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Short communication

Trypanosoma brucei solanesyl-diphosphate synthase localizes to the mitochondrion

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ABSTRACT

Polyprenyl-diphosphate synthase is a key enzyme in the biosynthesis of ubiquinone, a molecule considered essential for a typical eukaryotic cell. Its orthologue in the American stercorarian flagellate *Trypanosoma cruzi*, solanesyl diphosphate synthase, has been previously localized into the glycosomes. We wondered whether this unique cellular localization is shared by other trypanosome species. Using digitonin permeabilization, immunofluorescence and in situ tagging techniques, we show that in *Trypanosoma brucei*, the African salivarian flagellate, the enzyme localizes to the mitochondrion.

1. Introduction, results and discussion

Trypanosomes and related flagellates are well known for a range of unique biological features as well as for causing serious diseases. Members of the genera *Trypanosoma* and *Leishmania* are responsible for Chagas disease, sleeping sickness and leishmaniases. Current chemotherapeutic drugs for these diseases elicit numerous side effects and show poor effectivity and drug resistance [1]. The urgent need for new drugs against these pathogens drives the studies of their essential metabolic pathways, which are often subject to dramatic changes in the course of the life cycle. The procyclic (insect vector) stage of *Trypanosoma brucei* uses canonical electron transport chain to generate mitochondrial membrane potential and ATP, while the bloodstream (mammalian host) stage respires exclusively via terminal alternative oxidase [2]. However, since both pathways require a ubiquinone (UQ) pool for electron transport, the biosynthesis of UQ may constitute a suitable target for chemotherapeutic intervention against these parasites.

UQ contains a benzoquinone ring attached to an isoprenoid chain, which is of variable length in different organisms. In the well-studied *Saccharomyces cerevisiae*, the UQ contains six isoprene units in its chain and is hence labelled UQ-6, its biosynthesis requiring the products of at least nine genes (Coq1–Coq9). Coq1 encodes a polyprenyl-diphosphate synthase, Coq1p, which generates the isoprenyl moiety, while products of the other genes are responsible for modifications or transfer of the isoprenyl moiety to the benzoquinone ring. In yeast, all of these proteins are located in the mitochondrion, and all but Coq1p associate into a multi-subunit complex [3].

In *Trypanosoma cruzi*, the 9 units-long isoprenoid chain of UQ-9 is synthesized by the Coq1p orthologue, solanesyl-diphosphate synthase (TcSPPS), which has been reported to have an unusual localization in the glycosomes [4]. This feature may either be specific for *T. cruzi*, or may be shared by other trypanosomatids. *T. brucei*, the causative agent of African sleeping sickness, also possesses UQ-9 [5], and other Coq orthologues, such as TbSPPS, as confirmed by proteomic analyses [6,7]. In the case of TbSPPS, bioinformatic prediction by MitoProt II clearly indicated its mitochondial location with probability of 0.3562 [8]. The predicted signal peptide is 10 amino acids long. Therefore, we decided to carry out experiments to establish the subcellular localization of TbSPPS. To that end, specific antibodies against this protein were prepared and used in digitonin permeabilization studies of wild type and in situ tagged parasites.

Abbreviations: TbSPPS, *Trypanosoma brucei* solanesyl-diphosphate synthase; UQ, ubiquinone; ORF, open reading frame; FITC, fluorescein isothiocyanate.

