Phosphatidylethanolamine in *Trypanosoma brucei* Is Organized in Two Separate Pools and Is Synthesized Exclusively by the Kennedy Pathway*

Received for publication, May 12, 2008, and in revised form, June 20, 2008. Published, JBC Papers in Press, June 28, 2008, DOI 10.1074/jbc.M803600200

Aita Signorell,† Monika Rauch,‡ Jennifer Jelk,§ Michael A. J. Ferguson,§ and Peter Bütkofer

From the †Institute of Biochemistry and Molecular Medicine, University of Bern, Bühlerstrasse 28, 3012 Bern, Switzerland and ‡The Wellcome Trust Biocentre, Division of Biological Chemistry and Drug Discovery, University of Dundee, Dundee DD1 5EH, Scotland, United Kingdom

Phosphatidylethanolamine is a major phospholipid class of all eukaryotic cells. It can be synthesized via the CDP-ethanolamine branch of the Kennedy pathway, by decarboxylation of phosphatidylserine, or by base exchange with phosphatidylserine. The contributions of these pathways to total phosphatidylethanolamine synthesis have remained unclear. Although *Trypanosoma brucei*, the causative agent of human and animal trypanosomiasis, has served as a model organism to elucidate the entire reaction sequence for glycosylphosphatidylinositol biosynthesis, the pathways for the synthesis of the major phospholipid classes have received little attention. We now show that disruption of the CDP-ethanolamine branch of the Kennedy pathway using RNA interference results in dramatic changes in phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine. By targeting individual enzymes of the pathway, we demonstrate that de novo phosphatidylethanolamine synthesis in *T. brucei* procyclic forms is strictly dependent on the CDP-ethanolamine route. Interestingly, the last step in the Kennedy pathway can be mediated by two separate activities leading to two distinct pools of phosphatidylethanolamine, consisting of predominantly alk-1-enyl-acyl- or diacyl-type molecular species. In addition, we show that phosphatidylserine in *T. brucei* procyclic forms is synthesized exclusively by base exchange with phosphatidylethanolamine.

Phospholipids are major constituents of all biological membranes. In eukaryotic cells, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) represent the most abundant phospholipid classes, usually comprising 30–50 and 20–40%, respectively, of total phospholipid (1, 2). Besides being structural components, phospholipids are involved in various regulatory and trafficking processes and may affect protein function (3–7). Because the sites of phospholipid synthesis are localized in different organelles, a complex lipid trafficking system is needed to ensure proper distribution of all lipids within a cell (8).

Eukaryotic cells possess three different pathways for PE synthesis (1, 2, 9, 10). In the CDP-ethanolamine (CDP-Etn) branch of the Kennedy pathway (11), ethanolamine (Etn) is phosphorylated to ethanolamine-phosphate (Etn-P) by ethanolamine kinase (EK) and activated with CTP to CDP-Etn by ethanolamine-phosphate cytidylyltransferase (ET). Both reactions take place in the cytoplasm. CDP-Etn is subsequently transferred onto diradylglycerol by choline/ethanolamine-phosphotransferase (CEPT), a transmembrane protein localized to the endoplasmic reticulum and nuclear membrane. Many of the enzymes involved in PE synthesis by the Kennedy pathway in different cells show dual specificities for Etn and choline metabolites. However, evidence is emerging that specific enzymes for the CDP-Etn and CDP-choline branches also exist (12). In particular, a human Etn-specific phosphotransferase (EPT) has recently been cloned and characterized (13).

Alternatively, PE can be synthesized by decarboxylation of phosphatidylserine (PS) in mitochondria (14, 15). In mammalian cells, PS decarboxylation is mediated by a single gene product, whereas two separate enzymes have been identified in yeast (2). The relative contribution of PS decarboxylation to total PE synthesis varies between cells. Both the CDP-Etn and PS decarboxylase pathways for PE synthesis are essential for mouse development, however, their quantitative importance may depend on the cell type (2).

A third but quantitatively minor pathway for PE synthesis involves head group exchange (base exchange) with PS. This reaction is fully reversible and represents, together with the head group exchange with PC, the only pathway for PS synthesis in mammalian cells (2). In the mouse model, deletion of the two base exchange enzymes is lethal, whereas single knock-out animals are viable (16). In prokaryotes and yeast, PS is made by a completely different pathway involving transfer of serine to CDP-diacylglycerol (14, 17).

PE, which is preferentially located in the inner leaflet of plasma membranes, has a tendency to form non-bilayer structures and has been proposed to play important roles in mem-
brane fusion and budding events (10, 12). Remarkably, in most eukaryotic cells substantial fractions of PE consist of alk-1-enyl-acyl- (plasmalogen-) and alkyl-acyl-type PE species rather than the more common diacyl-type species in most other phospholipid classes (12). Plasmalogen-PE species may particularly be suited for membrane fusion events because they form non-bilayer structures at lower temperatures than diacyl-type PE species.

