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Farnesyl Diphosphate Synthase Localizes to the Cytoplasm of *Trypanosoma cruzi* and *T.brucei*

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Abstract

The farnesyl diphosphate synthase (FPPS) has previously been characterized in trypanosomes as an essential enzyme for their survival and as the target for bisphosphonates, drugs that are effective both *in vitro* and *in vivo* against these parasites. Enzymes from the isoprenoid pathway have been assigned to different compartments in eukaryotes, including trypanosomatids. We here report that FPPS localizes to the cytoplasm of both *Trypanosoma cruzi* and *T. brucei*, and is not present in other organelles such as the mitochondria and glycosomes.

Keywords

Bisphosphonates; farnesyl diphosphate synthase; mevalonate pathway; trypanosome

1. Introduction

Trypanosomiases are parasitic diseases affecting millions of people in the American and African continents. Recent work has shown that nitrogen-containing bisphosphonates, such as those used to treat bone resorption diseases, are competitive inhibitors of the farnesyl diphosphate synthase of both *Trypanosoma cruzi* (Montalvetti, 2001) and *T. brucei* (Montalvetti, 2003) and effective *in vitro* and *in vivo* against these parasites (Urbina, 1999; Szajnman, 2001 and 2003; Martin, 2001 and 2002; Garzoni, 2004; Bouzahzah, 2005). Farnesyl diphosphate synthase (FPPS) catalyzes the consecutive condensation of isopentenyl diphosphate (IPP, C_5) with dimethylallyl diphosphate (DMAPP, C_5), and with geranyl diphosphate (GPP, C_{10}), all products of the mevalonate pathway in trypanosomatids, to form the 15-carbon isoprenoid compound, farnesyl diphosphate (FPP, C_{15}). FPP is the substrate for enzymes catalyzing the first committed step for biosynthesis of sterols, ubiquinones, dolichols, heme a, and prenylated proteins. The three-dimensional structures of both *T. cruzi* (Gabelli, 2005) and *T. brucei* (Mao, 2004 and 2006) FPPS have been solved.

In trypanosomatids, the localization studies of some of the enzymes of the mevalonate pathway have yielded controversial results. 3-Hydroxy-3-methyl-glutaryl-CoA reductase (HMGR), the first enzyme of this pathway, was localized to the mitochondrion of *T. cruzi* and *L. major*

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(Pena-Diaz, 2004), although it was first reported to be glycosomal in *T. cruzi* (Concepcion, 1998), and was shown to be mitochondrial with some possible association to other organelles, such as the endoplasmic reticulum (ER) and glycosomes in *T. brucei* (Heise, 2000). We previously reported the presence of a long chain solanesyl diphosphate synthase (SPPS) in the glycosomes of *T. cruzi*, and detected the presence of another putative polyprenyl synthase in their cytoplasm (Ferella, 2006). Squalene synthase, which is involved in the first step of sterol biosynthesis, was reported to have multiple locations in *T. cruzi* and *Leishmania mexicana* (Urbina, 2002). The short chain farnesyl diphosphate synthase, described in all three trypanosomatids (Montalvetti, 2001 and 2003; Ortiz-Gomez, 2006), was reported to be a cytosolic enzyme in *L. major* (Ortiz-Gomez, 2006). An SKL-like sequence is present in the *T. cruzi* enzyme although it is not in the C-terminus of the protein as other motifs involved in glycosomal targeting (Montalvetti, 2001). In mammals and plants, there is a similar trend of different polyprenyl synthases located in different compartments. For example, FPPS has been found in the cytosol and associated with the endoplasmic reticulum (Hugueney, 1996), mitochondria (Cunillera, 1997), and plastids (Sanmiya, 1999) in plants, and the peroxisomes in animals (Biardi, 1996). These variable locations of the components of this pathway make it necessary to study this issue in detail in each organism.

