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Localization of a Trypanosome Peroxin to the Endoplasmic Reticulum

Sarah T. Bauer[a], Kelley E. McQueeney[a,b], Terral Patel[a] & Meredith T. Morris[a]

a Department of Genetics and Biochemistry, Eukaryotic Pathogens Innovation Center, Clemson University, Clemson, South Carolina 29634
b Department of Pharmacology, University of Virginia, 409 Lane Road, Charlottesville, Virginia 22908

ABSTRACT

Trypanosoma brucei is the causative agent of diseases that affect 30,000–50,000 people annually. *Trypanosoma brucei* harbors unique organelles named glycosomes that are essential to parasite survival, which requires growth under fluctuating environmental conditions. The mechanisms that govern the biogenesis of these organelles are poorly understood. Glycosomes are evolutionarily related to peroxisomes, which can proliferate de novo from the endoplasmic reticulum or through the growth and division of existing organelles depending on the organism and environmental conditions. The effect of environment on glycosome biogenesis is unknown. Here, we demonstrate that the glycosome membrane protein, TbPex13.1, is localized to glycosomes when cells are cultured under high glucose conditions and to the endoplasmic reticulum in low glucose conditions. This localization in low glucose was dependent on the presence of a C-terminal tripeptide sequence. Our findings suggest that glycosome biogenesis is influenced by extracellular glucose levels and adds to the growing body of evidence that de novo glycosome biogenesis occurs in trypanosomes. Because the movement of peroxisomal membrane proteins is a hallmark of ER-dependent peroxisome biogenesis, TbPex13.1 may be a useful marker for the study such processes in trypanosomes.

TRYPANOSOMA BRUCEI is a flagellated protozoan parasite that causes human African trypanosomiasis (HAT) and a wasting disease in cattle called nagana (Stuart et al. 2008). Parasite infection is lethal without treatment and current therapies are toxic and difficult to administer, making the search for new drug targets critical.

Trypanosomes have essential single-membrane bounded organelles called glycosomes that have been identified as good drug candidates (Michels et al. 2006; Szoor et al. 2014). Glycosomes lack DNA, import proteins posttranslationally, and are evolutionarily related to peroxisomes found in higher eukaryotes as evidenced by the conservation in protein import machinery. The mechanisms of glycosome biogenesis are not fully understood, although comparison of components required for peroxisome biosynthesis has been useful in resolving potential contributing proteins (Galland and Michels 2010; Kalel et al. 2015). For example, homologs of 10 of the more than 58 described eukaryotic peroxin (Pex) proteins have been identified in *T. brucei*. These proteins, which regulate peroxisome biogenesis in yeast, mammals, and plants are essential to parasites grown under standard conditions (Banerjee et al. 2005; Brennand et al. 2012; Furuya et al. 2002; Galland et al. 2007; Guerra-Giraldez et al. 2002; Kalel et al. 2015; Krazy and Michels 2006; Maier et al. 2001; Verplaetse et al. 2009, 2012; Voncken et al. 2003).

Peroxisomes are ubiquitous organelles found in most eukaryotic organisms (Smith and Aitchison 2013). They are functionally diverse and their composition varies with cell type and environment. Reflecting this, a number of different metabolic pathway components are contained within peroxisomes including enzymes involved in fatty acid catabolism, the pentose phosphate pathway, and reduction of reactive oxygen species. Glycosomes harbor similar pathways, but the trypanosome uniquely compartmentalizes most of glycolysis in the organelle as well (Szoor et al. 2014).

The processes that regulate peroxisome biogenesis and maintenance have been studied in yeast, plants, and mammals and include organelle proliferation,

posttranslational protein import, and organelle specific degradation via pexophagy (Smith and Aitchison 2009). Protein import has been relatively well studied in trypanosomes (Galland and Michels 2010) and glycosome turnover via organelle-specific autophagy, pexophagy, has also been documented (Brennand et al. 2011; Duszenko et al. 2011). In contrast, very little is known about glycosome biogenesis.