In *Trypanosoma brucei*, a protozoan parasite causing human African sleeping sickness and animal trypanosomiasis, PC and PE comprise the majority of phospholipids (18). A more detailed analysis revealed that both *T. brucei* bloodstream and procyclic forms are particularly rich in alk-1-enyl-acyl- and alkyl-acyl-type PE species (19). In contrast to other parasitic organisms, trypanosomes synthesize phospholipids *de novo*, however, the pathways for phospholipid synthesis in *T. brucei* have not been studied in great detail (20, 21). One exception is the biosynthesis of glycosylphosphatidylinositol (GPI) lipids, where *T. brucei* has been used as a model organism to delineate the individual steps leading to GPI assembly (22). Because GPI synthesis in *T. brucei* bloodstream forms is essential and differs in certain aspects from the pathway in mammalian cells and yeast (23), the GPI pathway has been validated as a potential drug target against *T. brucei* (24). In addition, the pathway for the synthesis of phosphatidylinositol in *T. brucei* has recently been delineated (25, 26).

Very recently, we have shown that the pathways for PE and GPI synthesis in *T. brucei* procyclic forms are tightly connected. Based on a previous observation showing that PE is the precursor of the Etn group linking the GPI anchor to protein (27), we were able to completely block GPI synthesis and assembly by protein in inhibiting *de novo* PE synthesis using RNA interference (RNAi) against ET (28). The importance of PE synthesis for GPI assembly and cell viability prompted us to elucidate the complete biosynthetic pathway in *T. brucei* procyclic forms. We found that PE is organized in two separate pools, both of which are dependent on the CDP-Etn pathway of the Kennedy pathway. Interestingly, the last step in PE biosynthesis by this pathway is mediated by two separate enzymes. Knocking down any of the enzymes involved in PE synthesis affects the rate of PE synthesis and the cellular phospholipid composition in *T. brucei* procyclic forms.

### EXPERIMENTAL PROCEDURES

Unless otherwise noted, all reagents were of analytical grade and were from Merck (Darmstadt, Germany), Sigma, or MP Biomedicals (Tägerig, Switzerland). [1-3H]Ethan-1-ol-2-amine hydrochloride ([3H]Etn, 60 Ci mmol⁻¹) was purchased from American Radiolabeled Chemicals Inc., St. Louis, MO. 1-[G-3H]Serine ([1H]serine, 29.5 Ci mmol⁻¹) was from PerkinElmer Life Sciences, [methyl-3H]Choline chloride ([3H]choline, 83 Ci mmol⁻¹), [α-32P]dCTP, and Bio-Max MS films were from GE Healthcare and Kodak MBX films from Kodak (Lausanne, Switzerland).

**Trypanosomes and Culture Conditions**—The procyclic *T. brucei* strain 29-13 co-expressing T7 polymerase and a tetracycline repressor (29) (obtained from Paul Englund, John Hopkins School of Medicine), which is derived from procyclic *T. brucei* 427, was cultured at 27 °C in SDM-79 (30) containing 15% heat-inactivated fetal bovine serum (Invitrogen), 25 μg/ml hygromycin, and 15 μg/ml G418 (Invitrogen). The derived clones AS0017, AS5730, AS1570, and AS0140 expressing different double strand RNA constructs were cultured in the presence of an additional 2 μg/ml puromycin. Generation of double-stranded RNA was induced by the addition of 1 μg/ml tetracycline.

**RNAi-mediated Gene Silencing**—The following gene products were down-regulated by RNAi using stem loop constructs containing a puromycin resistance gene: putative *T. brucei* ethanolamine kinase (GenDB accession number Tb11.18.0017), putative *T. brucei* ethanolamine-phosphate cytidylyltransferase (accession number Tb11.01.5730), putative *T. brucei* choline/ethanolamine phosphotransferase (accession number Tb10.6k15.1570), and the gene product of *Tb10.389.0140*, which shows 16% amino acid sequence identity with human ethanolamine phosphotransferase (Swiss-Prot accession number Q9C0D9). Cloning of the gene fragments into the vector pALC14 (a kind gift of André Schneider, University of Bern) was performed as described (31), using PCR products obtained with primers Tb0017 (spanning nucleotides 261-810 of Tb11.18.0017), Tb5730 (spanning nucleotides 51-585 of Tb11.01.5730), Tb1570 (spanning nucleotides 236-760 of Tb10.6k15.1570), and Tb0140 (spanning nucleotides 592-1147 of Tb10.389.0140) (Table 1), resulting in plasmids pAS0017, pAS5730, pAS1570, and pAS0140, respectively. For transfection of *T. brucei* procyclic forms, the vectors were linearized with NotI (New England Biolabs).