We here report that FPPS is localized in the cytoplasm of *T. cruzi* epimastigotes and *T. brucei* procyclic and bloodstream trypomastigotes, as demonstrated by its release from digitonin-permeabilized cells together with cytosolic markers, and by immunofluorescence microscopy localization studies.

2. Materials and methods

2.1. Cell cultures

Epimastigotes from *T. cruzi* CL Brener were grown at 28°C in Liver infusion tryptose (LIT) media (Bone, 1956) supplemented with 5% FBS (Gibco) and streptomycin /penicillin (Gibco), pH 7.3. Procyclic trypomastigotes from *T. brucei* were grown at 28°C in SDM-79 medium pH 7, supplemented with 10% FBS (Gibco) and streptomycin /penicillin (Gibco). Bloodstream trypomastigotes from *T. brucei* (*T. brucei brucei* strain) were obtained and cultured as described before (Hesse, 1995).

2.2. Digitonin permeabilization assay

Approximately 2-4 × 10^9 parasites were collected and washed twice by centrifugation at 2500 rpm (~1000 × g) for 10 min in buffer D (20 mM Tris-HCl, pH 7.2, 225 mM sucrose, 20 mM KCl, 10 mM KH_2PO_4, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT), and finally resuspended in 3 ml of the same buffer containing a protease inhibitor cocktail (P8340, Sigma). An aliquot of 50 μl was mixed with an equal volume of 0.2 % Triton X-100 in 0.3 M NaCl, incubated at room temperature for 20 min, and used to determine the total protein concentration per ml of total cell suspension, using the BioRad Protein Assay (BioRad). The cell suspension was diluted to 1 mg of total protein/ml and distributed into 12 tubes for the digitonin assays. Increasing amounts of digitonin were added to each tube and then incubated at 28°C for 5 min. The samples were centrifuged for 2 min at 14,000-x *g*, and the supernatants were transferred to new tubes. The pellets were resuspended in buffer D, 0.3 mg digitonin was added, and incubated at 28°C for 30 min to release the total soluble protein content.

2.3. Enzymatic activities

Activities for cytoplasmic, mitochondrial and glycosomal markers were measured in the supernatants and pellets, together with FPPS activity, as described previously (Montalvetti, 2001), using the purified recombinant *T. cruzi* FPPS (TcFPPS) (Montalvetti, 2001) as a positive control. Measurements were performed in duplicates for each of three permeabilization

experiments. Hexokinase was assayed as described before (Caceres, 2003) with minor modifications (higher $MgCl_2$ concentration, and use of 0.1 M triethanolamine buffer). The reaction mixture contained 0.1 M triethanolamine pH 7.5, 3 mM $MgCl_2$, 0.72 mM $NADP^+$, 4 mM glucose, 1.5 mM ATP and 0.2 units of glucose-6-phosphate dehydrogenase in a total volume of 100 μl. The reaction was started by the addition of the mixture to the samples already distributed in a multi-well plate, and the absorbance was followed at 340 nm, and at 30°C, for several minutes. Phosphoenolpyruvate carboxykinase was measured in a carboxylation reaction as described (Urbina, 1987) using $NaHCO_3$ instead of potassium carbonate. Glucose-6-phosphate dehydrogenase was assayed (Heise, 1999) in a reaction mixture containing 100 mM triethanolamine pH 7.5, 0.5 mM $MgCl_2$, 0.5 mM $NADP^+$, and 5 mM glucose-6-phosphate. The reaction was followed by absorbance at 340 nm at 30°C. For the citrate synthase activity (Adroher, 1988, with modifications) the reaction mixture contained 100 mM Tris-HCl, pH 8.1, 0.1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 0.2 mM acetyl-CoA, and water to a final volume of 80 μl. The mixture was added to wells containing the samples and incubated for 3 min. To start the citrate synthase reaction 10 μl of 5 mM oxaloacetate were added, and the absorbance was measured at 412 nm, and at 25°C instead of 37°C.