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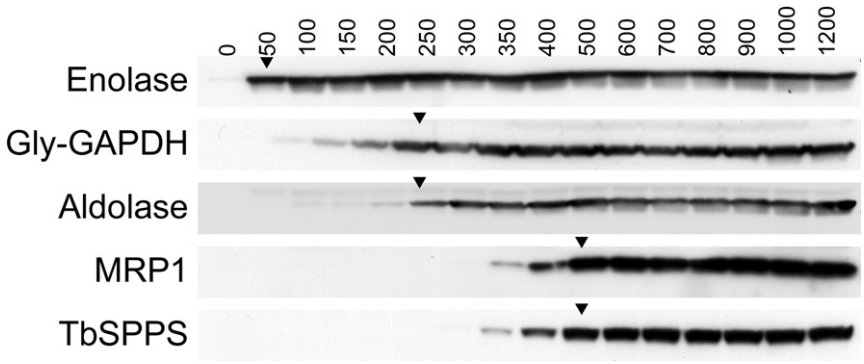

Fig. 1. Digitonin permeabilization. TREU927/4 procyclic cells were washed in SHE buffer (250 mM sucrose; 25 mM HEPES; 1 mM EDTA; pH 7.4) and resuspended at a density 5.4×10^9/ml (7 mg protein/ml) in STE buffer (250 mM sucrose; 25 mM Tris, pH 7.4; 1 mM EDTA; 150 mM NaCl). 140 μl of cells were resuspended in a total volume of 300 μl STE buffer, incubated for 5 min at room temperature and supplemented with increasing concentrations of digitonin per total protein (μg/mg). Supernatants were harvested by centrifugation at 10,000 × *g* for 2 min. Samples were boiled for 5 min in sample buffer (50 mM Tris, pH 6, 8; 100 mM DTT, 1.5% SDS) before being separated on SDS-PAGE gel, blotted and probed with antibodies against TbSPPS, MRP1 [24], enolase, aldolase, and glycosomal GAPDH at 1:500, 1:1000, 1:150,000, 1:20,000 and 1:20,000 dilutions, respectively. Arrowheads indicate the minimal concentration of digitonin needed to fully release the corresponding proteins.

The sequence of TbSPPS was found in *T. brucei* GeneDB, using TcSPPS as a query. A single gene (Tb09.160.4300) was identified, encoding a protein with calculated molecular weight of 39.2 kDa, displaying features essential for its activity, similar to those found in TcSPPS [4]. The full length TbSPPS protein was expressed with an N-terminal polyhistidine tag in a pQE30 vector (Qiagen). The insoluble recombinant protein was denatured with guanidinium chloride and purified by nickel affinity chromatography. After dialysis, the purified protein without adjuvant was, on a weekly basis, inoculated into the peritoneum of a mouse (100 μg/dose) for five consecutive weeks. Upon harvesting, the serum was shown by western blot analysis to recognize a band of 39 kDa (data not shown).

The *T. brucei* procyclic strain TREU 927 was used in the digitonin titration experiments. Cells kept in isotonic buffer supplemented with 150 mM NaCl were subjected to increasing concentrations of the detergent, resulting in the release of marker enzymes from the sequentially permeabilized cell compartments [9]. After SDS-PAGE and western blot, a set of representative proteins from the cytosol, glycosome and mitochondrion was detected by specific antibodies (Fig. 1). TbSPPS was released neither with the cytosolic marker enolase, nor with the glycosomal markers aldolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), but emerged together with the mitochondrial RNA-binding protein (MRP1) (Fig. 1). This result strongly indicates that TbSPPS is a mitochondrial protein.

To further confirm this observation, an in situ tagging technique was used, since the anti-TbSPPS antibody gave only a weak signal by immunofluorescence. We designed the tagged gene to remain under wild type regulation, avoiding the pitfall of overexpression and consequently increased risk of abnormal distribution of the tagged protein. The 5′ or 3′ untranslated region (UTR) of the *TbSPPS* gene was cloned into the pEnT6B-G vector [10] to generate an N- or C-terminally tagged construct, respectively. In our strategy the tag consisted of either the Ty1 epitope alone, or the same epitope was fused to the green fluorescent protein (GFP). The linearized constructs were transfected into the TREU 927 procyclics, where they integrated into the original *TbSPPS* locus via homologous recombination. Upon drug selection, the transfectants contained just one tagged allele of the gene (as shown below).

