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Identification and functional characterization of *Trypanosoma brucei* peroxin 16

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ABSTRACT

Protozoan parasites of the family Trypanosomatidae infect humans as well as livestock causing devastating diseases like sleeping sickness, Chagas disease, and Leishmaniasis. These parasites compartmentalize glycolytic enzymes within unique organelles, the glycosomes. Glycosomes represent a subclass of peroxisomes and they are essential for the parasite survival. Hence, disruption of glycosome biogenesis is an attractive drug target for these Neglected Tropical Diseases (NTDs). Peroxin 16 (PEX16) plays an essential role in peroxisomal membrane protein targeting and de novo biogenesis of peroxisomes from endoplasmic reticulum (ER). We identified trypanosomal PEX16 based on specific sequence characteristics and demonstrate that it is an integral glycosomal membrane protein of procyclic and bloodstream form trypanosomes. RNAi mediated partial knockdown of *Trypanosoma brucei* PEX16 in bloodstream form trypanosomes led to severe ATP depletion, motility defects and cell death. Microscopic and biochemical analysis revealed drastic reduction in glycosome number and mislocalization of the glycosomal matrix enzymes to the cytosol. Asymmetry of the localization of the remaining glycosomes was observed with a severe depletion in the posterior part. The results demonstrate that trypanosomal PEX16 is essential for glycosome biogenesis and thereby, provides a potential drug target for sleeping sickness and related diseases.

1. Introduction

Neglected Tropical Diseases (NTDs) comprise seventeen infectious parasitic diseases, affecting more than 1 billion people worldwide, mostly in developing countries [1,2]. Currently, more than 10 million people are infected with protozoan parasites of the family Trypanosomatidae [3,4]. African sleeping sickness is caused by *Trypanosoma brucei*, which is transmitted by the tsetse fly. Triatomine bugs transmit *T. cruzi*, the infectious parasite causing the American trypanosomiasis (Chagas disease). Leishmaniasis is caused by *Leishmania* species that are transmitted by sandflies. Without treatment, African sleeping sickness is fatal, with progressive mental deterioration leading to coma, systemic organ failure, and death [5]. The currently used drugs have several limitations such as toxicity, adverse side effects, difficult to administer. NTDs mainly affect countries, which only can provide limited resources for the development of new therapies. Hence, there is an urgent need for identification of novel drug targets and development of new affordable drugs against these diseases [6].

Trypanosomatid parasites harbor glycosomes, unique organelles that compartmentalize glycolytic enzymes and other metabolic pathways, which normally occur in the cytosol in other organisms [7]. This unique compartmentation is essential for the parasites, mislocalization of the glycosomal enzymes to the cytosol kills the parasite [8–10]. Glycosomes belong to the family of peroxisomes, sharing the same principles of biogenesis [11]. Proteins required for the biogenesis of these organelles are collectively called peroxins. Until now, thirty-three yeast peroxins and fifteen human and plant peroxins have been identified [12,13] but only 10 trypanosomatid counterparts have been discovered till date [14]. Since trypanosomatids diverged very early from other eukaryotes during evolution [15], the level of sequence similarity is low, making it difficult to identify orthologous peroxins of trypanosomatids by bioinformatic approaches.

Most of the few known peroxins of trypanosomatids are involved in glycosomal matrix protein import, which is reasonably well characterized [16]. However, our knowledge on glycosome membrane biogenesis is still scarce. In other organisms, peroxisome membrane biogenesis requires three peroxins, PEX19, PEX3, and PEX16 [17]. So far, only trypanosomatid PEX19 has been identified, while the corresponding PEX3 and PEX16 homologs are still unknown [18]. PEX19 is the cytosolic receptor for newly synthesized peroxisomal membrane proteins. The integral membrane protein PEX3 acts as anchor for PEX19 [19]. PEX16 is present in mammals