**Stable Transfection of Trypanosomes**—*T. brucei* 29-13 procyclic forms were harvested at mid-log phase, washed once in buffer (132 mM NaCl, 8 mM KCl, 8 mM Na,HPO₄, 1.5 mM KH₂PO₄, 0.5 mM magnesium acetate, 0.09 mM calcium acetate, pH 7.0), resuspended in 600 μl of the same buffer and mixed with 15 μg of linearized plasmids pAS0017, pAS5730, pAS1570, or pAS0140. Electroporation was performed with a BTX Electroporation 600 system (Axon Lab, Baden, Switzerland) with

### TABLE 1

| Primer | Direction | Sequence |
|--------|-----------|----------|
| Tb0017 | Sense     | 5′-GCCCAAGCTTTGAATCCGCAAGATGGAGGCC-3′ |
| Tb5730 | Sense     | 5′-GCCCAAGCTTTGAATCCGCAAGATGGAGGCC-3′ |
| Tb1570 | Sense     | 5′-GCCCAAGCTTTGAATCCGCAAGATGGAGGCC-3′ |
| Tb0140 | Sense     | 5′-GCCCAAGCTTTGAATCCGCAAGATGGAGGCC-3′ |

**Stable Transfection of Trypanosomes**—*T. brucei* 29-13 procyclic forms were harvested at mid-log phase, washed once in buffer (132 mM NaCl, 8 mM KCl, 8 mM Na,HPO₄, 1.5 mM KH₂PO₄, 0.5 mM magnesium acetate, 0.09 mM calcium acetate, pH 7.0), resuspended in 600 μl of the same buffer and mixed with 15 μg of linearized plasmids pAS0017, pAS5730, pAS1570, or pAS0140. Electroporation was performed with a BTX Electroporation 600 system (Axon Lab, Baden, Switzerland) with
PE Metabolism in T. brucei Procyclic Forms

one pulse (1.5 kV charging voltage, 2.5 kV resistance, 25 micro-
farads capacitance timing, and 186 Ω resistance timing) using a
0.2-cm pulse cuvette (Bio-Rad). Electroporated cells were
immediately inoculated in 10 ml of SDM-79 supplemented
with 20% conditioned medium. Dilutions of 1:5 and 1:25 were
plated into 24-well plates and after 24 h selected for antibiotic
resistance by addition of 2 μg/ml puromycin. Clones were
obtained by limiting dilution.

RNA Isolation and Northern Blot Analysis—Total RNA for
Northern blotting, prepared by the standard acidic gua-
nidium isothiocyanate method (32), was separated on form-
aldehyde-agarose gels (1% agarose, 2% formaldehyde in
MOPS) and transferred to GeneScreen Plus nylon mem-
branes (PerkinElmer Life Sciences). 32P-Labeled probes were
made by random priming the same PCR products used as
inserts in the stem-loop vector (Prime-a-Gene Labeling Sys-
tem, Promega, Madison, WI). Hybridization was performed
overnight at 60 °C in hybridization buffer containing 7% (w/v)
Na2HPO4, pH 7.2. Finally, the membrane was analyzed by auto-
radiography using Bio-Max MS film and a TransScreen-HE
intensifying screen (Kodak). Ribosomal RNA was visualized on
the same formaldehyde-agarose gel by ethidium bromide stain-
ing to control for equal loading.

Metabolic Labeling and Extractions—Metabolic labeling of
trypanosomes with [3H]Etn, [3H]serine, or [3H]choline was
done essentially as described earlier (33). Briefly, labeled comp-
pounds were added to procyclic form trypanosomes at a density of
0.7–10 × 106 cells/ml, and incubations were continued for
various times (16–20 h). Cells were spun down, washed with
ice-cold buffer (10 mM Tris, 144 mM NaCl, pH 7.4) to remove
unincorporated label, and phospholipids were extracted with
2 × 10 ml of chloroform:methanol (CM, 2:1, by volume).

[3H]Etn-labeled acid-soluble metabolites were extracted from
trypanosomes according to Ref. 34. Briefly, 4 volumes of
ice-cold perchloric acid were added to the washed trypano-
somes. After 30 min on ice, the insoluble material was removed
by centrifugation and the supernatant neutralized with 2.5 N
KHCO3. After another 30 min on ice, the KHClO4 precipitate
was removed by centrifugation and the supernatant dried
under N2 and resuspended in H2O.

Thin Layer Chromatography (TLC)—TLC was performed on
Silica Gel 60 plates. To analyze the major phospholipid classes,
CM extracts were separated by one-dimensional TLC using
solvent system 1, composed of chloroform:methanol:acetic
acid:water (25:15:4:2, by volume). Minor phospholipid classes
were separated by two-dimensional TLC using solvent system 2
(chloroform:methanol:ammonia:water, 90:74:12:9; by volume)
for the first and solvent system 3 (chloroform:methanol:aceto-
ne:acetic acid:water, 40:15:15:12:8; by volume) for the second
dimension (35). Etn metabolites were separated in solvent sys-
tem 4, composed of 25% ammonium hydroxide, MeOH, 0.6% 
NaCl in water (1:10:10, by volume). On each plate, appropriate
lipid standards were run alongside the samples to be analyzed.
Lipid spots were visualized by exposing the plates to iodine
crystal vapor. 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 EN3HANCE (PerkinElmer Life Sciences) and
exposed to MXB film at −70 °C.