In all the transfected cell lines, the expression of the Ty1-tagged protein was validated with the specific anti-Ty1 antibody. Depending on the introduced construct, a band of expected molecular weight of either ~70 kDa (Ty1::GFP::TbSPPS or TbSPPS::GFP::Ty1) or ~40 kDa (TbSPPS::Ty1) was immunodecorated (Fig. 2A; upper panel). Incubation with the polyclonal anti-TbSPPS antibody revealed both native and/or tagged proteins in the wild type and transfected flagellates, respectively, and allowed a raw quantitation of the amount of the tagged protein (Fig. 2A; medium panel). Protein loading was equalized as evaluated by the L8C4 antibody specific for the paraflagellar rod protein 2 (PFR2) (Fig. 2A; lower panel). In general, after one allele of gene is tagged, the presence of the recombinant protein is accompanied by a reduced expression of the native protein as is obvious from the comparison of the 39 kDa band among the lanes (Fig. 2A; lane 4). Next, we established the ratio of recombinant to native protein in each transfectant. The signal of the N-terminally tagged protein (Ty1::GFP::SPPS) was so weak that for its visualization longer exposures were needed (Fig. 2A; lane 1). The C-terminally tagged proteins were expressed at about the same level as the native one (both TbSPPS::GFP::Ty1 and TbSPPS::Ty1) (Fig. 2A; upper band in lane 3).

Moreover, the fluorescence (GFP) or immunofluorescence (anti-mouse FITC-conjugated secondary antibody) signals were examined by light microscopy (Fig. 2B). The Ty1::GFP::TbSPPS cells showed neither fluorescence (Fig. 2B; bottom row) nor immunofluorescence (data not shown), which is in accordance with the scant amount of the detected fusion protein (Fig. 2A). The transfected cells were also subjected to digitonin permeabilization. At low detergent concentrations the tagged protein appeared in the supernatant along with the cytosolic marker enolase. However, since the native TbSPPS protein behaved like MRP1 (Suppl. Fig. S1A), this result suggests that the N-terminal tag triggered mislocalization.

The transfectants expressing the C-terminally tagged proteins (both TbSPPS::Ty1 and TbSPPS::GFP::Ty1) displayed (immuno)fluorescence exclusively in the mitochondrion, overlapping with the MitoTracker Red signal (Fig. 2B; 1st and 3rd rows). In addition, by digitonin permeabilization, the fusion protein TbSPPS::Ty1 was released along with the native TbSPPS protein (Suppl. Fig. S1B). Therefore, the exclusive mitochondrial localization of the wild type TbSPPS was confirmed by the fusion proteins containing two different tags on their C-termini.

There is no canonical subcellular localization of polyprenyl-diphosphate synthases in a eukaryotic cell. Members of this protein family have been found associated with the inner mitochondrial membrane of yeast [3], mitochondria or plastids of *Oryza sativa* [11], or were present in both the endoplasmic reticulum and plastids of *Arabidopsis thaliana* [12]. The most complex distribution has so far been documented in rats, where the endoplasmic reticulum, golgi apparatus, and even peroxisomes contain polyprenyl-diphosphate synthases [13,14]. In trypanosomatids, TcSPPS was so far detected by immunofluorescence and immunogold labeling in the glycosomes of *T. cruzi* [4].