2.4. Western blot analysis

Equal sample volumes (25 μl sample per lane) were loaded on a SDS/10% PAGE, run and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked overnight at 4°C in PBS-0.1% Tween-20-5% skim milk and probed with rabbit anti-TcFPPS antibody (Montalvetti, 2001; 1:2,000), rabbit anti-TbgGAPDH (Rayyan, 1993; 1:3,000), and with secondary rabbit HRP-conjugated antibody (GE Healthcare; 1:20,000), as described before (Ferella, 2006). Immunoblots were visualized on radiographic film using the ECL enhanced chemiluminiscence detection kit according to the instructions of the manufacturer (GE Healthcare).

2.5. Immunofluorescence microscopy

Exponential cultures were pelleted and washed twice in PBS and fixed in 4% paraformaldehyde, 0.1 M cacodylate buffer and 0.1 % glutaraldehyde in PBS, pH 7.2. Fixed cells were adhered to polylysine coated cover slides, permeabilized with Triton X-100 in PBS, neutralized in 50 mM NH_4Cl and blocked with 3% BSA in PBS. Incubation with primary and secondary antibodies in 3% BSA-PBS was done for one hour. The antibodies used were rabbit anti-TcFPPS (1:600) and goat anti-rabbit Alexa 488 (1:1000) or goat anti-rabbit Alexa 546 (1:800). For the co-localization studies, primary rabbit anti-BiP antibody was labelled using the Zenon rabbit IgG labelling kit Alexa 546 (Molecular Probes). Slides were incubated with the anti-TcFPPS and then incubated with the pre-labelled antibody. Glycosomes were detected using mouse monoclonal anti-TbPPDK (1:4). For mitochondrial staining Mitotracker (Molecular Probes) was used at a concentration of 0.02 μM and 10 min incubation of live cells. The cells were counterstained with DAPI (4′,6-diamidino-2-phenylindole) when mounted with ProLong Gold antifade reagent with DAPI (Molecular Probes). Fluorescent optical images were captured under non-saturating conditions and identical exposure times using an Olympus IX-71 inverted fluorescence microscope with a Photometrix CoolSnapHQ CCD (charge-coupled device) camera driven by DeltaVision software (Applied Precision).

3. Results

3.1 Permeabilization assays and western blot analysis

We found that the release of FPPS from both trypanosomes coincided with the release of the cytoplasmic marker, glucose-6-phosphate dehydrogenase (Figs. 1A and 1B). Incubation for 5 min with 0.05 mg of digitonin per mg of total cellular protein was sufficient to initiate the

release of cytoplasmic components, while amounts of digitonin higher than 0.05 and 0.1 mg/ml were necessary to release the mitochondrial citrate synthase and the glycosomal content, respectively. The use of more than 0.5 mg/ml of digitonin partially inhibited the hexokinase (Hk) and phosphoenolpyruvate carboxykinase (Pepck) activities from both parasites.

To confirm these results, we performed western blot analyses of the supernatants after digitonin treatment using antibodies against TcFPPS (Montalvetti, 2003) and *T. brucei* glycosomal glyceraldehyde-3-phosphate dehydrogenase (TbgGAPDH) (Rayyan, 1993) (Figs. 1A and 1B). The results were in agreement with the results of the enzymatic activities. TcFPPS and TbFPPS appear to be cytosolic and a glycosomal location can be excluded. We also performed western blot analysis of the digitonin fractions from *T. cruzi* using the anti-TcSPPS (Ferella, 2006), as the primary antibody (data not shown). The SPPS was only present in the pellets and was not released in the supernatants, suggesting a membrane association.