Lipid Phosphorous Determination—Lipid phosphorous was
measured according to Rouser et al. (36). Briefly, phospholipids
in chromatographic fractions scraped from TLC plates were
digested by boiling in 70–72% perchloric acid for 30–45 min.
The released inorganic phosphate was reacted with ammonium
molybdate and the resulting blue color measured photometri-
cally. For quantification of phosphate, each determination was
accompanied by a series of inorganic phosphate standards. The
assay was linear between 0 and 200 nmol of phosphate per sam-
ple (linear correlation coefficients >0.99 in all experiments).

Enzymatic and Chemical Treatment of Lipid Extracts—[3H-
Labeled lipid extracts were treated with Bacillus cereus phos-
pholipase C or B. cereus phosphatidylinositol-specific phos-
pholipase C (Invitrogen) for 2 h at 37 °C in buffer containing
0.1% Triton X-100. After termination of the reaction, released
products were extracted with water-saturated butan-1-ol and
analyzed by TLC as described above. To test the susceptibility
of lipids to mild base, extracts were dried under a stream of
nitrogen, dissolved in 200 μl of chloroform:methanol:water (10:
10:3, by volume), and incubated with 40 μl of 0.6 M NaOH in
methanol for 1 h at 37 °C. Hydrolysis was stopped on ice by
addition of 40 μl of 0.8 M acetic acid. The lysate was dried under
N2 and partitioned between water and butan-1-ol.

Analysis of PE Molecular Species—The molecular species
composition of PE was analyzed by electrospray tandem
mass spectrometry (ES-MS-MS) in positive ion mode using a
Waters Quattro Ultima triple quadrupole machine. Dried
lipid extracts from ~108 cells were dissolved in 400 μl of
butan-1-ol with vortexing and residual salts extracted by vor-
texing with an equal volume of water. After centrifugation, 100
μl of the upper phase was infused, using a Harvard syringe
pump, into the mass spectrometer at 6 μl/min via a megaspray
source. The capillary and cone voltages were 2750 and 40 V,
respectively, and the collision energy was 22 V using argon
(2.9 × 10−3 mbar) as the collision gas. Spectra were collected in
neutral loss mode (neutral loss of m/z 141, i.e. loss of the Etn-P
head group) to selectively detect PE molecular species. Spectra
were collected over the m/z range of 400–1000 at 100 atomic
mass units/s and groups of 70 spectra were averaged and pro-
cessed using Waters MassLynx software.

RESULTS

PE Biosynthesis in T. brucei Procyclic Forms Depends on the
Kennedy Pathway—The T. brucei genome contains putative
homologues for all three enzymes involved in PE biosynthesis
by the Kennedy pathway. The sequence homologies between
the deduced T. brucei and human proteins are 24, 45, and 28%
for EK (TB11.18.0017), ET (TB11.01.5730), and CDP-alcohol
phosphatidyln transferase (or CEPT; Tb10.6k15.1570), respec-
tively. To determine the contribution of this pathway to the
synthesis of bulk PE in T. brucei procyclic forms and to study
the relative importance of the individual enzymatic reactions,
we generated a series of tetracycline-inducible mutant parasites
expressing double-stranded RNA against the three enzymes.
We found that all clones tested showed a clear growth phenotype
TABLE 2

| RNAi against | \(^{3}H\)-PE* | PE* | PC* |
|--------------|-------------|-----|-----|
| EK           | 15.1        | 62.3 ± 11.5 | 106.5 ± 20.3 |
| ET           | 22.7        | 22.5 ± 9.1  | 109.2 ± 21.1 |
| CEPT         | 113.4       | 69.4 ± 13.0  | 63.8 ± 10.0  |

* Incorporation of \(^{3}H\)-Etn into PE in cells after 3 days of RNAi against EK, ET, or CEPT, relative to control uninduced cells; numbers represent mean values from single scans from three independent experiments.

† PE or PC content in cells after 5 days of RNAi against EK, ET, or CEPT, relative to control uninduced cells; numbers represent mean ± S.D. from seven to eight independent experiments.

Identification of a Novel Enzymatic Activity in the PE Biosynthetic Pathway in T. brucei—Our observation that down-regulation of CEPT results in a reduction in total PE of 38, 78, and 31%, respectively (Table 2). In contrast, PC levels were not significantly affected by down-regulation of PE synthesis, except for a 37% decrease in cells after RNAi against CEPT (Table 2). Together, these results demonstrate that down-regulation of EK, ET, and CEPT leads to a clear reduction in cellular PE levels, indicating that the Kennedy pathway represents the major route for PE synthesis in T. brucei procyclic forms.