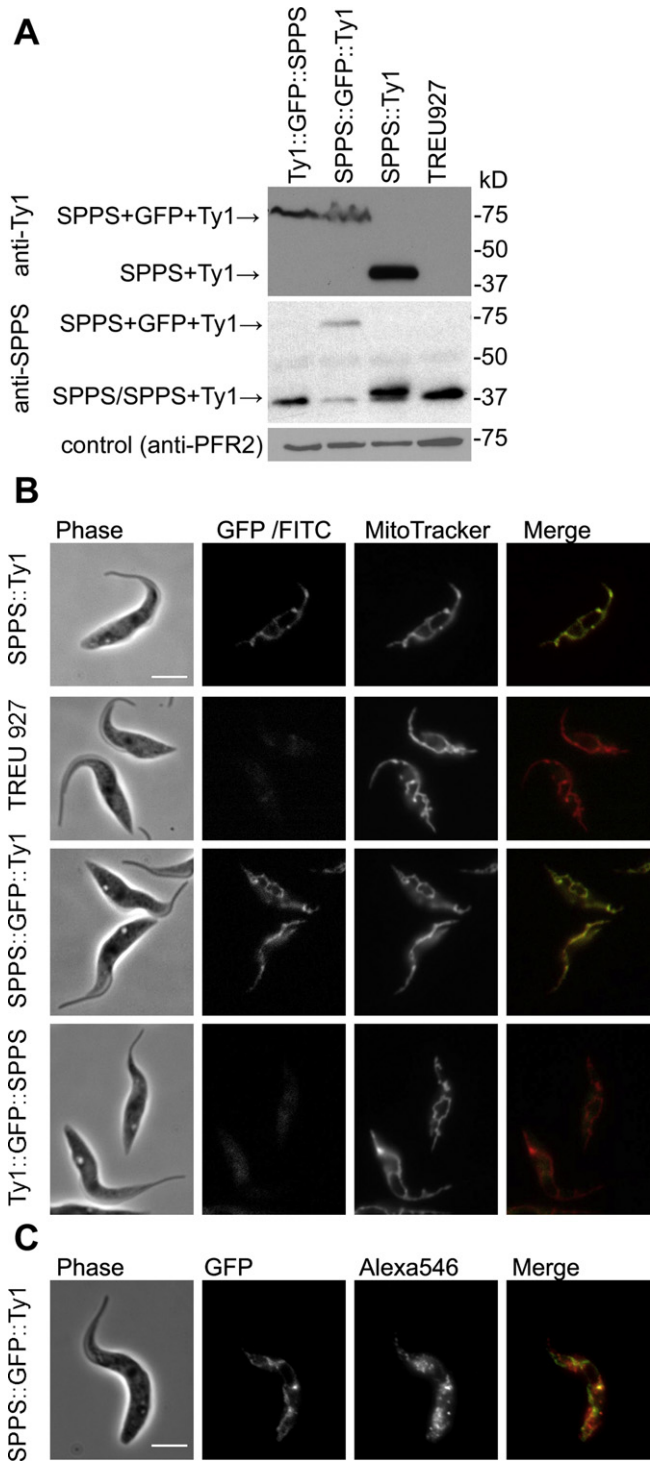

Fig. 2. Expression of tagged TbSPPS in *T. brucei*. The pEnT6B-G expression vector was used to generate recombinant TbSPPS proteins tagged either at its N- or C-terminus with enhanced GFP and/or Ty1 epitope [10]. The first 339 bp of the TbSPPS open reading frame (ORF) and 321 bp of respective 5′ UTR were amplified with the following primers that introduced restriction sites: TbSPPS-5′US-*Xho*I 5′-TGTCTCGAGTTTGTTTACCAATAGGAGACACA-3′; TbSPPS-5′UA-*Bam*HI 5′-GTGGGATCCAACACGCCGAAAGAAACAAC-3′; TbSPPS-5′S-*Xba*I 5′-CGTTCTAGAATGCACCGTGCTAATATTATATTAC-3′; TbSPPS-5′A-*Xho*I 5′-CTCCTCGAGCCGCAAAAAGGGGC-3′. The amplicons were digested with the corresponding restriction enzymes and cloned into pEnT6B-G vector to generate the N-terminally tagged TbSPPS. Another set of primers was used to amplify the 257 bp-long 3′ end ORF without the stop codon and 354 bp-long part of the 3′ UTR of TbSPPS as follows: TbSPPS-3′S-*Xho*I 5′-TGACTCGAGGAATCGCCACCTTACCCAC-3′; TbSPPS-3′A-*Spe*I (for GFP-TY) 5′-ATGACTAGTCAATTCCCGTGTCAGGAGGAGATT-3′; TbSPPS-3′A-*Xba*I (for TY) 5′-ATGTCTAGACAATTCCCGTGTCAGGAGGAGATT-3′; TbSPPS-3′US- *Hind*III 5′-GGGAAGCTTGAGCGCATCATTTTTACTCT-3′; TbSPPS-3′UA-*Xho*I 5′-AGGCTCGAGCGACGGACGATACGGGGA-3′. To generate C-terminally tagged TbSPPS, the amplicons were ligated into the pEnT6B-G vector. After confirmation by sequencing, all the constructs were linearized with *Xho*I before transfection into the TREU 927/4 procyclics. Selection for resistance to 10 μg/ml blasticidin started 8 h later. Stable transfectants were subjected to ten-fold serial dilutions with conditioned medium in 96-well plates. Clones were chosen from the most diluted plates and analysed by SDS-PAGE and immunofluorescence. (A) Western blots of selected clones immuno-decorated with anti-Ty1 (upper panel), anti-TbSPPS (medium panel) and anti-PFR2 antibody (lower panel). The latter was used as a loading control. (B) GFP fluorescence or FITC immunofluorescence of transfected or parental cells (TREU 927) was visualized with an Axioplan2 microscope (Zeiss, Germany). Mitochondrion was stained by incubating the cells with 50 nM MitoTracker Red for 20 min (Molecular Probes, Invitrogen, USA). The white scale bar equals 5 μm. (C) GFP fluorescence and AlexaFluor 546 (Invitrogen, USA) from GFP C-terminally tagged TbSPPS cell. 1st antibody against glycosomal GAPDH and 2nd Alexa546 labeled antibody were used for the glycosomes staining.