3.2. Immunofluorescence microscopy

To rule out a localization of TcFPPS and TbFPPS in the mitochondria, endoplasmic reticulum or glycosomes, we performed immunofluorescence microscopy studies of both, epimastigotes and procyclics trypomastigotes, using Mitotracker as a mitochondrial marker, and antibodies against BiP (Bangs, 1993) and PPDK (Bringaud, 1998), markers for the endoplasmic reticulum and glycosomes, respectively. No co-localization was detected with the glycosomal PPDK or in the mitochondrion, in neither epimastigotes (Fig. 2A) nor procyclic trypomastigotes (Fig. 2B). A weak labelling for FPPS was seen in the ER of both trypanosomes (Fig. 2A and B). The same pattern was observed for the bloodstream form in *T. brucei* (Supplementary Fig. 1).

4. Discussion

As pointed out in the introduction, the enzymes from the mevalonate pathway and particularly those of the isoprenoid biosynthetic route have been assigned to several different compartments in mammals and plants (20-23 Hugueney, 1996; Cunillera, 1997; Sanmiya, 1999; Biardi, 1996; Olivier, 2000; Hirooka, 2005; Leivar, 2005). Only a few enzymes of this pathway have been described in kinetoplastids (Montalvetti, 2001 and 2003; Pena-Diaz, 2004; Ferella, 2006; Urbina, 2002; Ortiz-Gomez, 2006), and have been located to the glycosome, mitochondria, and cytoplasm (Ferella, 2006; Pena-Diaz, 2004 and Ortiz-Gomez, 2006, respectively).

Although most of the genes are conserved among the three trypanosomatids, *T. cruzi*, *T. brucei* and *L. major*, and their metabolisms are similar, they still show some discrepancies.

Our results indicate that farnesyl diphosphate synthase, in both *T. cruzi* epimastigotes and *T. brucei* procyclic trypomastigotes, localizes mainly to the cytoplasm. This pattern can be assumed as well for the bloodstream forms of *T. brucei* as indicated by immunofluorescence microscopy analysis (Supplementary Fig. 1). This is in agreement with the cytosolic presence of FPPS in *L. major* (Ortiz-Gomez, 2006). And it can be extrapolated to the mammalian forms as seen by the immunofluorescence we performed over bloodstream T. brucei.

An internal SKL-like sequence, like those present in the C-terminus of other glycosomal proteins, was reported only in *T. cruzi* FPPS (Montalvetti et al., 2001) but not in *T. brucei* FPPS (Montalvetti et al., 2003). However this signal is not by itself a potential glycosomal targeting signal (Gatto, 2000 and 2003). The cytosolic localization described here, rules out a role for this putative signal in TcFPPS targeting, and confirms the conservation of the enzyme location throughout these three trypanosomes.

The localization of other enzymes of the isoprenoid pathway in other cell compartments, such as the TcSPPS present in the glycosome (Ferella, 2006), suggests that a mechanism must be available for the transport of the intermediates of this pathway between different cell compartments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Index descriptors and abbreviations

FPPS, farnesyl diphosphate synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; SPPS, solanesyl diphosphate synthase..