In higher eukaryotes, peroxisome proliferation occurs via the growth and division of existing organelles as well as de novo biogenesis via the endoplasmic reticulum (ER) (Nagotu et al. 2010; Smith and Aitchison 2013; Tabak et al. 2013). In the growth and division of existing organelles, peroxisomes grow through the incorporation of lipids from the ER followed by constriction and eventual fusion of the peroxisome membrane. ER-dependent peroxisome biogenesis involves preperoxisomal vesicles (PPV) that bud from the ER and mature via sequential protein import of peroxisomal membrane and matrix proteins or fusion with other PPVs. The degree to which each biogenesis mechanism occurs in wild-type cells is debated but likely differs with organism, cell type, and environment.

To date, ER-dependent glycosome proliferation has not been directly demonstrated in trypanosomes. However, recent proteomic studies suggest that one glycosome biogenesis protein, TbPex13.1, is present in vesicles also containing ER proteins (Guther et al. 2014). In other eukaryotes, Pex13 forms part of the membrane channel that facilitates posttranslational import of peroxisomal matrix proteins (Williams and Distel 2006). In addition to its role at the peroxisome membrane, this protein is also found in the ER (Geuze et al. 2003). It has been proposed that ER-localized Pex13 may play a role in ER-dependent peroxisomal biogenesis through mechanisms that are unclear.

While higher eukaryotes harbor a single *Pex13*, *T. brucei,* has two *Pex13* homologs, *TbPex13.1* and *13.2* (Brennand et al. 2012; Verplaetse et al. 2009, 2012). TbPex13.1 has a unique C-terminal peroxisomal targeting sequence type 1 (PTS1) sequence that is typically found on peroxisome matrix proteins. This sequence is usually recognized by the soluble receptor Pex5, which delivers the cargo to the peroxisome membrane for matrix localization. Peroxisomal membrane proteins (PMPs), however, are usually delivered to the peroxisome by the cytosolic chaperone Pex19 (Kim and Hettema 2015). Because TbPex13.1 harbors a putative Pex19-binding domain and a PTS1, the targeting of the protein is enigmatic. These two Pex13s are also found in other kinetoplastids including *Trypanosoma cruzi*, *Leishmania*, and *Crithidia*.

Trypanosoma brucei experiences fluctuating extracellular glucose levels during the lifecycle as it alternates between the mammalian bloodstream and the tsetse fly vector (Stuart et al. 2008; Vickerman et al. 1988). In the mammalian host, bloodstream form (BSF) parasites are exposed to relatively high glucose levels (~ 5 mM), while the insect or procyclic form (PCF) of the parasite spends most of its time in glucose-poor conditions. While the

parasites are exposed to changing nutrient availability in their natural environment, both BSF and PCF parasites are routinely cultured in media containing 5 mM glucose. In other eukaryotes, peroxisome composition and biogenesis are influenced by environmental conditions such as nutrient availability (Pieuchot and Jedd 2012). In trypanosomes, glycosome metabolism and the requirement for glycosomal protein import vary with extracellular glucose levels (Bringaud et al. 2006; Furuya et al. 2002) and compartmentalization of glycolytic enzymes is essential at high extracellular glucose levels (Furuya et al. 2002; Haanstra et al. 2008; Lamour et al. 2005). Here, we describe the localization of the glycosome membrane protein, TbPex13.1, in PCF parasites grown under low glucose conditions.

MATERIALS AND METHODS

Generation and transfection of epitope-tagged TbPex13.1 and epitope-tagged truncation variants

The DNA sequence encoding an HA epitope tag (YPYDVP-DYA) was fused to the 5′ end of different *TbPex13.1* variants, which included of the full-length open-reading frame of *TbPex13.1* (*HATbPEX13.1*), the *TbPex13.1* gene in which the nucleotides (973–1,150) encoding amino acids 325–384 of the SH3 domain were deleted (*HATbPex13.1ΔSH3*), and the *TbPex13.1* gene in which the nucleotides encoding the C-terminal tripeptide SKL were deleted (*HATbPex13.1ΔPTS*). These fusions were cloned into the vector pXS2 (Bangs et al. 1996), which integrates into the tubulin locus and directs constitutive expression by the procyclic acidic repetitive protein (PARP) promoter. After confirmation by sequencing, plasmids were linearized with *Mlu*1 and *T. brucei,* and *brucei* procyclic form (PCF) 29-13s (Wirtz and Clayton 1995) was transfected as previously described (Beverley and Clayton 1993) using a Bio-Rad Gene Pulser Xcell (Exponential, 1,500 V, 25 μF, 4 mm cuvette). Stably transfected cells were selected by culturing in media containing 15 μg/ml blasticidin.

