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Involvement of SNARE protein Ykt6 in glycosome biogenesis in *Trypanosoma brucei*

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ABSTRACT

The kinetoplastid parasites *Trypanosoma* and *Leishmania* are etiologic agents of diseases like African sleeping sickness, Chagas and leishmaniasis that inflict many tropical and subtropical parts of the world. These parasites are distinctive in that they compartmentalize most of the usually cytosolic enzymes of the glycolytic pathway within a peroxisome-like organelle called the glycosome. Functional glycosomes are essential in both the procyclic and bloodstream forms of trypanosomatid parasites, and mislocalization of glycosomal enzymes to the cytosol is fatal for the parasite. The life cycle of these parasites is intimately linked to their efficient protein and vesicular trafficking machinery that helps them in immune evasion, host-pathogen interaction and organelle biogenesis and integrity. Soluble *N*-ethylmaleimide sensitive factor attachment protein receptor (SNARE) proteins play important roles in vesicular trafficking and mediate a wide range of protein–protein interactions in eukaryotes. We show here that the SNARE protein Ykt6 is necessary for glycosome biogenesis and function in *Trypanosoma brucei*. RNAi-mediated depletion of Ykt6 in both the procyclic and bloodstream forms of *T. brucei* leads to mislocalization of glycosomal matrix proteins to the cytosol, pronounced reduction in glycosome number, and cell death. GFP-tagged Ykt6 appears as punctate structures in the *T. brucei* cell and colocalizes in part to glycosomes. Our results constitute the first demonstration of a role for SNARE proteins in the biogenesis of peroxisomal organelles.

1. Introduction

Trypanosoma brucei is the causative parasitic agent of African trypanosomiasis, more commonly known as African sleeping sickness. According to World Health Organization estimates, about 70 million people in sub-Saharan Africa are directly at risk for the disease, but indirectly the disease affects several hundred million individuals [1]. Most of this vulnerable population lives in remote areas, with more than 250 discrete disease foci in 36 African countries that have limited access to healthcare and therefore to timely diagnosis and treatment [2]. *T. brucei* also infects domesticated and wild animals, which act as a reservoir for the parasite. In its overall infectivity, the parasite constitutes a severe economic and health burden for the rural population of sub-Saharan Africa [1].

Specifically, sleeping sickness is caused by two subspecies of *T. brucei*, *T. brucei rhodesiense* and *T. brucei gambiense*. The parasites multiply in the blood and tissue fluids of their human host and are transmitted by the bite of the Tsetse fly, balancing a complicated life cycle between mammalian and insect hosts. The parasite adapts its metabolism in order to proliferate in different compartments of its hosts. The bloodstream form (BSF) of *T. brucei* uses glycolysis to produce ATP in the mammalian bloodstream with its availability of high glucose, while the procyclic form (PCF) uses an incomplete or modified version of the mitochondrial TCA cycle to catabolize the amino acids proline and threonine available in the Tsetse fly midgut [3]. Metabolic adaptations such as these help trypanosomatid parasites to survive in varied environments.

Protozoan parasites like *T. brucei* and *Trypanosoma cruzi*, which causes American trypanosomiasis or Chagas disease, as well as the various species of *Leishmania* that cause different manifestations of leishmaniasis, such as Kala Azar, have in common an organelle called the glycosome [3]. The glycosome is a specialized member of the peroxisome family of organelles. Like other peroxisome organelles, glycosomes sequester enzymes for the β-oxidation of fatty acids, the biosynthesis of ether lipids and purine salvage [3,4], but unlike other peroxisomes, glycosomes are distinctive because they house most of the enzymes of the glycolytic pathway, which are normally localized to the cytosol in other organisms [3,5]. Previous studies have shown that

Abbreviations: BSF, bloodstream form; ER, endoplasmic reticulum; PCF, procyclic form; RNAi, RNA interference; SNARE, soluble NSF attachment protein receptor

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mislocalization of glycolytic enzymes to the cytosol has fatal consequences for the parasites [6–9].

