. 20, 169–170
7. Gross, S. R., and Kinzy, T. G. (2005) Nat. Struct. Mol. Biol. 12, 772–778
8. Bouzaidi-Tiali, N., Aebly, E., Charriere, F., Pusnik, M., and Schneider, A. (2007) EMBO J. 26, 4302–4312
9. Rosenberry, T. L., Krall, J. A., Dever, T. E., Haas, R., Louvard, D., and Merrick, W. C. (1989) J. Biol. Chem. 264, 7096–7099
10. Whiteheart, S. W., Shenbagamurthi, P., Chen, L., Cotter, R. J., and Hart, G. W. (1989) J. Biol. Chem. 264, 14334–14341
11. Ransom, W. D., Lao, P.-C., Gage, D. A., and Boss, W. F. (1998) Plant Physiol. 117, 949–960
12. Cavalli, J., Zoll, W., Chakraburtty, K., and Merrick, W. C. (1993) Biochim. Biophys. Acta 1163, 75–80
13. Ferguson, M. A. J. (1999) J. Cell Sci. 112, 2799–2809
14. Eisenhaber, B., Maurer-Stroh, S., Novatchkova, M., Schneider, G., and Eisenhaber, F. (2003) BioEssays 25, 367–385
15. Kinoshita, T., and Inoue, N. (2000) Curr. Opin. Chem. Biol. 4, 632–638
16. Orlean, P., and Menon, A. K. (2007) J. Lipid Res. 48, 993–1011
17. Kabeya, Y., Mizushima, N., Yamamoto, A., Oshitani-Okamoto, S., Ohsumi, Y., and Yoshimori, T. (2004) J. Cell Sci. 117, 2805–2812
18. Ichimura, Y., Kirisako, T., Takao, T., Satomi, Y., Shimonishi, Y., Ishihara, N., Mizushima, N., Tanida, I., Kominami, E., Ohsumi, M., Noda, T., and Ohsumi, Y. (2000) Nature 408, 488–492
19. Nakatogawa, H., Ichimura, Y., and Ohsumi, Y. (2007) Cell 130, 165–178
20. Dixon, H., and Williamson, J. (1970) Comp. Biochem. Physiol. 33, 111–128
21. Patnaik, P. K., Field, M. C., Menon, A. K., Cross, G. A. M., Yee, M. C., and Bütköfer, P. (1993) Mol. Biochem. Parasitol. 58, 97–106
22. Vance, J. E. (2003) Prog. Nucleic Acids Res. Mol. Biol. 75, 69–111
23. Birner, R., and Daum, G. (2003) Int. Rev. Cytol. 225, 273–323
24. Kennedy, E. P., and Weiss, S. B. (1956) J. Biol. Chem. 222, 193–214
25. Vance, J. E., and Vance, D. E. (2004) Biochem. Cell Biol. 82, 113–128
26. Vance, J. E., and Steenbergen, R. (2005) Prog. Lipid Res. 44, 207–234
27. Choi, J. Y., Wu, W. L., and Voelker, D. R. (2005) Anal. Biochem. 347, 165–175
28. Vial, H. J., Eldin, P., Tielens, A. G. M., and van Hellemond, J. J. (2003) Mol. Biochem. Parasitol. 126, 143–154
29. van Hellemond, J. J., and Tielens, A. G. (2006) FEBS Lett. 580, 5552–5558
30. Wirtz, E., Bütköfer, P., Engstler, M., Jelk, J., and Roditi, I. (2003) Mol. Biol. Cell 14, 1308–1318
31. Wirtz, E., Leal, S., Ochatt, C., and Cross, G. A. (1999) Mol. Biochem. Parasitol. 99, 89–101
32. Bütköfer, P., Vassella, E., Ruepp, S., Boschung, M., Civenni, M., Seebeck, T., Hemphill, A., Mookherjee, N., Pearson, T. W., and Roditi, I. (1999) J. Cell Sci. 112, 1785–1795
33. Ruepp, S., Furger, A., Kurath, U., Kunz Renggli, C., Hemphill, A., Brun, R., and Roditi, I. (1997) J. Cell Biol. 137, 1369–1379
34. Bochud-Allemann, N., and Schneider, A. (2002) J. Biol. Chem. 277, 32849–32854
35. Chomczynski, P., and Sacchi, N. (2006) Nat. Protoc. 1, 581–585
36. Bütköfer, P., Ruepp, S., Boschung, M., and Roditi, I. (1997) Biochem. J. 326, 415–423
37. Field, M. C., and Menon, A. K. (1992) in Lipid Modification of Proteins: A Practical Approach (Hooper, N. M., and Turner, A. J., eds) pp. 155–190, IRL Press, Oxford
38. Bütköfer, P., Boschung, M., Brodbeck, U., and Menon, A. K. (1996) J. Biol. Chem. 271, 15533–15541
39. Rifkin, M. R., Strobos, C. A. M., and Fairlamb, A. H. (1995) J. Biol. Chem. 270, 16220–16226
40. Rouser, G., Fleischer, S., and Yamamoto, A. (1970) Lipids 5, 494–496
41. Laemmli, U. K. (1970) Nature 227, 680–685
42. Field, M. C., Menon, A. K., and Cross, G. A. M. (1991) EMBO J. 10, 2731–2739
43. Roditi, I., and Liniger, M. (2002) Trends Microbiol. 10, 128–134
44. Inglis, A. S. (1983) Methods Enzymol. 91, 324–332
45. Acosta Serrano, A., Cole, R. N., and Englund, P. T. (2000) J. Mol. Biol. 304, 633–644
46. Menon, A. K., Epping, M., Mayor, S., and Schwarz, R. T. (1993) EMBOJ. 12, 1907–1914
47. Whiteheart, S. W., and Hart, G. W. (1994) Arch. Biochem. Biophys. 309, 387–391
48. Menon, A. K., and Stevens, V. L. (1992) J. Biol. Chem. 267, 15277–15280
49. Stephens, J. L., Lee, S. H., Paul, K. S., and Englund, P. T. (2007) J. Biol. Chem. 282, 4427–4436
50. Leclercq, T. M., Moretti, P. A., Vadas, M. A., and Pitson, S. M. (2008) J. Biol. Chem. 283, 9606–9614
51. Panasyuk, G., Nemazanyy, I., Filonenko, V., Negrutskii, B., and El’skaya, A. V. (2008) Int. J. Biochem. Cell Biol. 40, 63–71
52. Kiel, J. A., Titorenko, V. L., van der Klei, I. J., and Veenhuis, M. (2007) FEMS Yeast Res. 7, 1114–1125