Growth of parasites

PCF 29-13 cells were grown in SDM79 (5 mM glucose) or SDM80 (< 0.5 mM glucose, 5.3 mM proline) as previously described (Lamour et al. 2005). PCF 29-13s expressing HATbPex13.1, HATbPex13.1ΔSH3, or HATbPex13.1ΔPTS1 were maintained in either SDM79 or SDM80 containing hygromycin (50 μg/ml), G418 (15 μg/ml), and blasticidin (15 μg/ml).

Glycosome isolation and western blot analysis

Glycosomes were isolated via density centrifugation of glycosome-rich postnuclear fractions over an Optiprep gradient (Sigma Aldrich, St. Louis, MO) as described previously (Colasante et al. 2006). Fractions (1 ml) were collected from the bottom of the gradient, protein concentration determined by Bradford assay (Pierce, Life

Technologies, Grand Island, NY), and samples processed by acetone precipitation. Briefly, four volumes of ice-cold acetone was added to each fraction, vortexed, and incubated at −20 °C for 1 h. Afterwards, fractions were centrifuged at 15,000 g for 10 min, the supernatant decanted, 2.5 μg of each fraction resolved by SDS-PAGE, and probed with antibodies against the glycosome proteins aldolase (1:20,000), hexokinase (1:100,000), and TbPex13.1 (1:10,000) which were provided by Dr. Paul Michels (de Duve Institute and Universite Catholique de Louvain, Brussels, Belgium)(Verplaetse et al. 2009), TbPex11 (1:4,000) provided by Dr. Christine Clayton (Zentrum für Molekulare Biologie der Universität Heidelberg, Germany) (Lorenz et al. 1998), and TbBiP (1:100,000) provided by Dr. Jay Bangs (University at Buffalo, Buffalo, NY) (Bangs et al. 1996).

Immunofluorescence microscopy

Immunofluorescence assay (IFA) conditions were modified from that previously described by (Field et al. 2004). Cells were harvested at 800 g for 5 min and resuspended in fresh media. Paraformaldehyde (1%) was added to cells and incubated on ice for 5 min. Cells were then washed in ice cold Voorheis's modified PBS (vPBS;10 mM glucose, 46 mM sucrose, 3 mM NaCl, 3 mM KCl, 3 mM KH_2PO_4, 16 mM Na_2HPO_4, 137 mM NaCl, pH 7.6), incubated on poly-L-lysine slides for 20 min, and permeabilized with 0.1% Triton X-100 in PBS. Slides were washed with vPBS and block (0.25% Tween in 1X vPBS) applied. Primary mouse anti-HA (H9658) (Sigma-Aldrich) was used to determine the localization of HATbPex13.1 variants. Rabbit anti-aldolase and rabbit anti-BiP were used as glycosomal and ER markers, respectively. Alexa Fluor 488-conjugated goat anti-rabbit and Alexa Fluor 568-conjugated goat anti-mouse antibodies (Life Technologies) were used to detect primary antibodies. Slides were mounted with vectashield containing DAPI (Vector Laboratories, Youngstown, OH) and visualized on a Zeiss Axiovert 200M using AxioVision software version 4.8.2 for image analysis.

RESULTS

TbPex13.1 localizes extraglycosomally in low glucose conditions

Glycosomes and other organelles such as ER and mitochondria can be isolated via density centrifugation (Colasante et al. 2006). We utilized this protocol to examine glycosome composition under high and low glucose conditions. In agreement with published data (Brennand et al. 2012; Krazy and Michels 2006; Verplaetse et al. 2009), the glycosome matrix proteins aldolase and hexokinase and the peroxisome membrane proteins TbPex11 and TbPex13.1 were all detected in fractions 14–22 when cells were grown in high glucose media (Fig. 1A, left panel). Interestingly, when cells were grown in low glucose media, TbPex13.1 was no longer detected in fractions harboring other glycosome-resident proteins (Fig. 1A, right panel). Instead, the protein was found in higher fractions (26–30), which also contained the ER resident protein BiP.