Herein, we describe that in *T. brucei*, TbSPPS is localized solely in the mitochondrion. This unexpected heterogeneity in the intracellular localization of an essential protein could be attributed to the fact that regardless of the location in the cell the UQ side chain synthesis takes place, the product can traverse membranes and will be delivered to the target compartment by a carrier protein or a still unidentified mechanism. In a typical eukaryotic cell, the predominant localization where the hydrophobic isoprenoid side chain is condensed with p-hydroxybenzoic acid is in the mitochondrion, yet other cellular compartments such as the microsomes, have also been implicated [13,15]. Little is known about the transport of the final product of this biosynthetic pathway. Cop10p in the inner mitochondrial membrane of yeast [16], and human lysosomal protein saposin B [17] has been shown to bind UQ-6 and UQ-10 respectively, but details on their transport mechanism are currently unknown. Until a general idea of the transport of isoprenoid chains emerges, it may be premature to interpret the differences in the intracellular localization of SPPS in different organisms.

Since bisphosphonate inhibitors against TcSPPS have already been synthesized and tested [18], establishing its localization in *T. brucei* has an added value in terms of developing more efficient and less toxic chemotherapeutic drugs. Short chain nitrogen-containing bisphosphonates are traditionally used to treat osteoporosis. Their high affinity binding to bone hydroxyapatite is an advantage when targeting farnesyl-diphosphate synthase of the bone-remodelling osteoclasts. However, it is a disadvantage when tumor cells [19] or the liver-stage of *Plasmodium* are targeted [20], in which case special functional groups are attached to the active compound in order to increase their lipophilicity and decrease their affinity for bone [19,20]. Inside trypanosomatids, acidocalcisomes are the largest store of calcium, also containing pyrophosphate and polyphosphate [21], although the presence of hydroxyapatite has not been reported, as suggested for an apicomplexan [22]. This confluence of elements led to the hypothesis that acidocalcisomes could preferentially concentrate the bisphosphonates [21]. Based on the present report, increasing the lipophilicity of the inhibitors we are developing [18], or even targeting them to specific organelles [23] could increase their efficacy by making them more bioavailable.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2004.08.011.10.1016/j.molbiopara.2012.02.011

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