The biosynthesis of PE in T. brucei procyclic forms after induction of RNAi was analyzed by measuring incorporation of \(^{3}H\)-Etn into the phospholipid pool. Our results showed that labeling of cells in the absence of tetracycline results in incorporation of radioactivity into a single lipid class that co-migrates on TLC with a PE standard (result not shown; see also Ref. 28). The identification of the radioactive lipid as PE was confirmed by TLC in two additional solvent systems and its complete susceptibility to bacterial phospholipase C (results not shown). After 3 days of incubation in the presence of tetracycline to induce RNAi, incorporation of \(^{3}H\)-Etn into PE dropped to 15 or 22% of control levels when EK or ET, respectively, were down-regulated (Table 2). In contrast, RNAi against CEPT led to a slight increase in \(^{3}H\)-Etn incorporation into PE (Table 2). Although these results demonstrate changes in \(^{3}H\)-incorporation into PE after RNAi against individual enzymes of the Kennedy pathway, they do not provide information on possible changes in the absolute amounts of PE. Therefore, we determined the total PE levels in trypanosomes after RNAi against the three enzymes using TLC separation followed by lipid phosphorous analysis. The results show that RNAi against EK, ET, and CEPT resulted in a reduction in total PE of 38, 78, and 31%, respectively (Table 2). In contrast, PC levels were not significantly affected by down-regulation of PE synthesis, except for a 37% decrease in cells after RNAi against CEPT (Table 2). Together, these results demonstrate that down-regulation of EK, ET, and CEPT leads to a clear reduction in cellular PE levels, indicating that the Kennedy pathway represents the major route for PE synthesis in T. brucei procyclic forms.
or ET had no effect on incorporation of [3H]choline into PC. The phospholipid data shown in Table 2, down-regulation of EPT only slightly affects the total PE content in procyclic forms but interferes dramatically with cellular regulation of EPT was verified by labeling cells with [3H]choline-phosphotransferase (Swiss-prot accession number Q9C0D9) was done using the ClustalW algorithm. White letters on black background indicate amino acid identity between the two sequences, black letters on gray background indicate similarity. The CDP-alcohol phosphatidyltransferase motif (DQG[X]ARXG[C(X)]D*X) is highlighted by boxes, predicted transmembrane domains are indicated with black bars.

pathway, we generated RNAi cells expressing double-stranded RNA against EPT. The results show that down-regulation of EPT led to the disappearance of the corresponding RNA and a reduction in cell growth (Fig. 3A). In addition, we found that upon RNAi induction incorporation of [3H]Etn into PE slightly decreased, whereas total PE slightly increased compared with control cells (Fig. 3, B and C). Interestingly, RNAi of EPT caused a dramatic increase in the cellular PC levels by more than 100% (Fig. 3C). The unexpected increase in cellular PE after down-regulation of EPT was verified by labeling cells with [3H]choline. We found that addition of [3H]choline to T. brucei procyclic forms results in incorporation of radioactivity into two bands; the major product co-migrates with a PC standard run on the same TLC plate, whereas the minor band co-migrates with sphingomyelin (Fig. 4, A–D, left panels). Consistent with the phospholipid data shown in Table 2, down-regulation of EK or ET had no effect on incorporation of [3H]choline into PC (Fig. 4, A and B). Similarly, the 60% decrease in the generation of [3H]PC after RNAi against CEPT (Fig. 4C) is consistent with the observed decrease in total PC in these cells (Table 2). At present, it is unclear why the incorporation of radioactivity into PC is not completely blocked under these conditions; possible reasons may include a residual activity of CEPT, or the presence of a second enzyme that catalyzes the reaction. The dramatic increase in cellular PC content observed in RNAi cells against EPT (Fig. 3C) was paralleled by a massive increase in [3H]PC formation (Fig. 4D). Together, these findings show that down-regulation of EPT only slightly affects the total PE content in T. brucei procyclic forms but interferes dramatically with cellular PC levels.

CEPT and EPT Are Responsible for the Synthesis of Different PE Molecular Species—The above results indicate that both CEPT and EPT are involved in PE synthesis in T. brucei procyclic forms. To study if the two enzymes generate different pools of PE, the molecular species composition of PE was analyzed in RNAi cells after down-regulation of CEPT or EPT. ES-MS-MS analysis of PE in uninucleated cells shows that PE is composed of a set of alk-1-enyl-acyl-type molecular species in the [M + H]+ range 728.5–732.5, with 18:1/18:1-, 18:0/18:1-, and 18:0/18:0-PE as the most prominent species (Fig. 5, A–D, top panels, and Table 3). The relative amounts of diacyl-type PE molecular species (peaks in the [M + H]+ range 716.5–718.5 and 740.5–746.5) are low compared with the ether-type species (Table 3). These results are in good agreement with a previous study (19). The PE molecular species composition was unchanged in parasites after down-regulation of EK, ET, or CEPT, except for a single species (C18:1/C18:1 alk-1-enyl-acyl-PE; [M + H]+ 728.5) that seems slightly increased in RNAi cells against EK, ET, and CEPT (Fig. 5, B and C, and Table 3). In contrast, the molecular species composition of PE was completely different in cells after down-regulation of EPT (Fig. 5D and Table 3). The diacyl-type species (peaks in the [M + H]+ range 740.5–746.6, i.e. 18:2/18:2-, 18:1/18:1-(and 18:0/18:2-), and 18:0/18:1-diacyl-PE), which represent a minor fraction in control cells (~20% of total), clearly are the predominant species in EPT-depleted parasites (>50% of total). In contrast, the relative amounts of alk-1-enyl-acyl-PE species (peaks in the [M + H]+ range 726.4–732.6, i.e. 18:1/18:2-, 18:1/18:1-(and 18:0/18:2-), and 18:0/18:0-alk-1-enyl-acyl-PE) in EPT RNAi cells decreased from >60% in uninucleated to <20% in induced cells. These data indicate that CEPT and EPT are involved in the synthesis of different pools of PE in T. brucei procyclic forms.