TbPex13.1 has a unique domain structure

In contrast to higher eukaryotes that have a single *Pex13* gene, *T. brucei* has two *Pex13* genes, *TbPex13.1* and *TbPex13.2* (Brennand et al. 2012). Both are expressed and silencing of either gene slows parasite growth and results in glycosome protein import defects. It is unknown why trypanosomes have two *Pex13*s while higher eukaryotes have only one. One unique aspect of the TbPex13.1 predicted protein sequence is the presence of a C-terminal peroxisome targeting sequence 1 (PTS1) (Fig. 1B). PTS1 sequences target soluble matrix proteins to peroxisomes and glycosomes through binding with the soluble receptor protein Pex5. This mechanism of targeting is distinct from that of peroxisomal membrane proteins such as Pex13 that have two TM domains and a chaperone binding site. These peroxisome membrane proteins (PMPs) are delivered to the peroxisome membrane through the action of the soluble chaperone, Pex19 (Kim and Hettema 2015). Because TbPex13.1 harbors a putative TbPex19 binding sequence, we predict it to traffic to glycosomes in a TbPex19-dependent process. Therefore, the presence of a PTS1-like sequence is intriguing and its function is unclear.

Glycosome and ER morphology is maintained in low glucose conditions

We used indirect immunofluorescence assays to determine if ER or glycosome morphology was altered in low glucose conditions. Cells grown in high glucose or low glucose media were fixed and stained with antibodies against the ER marker, BiP, or the glycosome marker protein, aldolase. Under both conditions, aldolase staining was observed in punctate structures that were distributed throughout the cells (Fig. 2A) as is typically observed with *T. brucei* glycosomes (Verplaetse et al. 2009). Additionally, BiP localization did not change with extracellular glucose levels. In both high and low conditions, BiP antibodies labeled tubular structures (Fig. 2B) consistent with ER (Bangs et al. 1993).

HATbPex13.1 localizes to glycosomes when cells are grown in high glucose conditions

Because our TbPex13.1 antibodies do not work under immunofluorescence assay (IFA) conditions, we fused an HA epitope tag to the N-terminus of TbPex13.1 and followed the cellular localization of HATbPex13.1 in high and low glucose. In agreement with previous reports from cells grown in high glucose (Verplaetse et al. 2009) and our fractionation experiments (Fig. 1A), HATbPex13.1 localized to punctate structures distributed throughout the cytoplasm in a pattern that largely overlapped with the glycosome-resident protein aldolase (Fig. 3A). Cells expressing HATbPex13.1 were also stained with antibodies against the ER protein BiP, which labeled reticular structures typical of ER

A
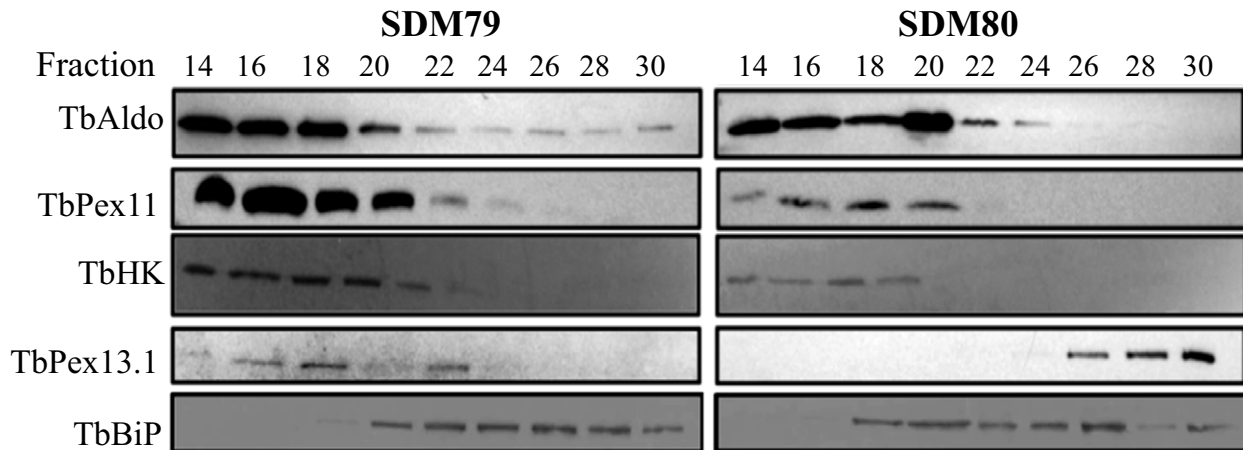
B
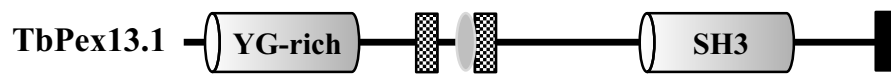
Figure 1 TbPex13.1 localizes with ER marker proteins in density gradient and has multiple domains. (**A**) Procyclic form parasites were grown in high glucose SDM79 (5 mM glucose) or low glucose SDM80 (< 0.5 mM glucose). Postnuclear supernatant was resolved by density centrifugation over an Optiprep gradient. One milliliter fractions were collected from the bottom of the gradient, and 2.5 µg protein from each fraction was resolved by SDS-PAGE and assayed by western blotting with antibodies that recognize, aldolase (TbAldo), TbPEX11, hexokinase (TbHK), and TbPex13.1 as well as the ER resident protein TbBiP. (**B**) TbPex13.1 has multiple structural domains. TbPex13.1 contains an YG-rich region, two transmembrane domains (hatched rectangles), a Pex19 binding sequence (gray oval), an SH3 domain and a PTS1 sequence (black rectangle).