Studies of peroxisome biogenesis in a variety of organisms, most notably different species of yeast, have identified 35 genes termed PEX genes that encode peroxins, which are proteins required for peroxisome biogenesis [4]. These proteins function in the assembly of the peroxisomal membrane, in the production of early peroxisome biogenic structures, soluble matrix protein import into the peroxisome, and control of peroxisome size and number [4]. Fifteen of these PEX genes function in peroxisome biogenesis in humans, while 12 trypanosomatid counterparts of these human PEX genes have been identified that are involved in glycosome biogenesis [3]. Although the later stages of import of soluble glycosomal matrix proteins are relatively well understood, knowledge of the early steps in the biogenesis of the glycosome is still relatively scant. As the sequences of peroxins are often poorly conserved between different organisms, it is often difficult to find peroxin homologues in distantly related organisms like trypanosomatids and mammals or yeasts. Of the peroxins that function in glycosome membrane assembly, only Pex16 [10] and Pex19 [11] have been identified. Other proteins functioning in this early event of glycosome assembly surely await discovery.

The Pex19 protein functions in peroxisomal membrane assembly by acting as a chaperone that transports proteins to, and facilitates their integration into, the peroxisomal membrane [4,12,13]. In most organisms, but notably not in trypanosomatids [11], Pex19 proteins contain a CAAX motif at or near their C-termini which allows for their prenylation and enhanced interaction with the peroxisomal membrane [12]. In this study, we focused on identifying proteins with CAAX or similar motifs in *T. brucei* with the aim of determining whether any one of these proteins might function in glycosome biogenesis. We show here that the CAAX motif-containing *T. brucei* homologue of the yeast SNARE (soluble NSF attachment protein receptor) protein Ykt6 involved in vesicular trafficking to and within the Golgi complex and to the vacuole (lysosome) functions in glycosome biogenesis. A fraction of *T. brucei* Ykt6 is found in association with glycosomes, and a reduction in Ykt6 protein levels leads to reduced numbers of glycosomes, mislocalization of glycosomal enzymes to the cytosol, and ultimately death of the parasites in either the procyclic or bloodstream form. Ykt6 is the first SNARE protein to be shown to have a role in the biogenesis of a member of the peroxisome family of organelles.

2. Materials and methods

2.1. Trypanosome culturing and transgenic lines

PCF *T. brucei* 29-13 [14] co-expressing T7 RNA polymerase and the tetracycline (Tet) repressor was maintained in SDM-79 medium (Invitrogen) containing 10% fetal bovine serum, 50 μg hygromycin/mL and 2.5 μg G-418/mL. Cultures were maintained at 25 °C with 5% CO_2 in a water-saturated incubator. Transgenic lines were generated by the limiting dilution method [15] and selection with 2.5 μg phleomycin/mL. For RNAi studies, procyclic forms were grown in glucose-free medium [7].

BSF *T. brucei* NR-42011 co-expressing T7 RNA polymerase and the Tet repressor [14] was maintained in HMI-9 medium containing 10% fetal bovine serum, 10% serum plus (Sigma-Aldrich) and 2.5 μg G-418/mL. Cultures were maintained at 37 °C with 5% CO_2 in a water-saturated incubator. Transgenic lines were obtained by the limiting dilution method and selection using 2.5 μg phleomycin/mL.

2.2. Cloning and plasmid constructs

The *T. brucei YKT6* gene (Tb927.9.14080) was amplified from genomic DNA using primers 5′-GCTCTAGAATGAAGCTTTACTCCCTGGCAATTG and 5′-GCGGGATCCTCACATGACGGTGCAACACCC. Sequence encoding GFP from *Aequoria victoria* was ligated in-frame and upstream of the *YKT6* gene, and the chimeric gene was cloned into vector pDEX577-C [16] for expression of GFP-Ykt6. An RNAi stem-loop construct for the *YKT6* gene was made by PCR amplification using primers 5′-GCGGGATCCGGCAAATACGACCTCGTTGATG and 5′-GGAATTCTGTAGGCACN$_{50}$CTTGGCTTGCTTGTAGAAGG. The PCR product, which has a randomized 3′-end containing an EcoRI site, was digested with EcoRI and ligated to form a stem-loop region. The ligated product was purified, digested with BamHI, and inserted into vector p2T7-177 [17] at the BamHI site.