Analysis of [3H]-labeled acid-soluble metabolites showed that down-regulation of CEPT and EPT did not significantly change the amounts of [3H]Etn-P and CDP-[3H]Etn compared with uninucleated cells (results not shown). In contrast, RNAi against ET resulted in accumulation of [3H]Etn-P and a complete lack of CDP-[3H]Etn (result not shown; see also Ref. 28).

PS Is Generated by Head Group Exchange with PE in T. brucei Procyclic Forms—Our observation that the synthesis and cellular amount of PE is severely affected by down-regulation of EK or ET indicates that the Kennedy pathway provides most of the PE in T. brucei procyclic forms. However, because in many cells, the majority of PE is generated by decarboxylation of PS, we studied the importance of this pathway in T. brucei by labeling RNAi cells with [3H]serine. Incubation of control trypanosomes with [3H]serine showed that four major lipids were labeled (Fig. 6, bands labeled I–IV). Enzymatic and chemical treatment of the labeled CM extracts using phospholipase C, phosphatidylinositol-specific phospholipase C, and mild base, in combination with the migration of appropriate lipid stand-
The results showed that in RNAi cells against ET the loss of PE and PS, the relative amounts of the other phospholipids slightly increased (PC, sphingomyelin, and cardiolipin) or remained unchanged (phosphatidylinositol, inositol phosphoceramide). The PS contents in parasites after RNAi against EK, CEPT, and EPT showed no differences to control uninduced cells (results not shown).

**DISCUSSION**

*T. brucei* procyclic and bloodstream forms contain all major phospholipid classes present in mammalian cells, with PC and PE comprising about 70% of total phospholipids (18, 19). However, except for the pathways for GPI (22) and phosphatidylinositol (25, 26) synthesis, little is known about the reaction sequences involved in phospholipid synthesis. Earlier reports, aimed at identifying the precursor of the Etn moiety in the GPI core structure (27) and to study Etn uptake and metabolism (34), showed that the CDP-Etn branch of the Kennedy pathway is functional in *T. brucei* bloodstream forms. However, the con-
PE Metabolism in T. brucei Procyclic Forms

In the present report, we studied the synthesis of PE in T. brucei procyclic forms in culture using a series of RNAi cell lines and found that PE is exclusively synthesized by the CDP-Etn branch of the Kennedy pathway (Fig. 7). RNAi-mediated gene silencing of EK or ET, the first two enzymes of the Kennedy pathway, caused a severe reduction in the cellular PE content and a growth arrest of the mutant cells. These findings are surprising because bulk PE in eukaryotic cells is usually synthesized by decarboxylation of PS (1, 2, 10). In addition, our results seem to contradict previous reports showing that PE in T. brucei bloodstream and procyclic forms can also be generated by decarboxylation of PS (27, 34, 37), in particular because a homologue for PS decarboxylase is present in the T. brucei genome (gene accession number Tb927.7.3760). We confirmed the presence of an active PS decarboxylase in T. brucei procyclic forms by labeling trypanosomes with [3H]Etn, which was readily converted to [3H]PE. However, the formation of [3H]PS, and accordingly the generation of [3H]PE from [3H]PS, was completely blocked when PE synthesis was inhibited by RNAi against ET, demonstrating that PS in T. brucei procyclic forms is synthesized by base exchange with PE and can subsequently be converted (back) to PE by PS decarboxylation. A gene homologue for PS synthase 2, catalyzing PE/PS base exchange, is present in the T. brucei genome.

Unexpectedly, our results show that the last step in the CDP-Etn branch of the Kennedy pathway is mediated by two separate activities in T. brucei procyclic forms. Based on current schemes for PE and PC synthesis by the Kennedy pathway, transfer of CDP-Etn and CDP-choline to diradylglycerol is mediated by CEPT, an enzyme showing dual specificity for Etn and choline metabolites (2). Accordingly, we found no homologues of the corresponding methyltransferases in the T. brucei genome.

In another protozoan parasite, Plasmodium falciparum, PE is converted to PC by methylation (38). Clearly, this pathway is not functional in T. brucei because incubation of cells with [3H]Etn shows no incorporation of label into PC (see also Refs. 27 and 34). In addition, we found no homologues of the corresponding methyltransferases in the T. brucei genome.

FIGURE 5. Analysis of PE molecular species. T. brucei procyclic RNAi clones were incubated in the absence (−Tet) or presence (+ Tet) of tetracycline for 5 days to induce RNAi against EK (panel A), ET (panel B), CEPT (panel C), and EPT (panel D). Lipid extracts were analyzed for PE molecular species by ES-MS-MS in positive ion mode and scanning for neutral loss of 141 Da (Etn-P). The masses of the major species in control untreated cells are indicated in panel A; the masses of the species reflecting the major differences compared with control cells are indicated in panels B and D.