staining (Fig. 3B). Under these high glucose conditions, HATbPex13.1 staining exhibits more overlap with glycosomal aldolase than with ER-localized BiP.

Cells expressing HATbPex13.1 have altered glycosome and ER morphology under low glucose conditions

When cells were grown in low glucose media, HATbPex13.1 staining was no longer found in punctate structures distributed evenly throughout the cells but rather was detected in larger foci concentrated within discrete regions of the cell (Fig. 4). To assess glycosome morphology, we stained these cells with antibodies against aldolase. Interestingly, aldolase staining was also altered in these cells under low glucose conditions and exhibited a staining pattern very similar to that observed with anti-HA antibodies (Fig. 4A). Because Pex13s localize to the ER in other systems, we assessed BiP localization in these cells. Like aldolase and HATbPex13.1, BiP staining exhibited glucose-dependent localization. In low glucose conditions, BiP staining was no longer reticular but contained within discrete areas of the cell that labeled with antibodies that recognize HATbPex13.1 and aldolase (Fig. 4B).

Deletion of the SH3 domain does not alter localization

SH3 domains mediate protein–protein interactions. To assess the contribution of this domain to the glucose-dependent localization of HATbPex13.1, we performed

IFA on cells expressing the HATbPex13.1 in which the SH3 domain was deleted (HATbPex13.1ΔSH3). Staining of the HATbPex13.1ΔSH3 variant was the same as observed with the HATbPex13.1 (Fig. 4, 5). In high glucose, HATbPex13.1ΔSH3 was detected in glycosomes and the anti-HA staining overlapped to a greater extent with aldolase than with the ER marker BiP (Fig. 5A). In low glucose, however, HATbPex13.1ΔSH3 staining was restricted to foci that also stained with aldolase and BiP (Fig. 5B).

The PTS1 domain is necessary for ER localization of HATbPex13.1 in low glucose conditions

TbPex13.1 is unique in that it harbors a PTS1 sequence that is not present in Pex13 sequences of other eukaryotes. To assess its role in the localization of HATbPex13.1, we performed IFA on cells in which the PTS1 was deleted (HATbPex13.1ΔPTS1). In contrast to HATbPex13.1 and HATbPex13.1ΔSH3, HATbPex13.1ΔPTS1 localized to glycosomes under both high and low glucose conditions (Fig. 6). Under both conditions, anti-HA antibodies labeled punctate structures containing aldolase and did not label reticular structures recognized by anti-BiP antibodies. In all cases, HA staining overlapped more with aldolase than BiP.