2.3. Fluorescence microscopy

Images were acquired using an LSM510 AxioObserver.Z1 confocal microscope (Carl Zeiss) equipped with an oil immersion objective and ZEN 2009 acquisition software (Carl Zeiss). Image contrast was adjusted with Imaris software and processed in Adobe Photoshop.

For immunofluorescence microscopy, PCF or BSF cells were harvested by centrifugation, washed in PBS, pH 7.4, and spread onto coverslips coated with poly-L-lysine. Cells were fixed in PBS containing 4% paraformaldehyde for 10 min and permeabilized/blocked using 50 mM Tris-HCl, pH 7.5, 0.25% Triton X-100, 2% FBS for 30 min. Cells were washed with PBS, incubated with primary antibody in PBS containing 2% FBS for 2 h, washed with PBS, and incubated with fluorescein-conjugated secondary antibody (Alexa Fluor 488 anti-rabbit IgG or Alexa Fluor 568 anti-rabbit IgG) for 1 h. Cells were washed in PBS, pH 7.4, and mounted in 50% glycerol/0.4% *n*-propyl galate.

Lysosomes and endosomes were visualized by staining cells with 0.5 μM Lysotracker Red DND-99 (Thermo-Fisher) for 30 min to 1 h.

2.4. Subcellular fractionation and immunoblot analysis

PCF cells were pelleted by centrifugation and washed once with PBS, pH 7.4, and once with homogenization buffer (25 mM Tris-HCl, pH 8.0, 250 mM sucrose, 1 mM EDTA, 1 mM DTT, 1 × complete protease inhibitors (Roche)). Washed cells were disrupted in homogenization buffer containing silicon carbide (400 mesh, Sigma-Aldrich) and subjected to centrifugation at 1000 × *g* for 10 min at 4 °C to remove cell debris and unbroken cells. The postnuclear supernatant was loaded onto a 30-mL discontinuous sucrose gradient consisting of 25%, 50% and 60% sucrose steps and a 70% sucrose cushion (all sucrose solutions, w/v) and subjected to centrifugation at 216,000 × *g* for 90 min in a VTi50 rotor (Beckman) at 4 °C. Equivolume fractions were collected, and proteins were precipitated by addition of TCA.

Immunoblot analysis was done using standard protocols. Immunoreactive proteins were visualized with the SuperSignal West Femto Maximum Sensitivity Substrate chemiluminescence detection system (Thermo-Fisher).

2.5. Digitonin permeabilization

Digitonin permeabilization was performed essentially as described [18,19]. Specifically, PCF or BSF cells were pelleted by centrifugation and washed once in PBS, pH 7.4, and once in 25 mM Tris-HCl, pH 7.4, 250 mM sucrose, 150 mM NaCl, 1 mM EDTA (STES). Cells were resuspended in STES, and aliquots of cells corresponding to 25 μg of protein were mixed with an equal volume of STES containing increasing concentrations of digitonin to yield final amounts of 0.008–1.6 mg digitonin/mg protein, as indicated in Fig. 7, and incubated at room temperature for 4 min and on ice for 5 min. The mixture was subjected to centrifugation at 13,000 × *g* at 4 °C for 2 min, and proteins were precipitated from the supernatant by addition of TCA and analyzed by immunoblotting with antibodies to aldolase, GAPDH and α-tubulin.

2.6. RNAi analysis and semi-quantitative RT-PCR analysis

RNA interference (RNAi) was induced in PCF cells by addition of tetracycline to 2 μg/mL in glucose-free medium essentially as described