TABLE 3
ES-MS-MS analysis of PE molecular species in trypanosomes after down-regulation of EK, ET, CEPT, and EPT

| [M+H]+ | Molecular species | Abundance (% of total) |
|--------|-----------------|-----------------------|
| Subclass | Composition | Control | ET | CEPT | EPT |
| 702.5 | AA | C34:1 | 0.8 | 0.0 | 1.2 | 2.7 | 0.9 |
| 704.5 | AA | C34:0 | 0.6 | 0.6 | 0.6 | 3.3 | 0.5 |
| 714.5 | DA | C34:3 | 0.7 | 0.7 | 2.0 | 1.4 | 1.1 |
| 716.6 | DA | C34:2 | 3.2 | 2.2 | 3.5 | 4.4 | 8.0 |
| 718.5 | DA | C34:1 | 3.0 | 2.3 | 1.1 | 2.0 | 6.4 |
| 726.4 | AA | C36:3 | 0.3 | 0.5 | 1.7 | 1.2 | 0.5 |
| 728.6 | AA | C36:2 | 6.5 | 11.6 | 17.2 | 14.8 | 26.6 |
| 730.6 | AA | C36:1 | 38.0 | 37.5 | 33.4 | 20.6 | 11.2 |
| 732.6 | AA | C36:0 | 19.2 | 23.0 | 16.0 | 22.4 | 3.2 |
| 740.5 | AA | C36:4 | 3.2 | 3.2 | 4.7 | 1.2 | 11.3 |
| 742.5 | AA | C36:3 | 6.4 | 5.0 | 3.0 | 3.4 | 15.6 |
| 744.6 | AA | C36:2 | 6.2 | 4.6 | 4.9 | 4.9 | 16.3 |
| 746.6 | AA | C36:1 | 4.8 | 2.6 | 2.4 | 3.1 | 10.1 |
| 756.7 | AA | C38:3 | 0.7 | 0.6 | 0.5 | 2.1 | 1.1 |
| 758.4 | AA | C38:2 | 1.5 | 0.7 | 1.8 | 2.9 | 0.9 |
| 760.6 | AA | C38:1 | 0.8 | 0.7 | 1.2 | 4.6 | 0.7 |
| 764.5 | AA | C38:0 | 1.1 | 1.5 | 1.6 | 1.7 | 2.9 |
| 766.6 | AA | C38:5 | 1.2 | 1.3 | 1.1 | 0.6 | 3.2 |
| 768.5 | AA | C38:4 | 1.1 | 1.2 | 1.4 | 1.6 | 2.6 |
| 770.6 | AA | C38:6 | 0.7 | 0.0 | 0.6 | 1.1 | 1.1 |

a AA, alk-1-enyl-acyl; DA, diacyl.

b Total number of carbon atoms and double bonds.

The total number of carbon atoms and double bonds.

tribution of this pathway to bulk PE synthesis was not determined and no information is available on PE synthesis in T. brucei procyclic forms.

The contribution of this pathway to bulk PE synthesis was not determined and no information is available on PE synthesis in T. brucei procyclic forms.

In another protozoan parasite, Plasmodium falciparum, PE is converted to PC by methylation (38). Clearly, this pathway is not functional in T. brucei because incubation of cells with [3H]Etn shows no incorporation of label into PC (see also Refs. 27 and 34). In addition, we found no homologues of the corresponding methyltransferases in the T. brucei genome.
EPT, a protein containing 8 predicted transmembrane domains, is embedded into the glycosomal membrane. In contrast, bulk PC in *T. brucei* procyclic forms is synthesized by CEPT, which has been found to associate with the endoplasmic reticulum in human cells (40). Experiments to study the localization of EPT and CEPT in *T. brucei* procyclic forms are currently under way. Because *T. brucei* CEPT shows dual specificity for CDP-Etn and CDP-choline, it may take over PE production in the case when EPT is absent, i.e. down-regulated by RNAi. However, due to its (non-glycosomal) localization, CEPT will transfer CDP-Etn not to ether-type lipid precursors but to diacylglycerols, resulting in mostly diacyl-PE species that resemble the predominantly diacyl-type PC species in *T. brucei* procyclic forms (19). In addition, because CEPT shows higher affinity toward CDP-choline than CDP-Etn, increased CEPT activity results in the elevated PC content we find in these cells. This interpretation is in line with a previous report showing that two different enzyme activities are responsible for the synthesis of alk-1-enyl-acyl-PE and diacyl-PE in rabbit heart membranes (41). Separate pools of PE consisting of different molecular species have also been reported in other mammalian cells (42) and yeast (43).

**Acknowledgments—**We thank A. Schneider for plasmids and cell lines. P. B. thanks C. Nobs and the reviewers for valuable input.