DISCUSSION

Glycosomes are highly specialized peroxisomes found only in kinetoplastid parasites (Michels et al. 2005, 2006;
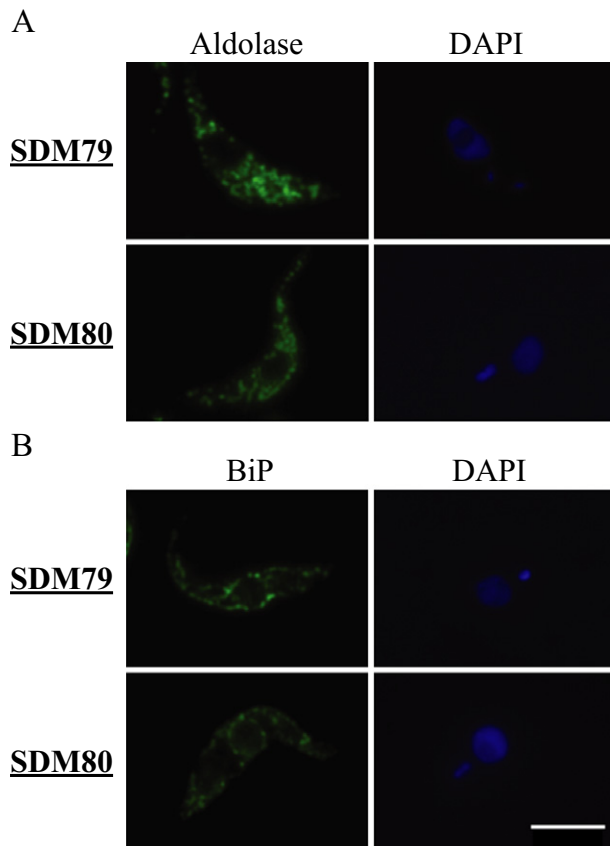
Figure 2 Glycosome and ER morphology is maintained in low glucose conditions. (**A**) Cells were grown in SDM79 or SDM80, fixed, permeabilized, and stained with antibodies against the glycosome protein, aldolase. (**B**) Cells were grown in SDM79 or SDM80, fixed, permeabilized, and stained with antibodies against the ER protein, BiP.
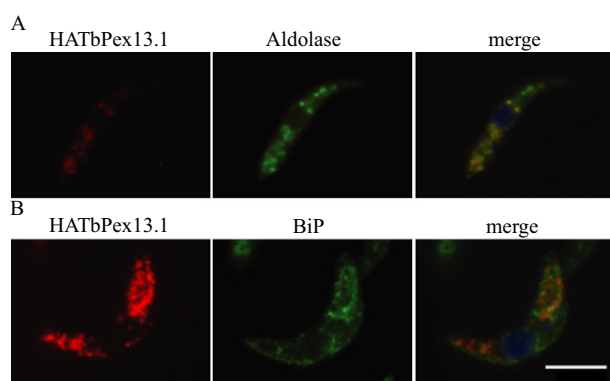
Figure 3 HATbPex13.1 localizes to glycosomes in SDM79 glucose rich media. Procyclic form parasites expressing HATbPex13.1 were, fixed, permeabilized, and stained with mouse anti-HA antibodies, which were detected with Alexa Fluor 568-conjugated goat anti-mouse antibodies and rabbit anti-aldolase or rabbit anti-BiP which were detected with Alexa Fluor 488-conjugated goat anti-rabbit antibodies. (**A**) Cells labeled with HATbPex13.1 (red) and aldolase (green). (**B**) Cells labeled with HATbPex13.1 (red) and BiP (green).
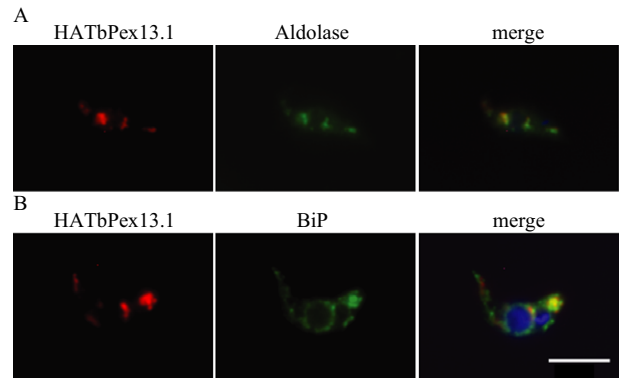