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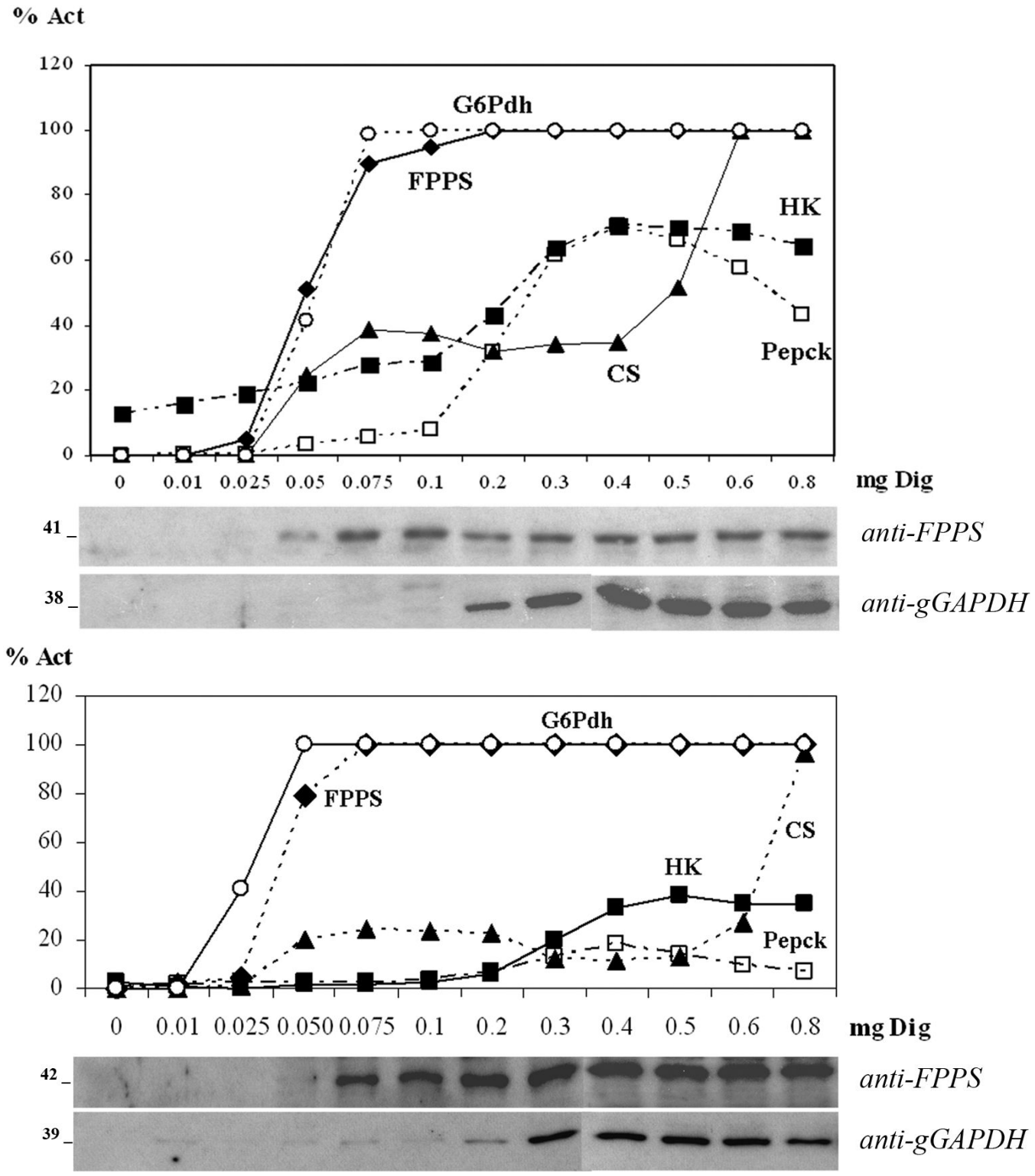
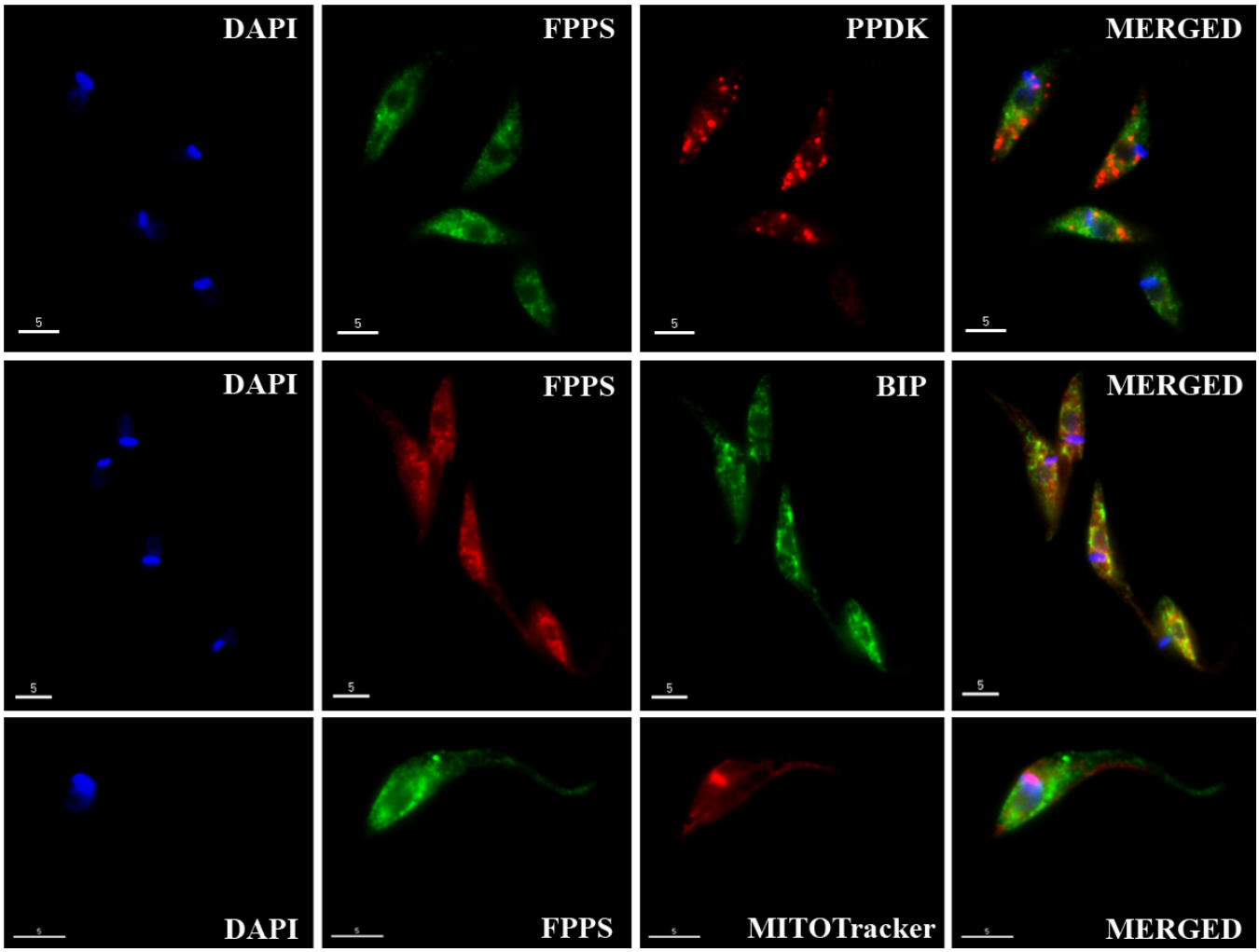