**REFERENCES**

1. Vance, J. E., and Vance, D. E. (2004) *Biochem. Cell Biol.* 82, 113–128
2. Vance, J. E. (2008) *J. Lipid Res.* 49, 1377–1387
3. Dowhan, W. (1997) *Ann. Rev. Biochem.* 66, 199–232
4. Tillman, T. S., and Cascio, M. (2003) *Cell Biochem. Biophys.* 38, 161–190
5. Munro, S. (2004) *Nat. Cell Biol.* 6, 869–872
6. De Matteis, M. A., and Godi, A. (2004) *Nat. Cell Biol.* 6, 487–492
7. Wang, X., Devaiah, S. P., Zhang, W., and Welti, R. (2006) *Prog. Lipid Res.* 45, 250–278
8. Voelker, D. R. (2003) *J. Lipid Res.* 44, 441–449
9. Carman, G. M., and Henry, S. A. (1999) *Prostaglandins Leukot. Med.* 64, 87–97
10. Birner, R., and Daum, G. (2003) *Bioorg. Med. Chem. Lett.* 13, 2629–2632
11. Kennedy, E. P., and Weiss, S. B. (1956) *J. Biol. Chem.* 218, 149–158
12. Arikketh, D., Nelson, R., and Vance, J. E. (2008) *J. Cell Sci.* 121, 245–255
13. van Hellemond, J. J., and Tielens, A. G. (2006) *Comp. Biochem. Physiol.* 144C, 111–118
14. Matsumoto, S., and Williamson, J. (1970) *J. Biol. Chem.* 245, 361–399
15. Voelker, D. R. (1984) *Proc. Natl. Acad. Sci. U. S. A.* 81, 2639–2673
16. Arikette, D., Nelson, R., and Vance, J. E. (2007) *J. Biol. Chem.* 282, 12888–12897
17. Nikawa, J. I., and Yamashita, S. (1981) *Biochim. Biophys. Acta* 665, 420–426
18. Dixon, H., and Williamson, J. (1970) *Comp. Biochem. Physiol.* 33, 111–128
19. Patnaik, P. K., Field, M. C., Menon, A. K., Cross, G. A. M., Yee, M. C., and Blackwelder, P. (1993) *J. Biol. Chem.* 268, 10086–10092
20. Vial, H. J., Eldin, P., Tielens, A. G. M., and van Hellemond, J. J. (2003) *Mol. Biochem. Parasitol.* 126, 143–154
21. van Hellemond, J. J., and Tielens, A. G. (2006) *FEBS Lett.* 580, 5552–5558
22. Ferguson, M. A. J. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 10673–10675
23. Martin, K. L., and Smith, T. K. (2006) *Biochem. J.* 396, 287–295
24. Martin, K. L., and Smith, T. K. (2006) *Mol. Microbiol.* 61, 89–105
25. Menon, A. K., Eppinger, M., Mayor, S., and Schwarz, R. T. (1993) *EMBO J.* 12, 1907–1914
26. Signorell, A., Jelk, J., Rauch, M., and Bulteau, P. (2008) *J. Biol. Chem.* 23643.
283, 20320–20329
29. Wirtz, E., Leal, S., Ochatt, C., and Cross, G. A. (1999) Mol. Biochem. Parasitol. 99, 89–101
30. Brun, R., and Schönenberger, M. (1979) Acta Trop. 36, 289–292
31. Bochud-Allemann, N., and Schneider, A. (2002) J. Biol. Chem. 277, 32849–32854
32. Chomczynski, P., and Sacchi, N. (2006) Nat. Protoc. 1, 581–585
33. Bütikofer, P., Ruepp, S., Boschung, M., and Roditi, I. (1997) Biochem. J. 326, 415–423
34. Rifkin, M. R., Strobos, C. A. M., and Fairlamb, A. H. (1995) J. Biol. Chem. 270, 16160–16166
35. Bütikofer, P., Lin, Z. W., Kuypers, F. A., Scott, M. D., Xu, C., Wagner, G. M., Chiu, D. T. Y., and Lubin, B. (1989) Blood 73, 1699–1704
36. Rouser, G., Fleischer, S., and Yamamoto, A. (1970) Lipids 5, 494–496
37. Guler, J. L., Kriegova, E., Smith, T. K., Lukes, J., and Englund, P. T. (2008) Mol. Microbiol. 67, 1125–1142
38. Pessi, G., Choi, J. Y., Reynolds, J. M., Voelker, D. R., and Mamoun, C. B. (2005) J. Biol. Chem. 280, 12461–12466
39. Opperdoes, F. R. (1984) FEBS Lett. 169, 35–39
40. Henneberry, A. L., Wright, M. M., and McMaster, C. R. (2002) Mol. Biol. Cell 13, 3148–3161
41. Ford, D. A. (2003) J. Lipid Res. 44, 554–559
42. Bleijerveld, O. B., Brouwers, J. F., Vaandrager, A. B., Helms, J. B., and Houweling, M. (2007) J. Biol. Chem. 282, 28362–28372
43. Bürgermeister, M., Birner-Grunberger, R., Heyn, M., and Daum, G. (2004) Biochim. Biophys. Acta 1686, 148–160