Figure 4 HATbPex13.1 cells have altered glycosome and ER morphology when grown in SDM80 low glucose media. (**A**) HATbPex13.1 cells were fixed, permeabilized, and labeled with mouse anti-HA antibodies detected with Alexa Fluor 568-conjugated goat anti-mouse and rabbit anti-aldolase antibodies detected with Alexa Fluor 488-conjugated goat anti-rabbit antibodies. (**B**) HATbPex13.1 cells were fixed, permeabilized, and labeled with mouse anti-HA antibodies detected with Alexa Fluor 568-conjugated goat anti-mouse and rabbit anti-BiP antibodies detected with Alexa Fluor 488-conjugated goat anti-rabbit antibodies.
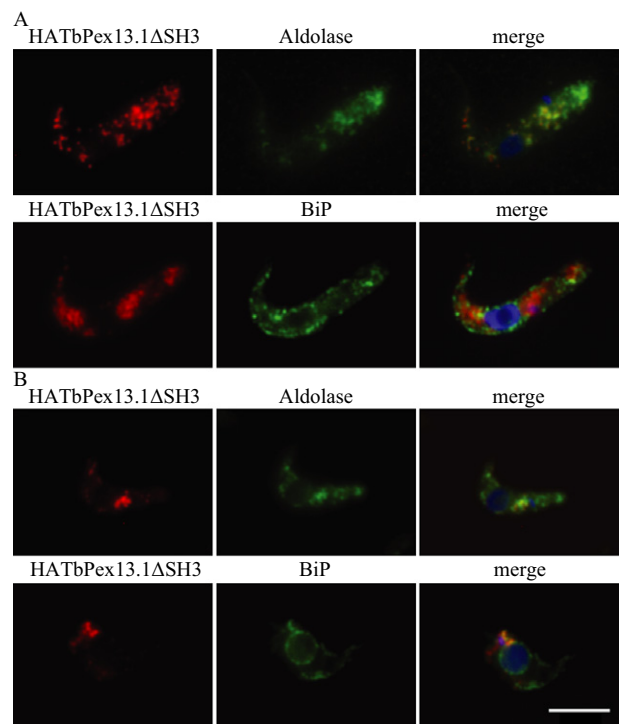
Figure 5 Deletion of the SH3 domain does not alter the glucose-dependent localization of HAPex13.1. (**A**) Cells grown in SDM79 were stained for HATbPex13.1 (red) and aldolase (green). (**B**) Cells grown in SDM80 were stained for HATbPex13.1 (red) and aldolase (green).

Moyersoen et al. 2004; Parsons et al. 2001). Because of their essential nature, they have been identified as good drug candidates. Peroxisome maintenance has been studied in yeast, mammals, and plants and involves the
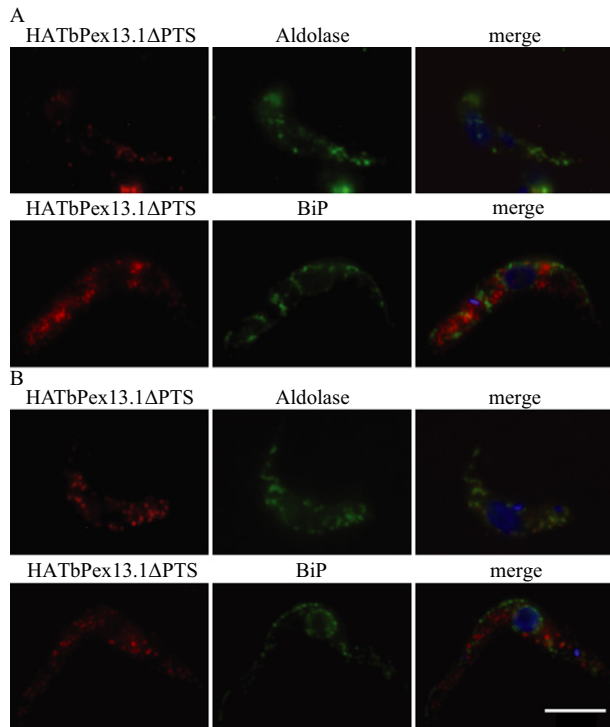
Figure 6 HATbPex13.1ΔPTS localizes to glycosomes in high and low glucose conditions. (**A**) Cells grown in SDM79 were stained for HAPex13.1ΔPTS (red) and aldolase (green). (**B**) Cells grown in SDM80 were stained for HAPex13.1ΔPTS (red) and aldolase (green).