Fig. 1.

Digitonin permeabilization assays. The graphs show the percentage of total recovered activity in the supernatants for each amount of digitonin used. **A.** *T cruzi* epimastigotes. **B.** *T brucei*

procyclic trypomastigotes. Glucose-6-Phosphate dehydrogenase (G6PDH; ○); farnesyl diphosphate synthase (FPPS, ◆); citrate synthase (CS, ▲); hexokinase (Hk; □); phosphoenolpyruvate kinase (Pepck; ■). *Immunoblot analyses of supernatants* (25 μl sample per lane) are shown at the bottom of each figure. M_r is indicated at the left.



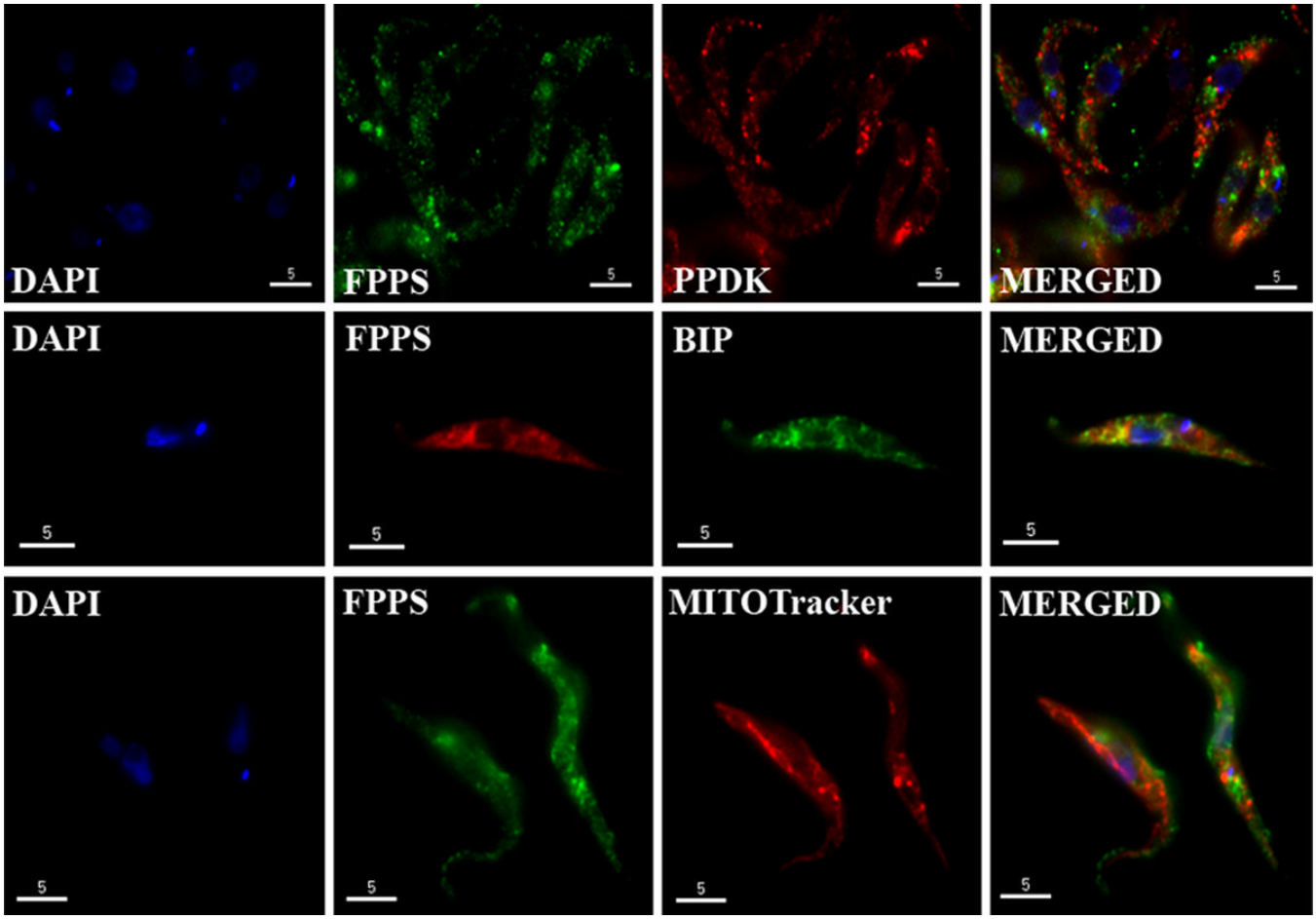


Fig. 2.

Immunofluorescence microscopy analysis. **A.** *T. cruzi* epimastigotes. **B.** *T. brucei* procyclic trypomastigotes. Colocalization of FPPS with: PPDK (glycosomal marker); BiP (endoplasmic reticulum marker) and Mitotracker (mitochondrion and kinetoplast stain). DAPI staining of the nuclei and kinetoplast is shown in blue. Bars = 5 μm.