Abbreviations: PEX, peroxin; PTS, peroxisomal targeting signal; DAPI, 4′,6′-diamidino-2-phenylindole; GAPDH, glycosomal glyceraldehyde-3 phosphate dehydrogenase; PFK, phosphofructokinase; DIC, differential interference contrast; *Tb*PEX16, *Trypanosoma brucei* PEX16

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and plants and in some yeasts like *Yarrowia lipolytica*, though in *Saccharomyces cerevisiae* it seems to be absent. The functional role of PEX16 still remains elusive. Evidence has been provided suggesting a role of human PEX16 for proper targeting of PEX3 to the peroxisomes [20]. Mutation in peroxins required for membrane formation leads to complete loss of peroxisomes. In humans, such mutations are responsible for inherited severe metabolic disorders, like the Zellweger syndrome [21]. Since glycosomes are essential for trypanosomatid parasites, the parasite peroxins might provide suitable novel drug targets, especially as the degree of conservation between the parasite human proteins is low.

Here we identified and functionally characterized PEX16 of *T. brucei*. We show that *Tb*PEX16 is a glycosomal membrane protein. PEX16 specific RNAi, which results in a partial knockdown of PEX16, kills the bloodstream form of the parasites in culture. Reduced *Tb*PEX16 expression directly affects glycosomes leading to drastic reduction in their number and mislocalization of glycosomal matrix proteins to the cytosol. The remaining glycosomes were asymmetrically distributed in the cell, most localized in the anterior part. The glycosome biogenesis defect leads to a mislocalization of glycolytic enzymes, depletion in ATP levels, motility defects, and cell death.

2. Materials and methods

2.1. Trypanosome strains, growth conditions, and transfection

Bloodstream and procyclic form cell line 449 (*T. brucei* 427 strain stably transfected with pHD449 plasmid, thus stably expressing Tet repressor) were used in this study [22]. The bloodstream form was grown in HMI-11 medium containing 10% fetal bovine serum (FBS, Sigma) at 37 °C in humidified incubator with 5% CO2 [23]. The procyclic form was grown in SDM-79 medium supplemented with 15% FBS at 28 °C in humidified 5% CO_2 incubator [24]. Bloodstream form cells were maintained in logarithmic phase (below 2×10^6 cells/ml) and procyclic form cells were maintained at 5×10^5–5×10^8 cells/ml.

2.2. Bioinformatics

BLAST searches were performed against trypanosomatid genomes at tritrypdb.org. Pfam domain search was done at http://pfam.xfam.org. Multiple sequence alignment was generated using the Clustal Omega online server and FASTA aligned sequences were visualized using JALVIEW with Clustalx color code. Amino acid percentage identity and similarity matrix were generated using MatGAT2.01 with BLOSUM62 scores. Prediction of transmembrane domains and topology was done using Phobius prediction software (http://phobius.sbc.su.se).

2.3. Cloning

2.3.1. Primers are listed in Supplementary Table S1

Genomic DNA of *T. brucei* 449 cell line or *Leishmania major* was used as template for PCR amplifications of desired genes using peqGOLD *Pwo*-DNA-Polymerase (Peqlab).

*Tb*PEX16 was C-terminally fused with protein-A tag by cloning the blunt PCR product of RE3135–RE3651 in HpaI digested pHD918 (kindly donated by Dr. F. Voncken, The University of Hull). N- or C-terminally GFP tagged *Tb*PEX16 was generated by cloning BamHI digested PCR products of RE3135–RE3130 and RE3135–RE3131 into BamHI site in trypanosome specific expression vectors pGC1 and pGN1 derived from plasmid pHD1336 [25], respectively. LmPEX16 was C-terminally GFP-tagged by cloning of the HindIII–BglII double digested PCR product of RE3144–RE3145 into HindIII–BamHI sites of pGN1. Stem loop construct for *Tb*PEX16 RNAi was generated by cloning the 550 bp fragment (RE3285–RE3286, digested with HindIII–NcoI) and the complementary 500 bp fragment (RE3287–RE3288, digested with NcoI–BamHI) in tandem but opposite orientation in HindIII–BamHI Site in pHD677 [22]. For generation of fluorescent glycosomal marker PTS2-GFP, complementary oligonucleotides RE3474 and RE3475 (coding for first 15 amino acids of aldolase including the PTS2 signal), containing HindIII and BamHI overhangs were annealed and ligated into HindIII–BamHI site in pGN1.

2.4. Trypanosome stable transfection

All transfections were genomic integrations of NotI linearized plasmids and stably transfected clones were selected by limiting dilution. Procyclic trypanosome transfection was performed as described in [26]. Bloodstream form trypanosomes were transfected using Human T-Cell nucleofector Kit (Lonza), the Amaxa Nucleofector II device and program X-001. 30 million Log phase cells were used for each transfection. The transformation mixture was transferred to 30 ml HMI11 medium (see Section 2.1) and serially diluted 1:10 and 1:100 to a final volume of 30 ml. 1 ml aliquots of the cultures were transferred to three 24 well plates. Antibiotics were added after incubation for 8 h or overnight (5 μg/ml blasticidin from InvivoGen for pGC1, pGN1 and pHD1336 constructs; 5 μg/ml hygromycin from Invitrogen for pHD677 and pHD918 constructs). Transformed cells were monitored 5–6 days after transfection. The clones were induced with tetracycline (1 μg/ml) and positive clones were selected and further cultivated. Glycerol stocks of positive clones were stored at −80 °C in appropriate medium containing 12% glycerol.

2.5. RNAi, RT-PCR, and qRT-PCR

Double stranded (stem-loop) RNA was inducibly expressed from genomically integrated pHD677 construct described in Section 2.3, bearing a tetracycline regulated trypanosome-specific promoter. RNAi was induced by addition of tetracycline (1 μg/ml) to the cultures of density 2×10^5 cells/ml. Cells were diluted back to 2×10^5 every 24 h and fresh tetracycline was added. The growth of uninduced and RNAi-induced cultures was monitored up to 8 days. RNA was isolated from day-3 and day-7 cultures using RNAeasy Mini Kit (Qiagen), transcribed to cDNA using Oligo$(dT)_{18}$ primers with RevertAid First Strand cDNA Synthesis Kit (Thermo scientific). Quantitative Realtime PCR was performed using MESA GREEN qPCR masterMix Plus on MJ Research DNA Engine MJ Research DNA Engine Opticon thermal cycler. RT-PCR primers for tubulin are described in [27].

2.6. Carbonate extraction

Carbonate extraction was essentially performed as described by Lorenz et al. [28], with the following modifications. 10×10^7 bloodstream form cells of Protein A- or *Tb*PEX16-Protein A-expressing cell line 449, induced with 0.1 μg/ml tetracycline for 24 h, were used. Extracts were directly denatured with Laemmli buffer and subjected to SDS-PAGE. PEX11 (1:5000) and GIM5 (1:5000) antisera were used as integral membrane protein marker, enolase (1:75,000) as cytosolic marker and aldolase (1:75,000) as glycosomal marker [29].

2.7. Microscopy

Trypanosomes were sedimented by centrifugation and resuspended in 4% paraformaldehyde in PBS (supplemented with 250 mM sucrose in case of RNAi experiments) and incubated on rotatory wheel for 20 min. Fixed trypanosomes were washed two times with PBS and immobilized on poly-L-lysine coated wells. Aldolase (1:1500) and GAPDH (1:5000) antibodies were used as glycosomal markers. PEX11 and GIM5 antibodies (1:200) were used as glycosomal membrane markers. Goat anti-rabbit Alexa 594 was used as secondary antibody. Nuclear and kinetoplast DNA were stained with DAPI. Co-staining in Fig. 4C was done sequentially, first with PEX11 and Alexa 488 (green) as secondary antibody, thoroughly washed with PBS supplemented with 250 mM sucrose (5 min, 6 times) followed by staining with aldolase and Alexa 594 (red) as secondary antibody. Since both PEX11 and aldolase antibodies were raised in rabbit,

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