coordination of peroxisome protein import, organelle biogenesis and organelle degradation (Smith and Aitchison 2013). Each process is governed by a set of proteins called peroxins (Pex). Approximately 17% of the 58 PEX genes identified in yeast, mammals, and plants have been identified in the trypanosome genome databases. Highlighting the importance of these organelles, the silencing of any of these PEX genes is lethal in *T. brucei* grown under standard conditions (Banerjee et al. 2005; Brennand et al. 2012; Furuya et al. 2002; Galland et al. 2007; Guerra-Giraldez et al. 2002; Kalel et al. 2015; Krazy and Michels 2006; Maier et al. 2001; Verplaetse et al. 2009, 2012; Voncken et al. 2003).

The overall mechanism of protein import into glycosomes appears to be conserved with higher eukaryotes (Galland and Michels 2010). In contrast, very little is known about the mechanisms that regulate glycosome biogenesis. In yeast and mammalian cells, peroxisome proliferation is a balance between the growth and division of existing organelles or de novo biogenesis (Smith and Aitchison 2009).

The extent to which each proliferation pathway predominates varies with organism, cell type, life cycle stage, and environmental regulation (Smith and Aitchison 2009). In yeast, peroxisomes are usually formed by fission of existing organelles (Motley and Hettema 2007) except when cells are temporarily depleted of peroxisomes through deletion of PEX genes essential for peroxisome biogenesis

(Hoepfner et al. 2005; Kragt et al. 2005). In the absence of existing peroxisomes, yeast can synthesize them de novo upon reintroduction of the deleted *Pex* gene. In mammalian cells, it has been proposed that peroxisomes multiply predominately through the de novo pathway (Kim et al. 2006). Recent studies suggest that glycosomes may be formed de novo in the African trypanosomes. First, ER proteins have been identified in membrane vesicles harboring GFPTbPex13.1 fusions (Guther et al. 2014). Additionally, a Pex16 homolog was recently identified in *T. brucei* and silencing of this glycosome protein results in glycosome abnormalities with accumulation of structures in the anterior part of the cells where ER exit sites are located (Kalel et al. 2015).

Trypanosoma brucei encounters a number of different environmental conditions as it alternates between the mammalian bloodstream and the insect vector (Stuart et al. 2008). In the mammalian bloodstream, glucose levels are present at relatively constant and high levels (~ 5 mM). After the parasites are taken up during a blood meal, glucose levels fall to undetectable levels within 15 min (Vickerman 1985). Glycosome composition of bloodstream form (BSF) parasites differs significantly from insect, procyclic form (PCF) parasites (Colasante et al. 2006). Furthermore, glycosome metabolism (Haanstra et al. 2008; Lamour et al. 2005), glycosome composition (Bauer et al. 2013) and the requirement for glycosome protein import in the PCF parasite (Furuya et al. 2002) is influenced by extracellular glucose levels. Despite these observations, most glycosome studies in PCF parasites have been performed under high glucose conditions and we were curious to know if glycosome biogenesis varied with extracellular glucose levels. In this work, we focus on PCF parasites because BSF parasites are not viable in glucose-free media.

In our biochemical fractionations, the sedimentation of TbPex13.1 changes with variation in extracellular glucose levels. Under high glucose conditions, TbPex13.1 equilibrated in fractions containing other glycosome-resident proteins. This is in agreement with previous work (Colasante et al. 2006; Verplaetse et al. 2009). We were surprised to find, however, that in low glucose media, TbPex13.1 was found in fractions of lighter density, which also contained the ER protein TbBiP. We have repeated these fractionations multiple times with different cultures, and in all cases, this glucose-dependent localization was observed.

While BiP was detected in all fractions in which TbPex13.1 was present, the distribution of these two proteins did not overlap completely. We believe there are several reasons for this behavior. First, the ER is a heterogeneous, branched organelle with multiple subdomains. The heterogeneous behavior in biochemical fractionations has also been reported by elsewhere (Rout and Field 2001; Yonekawa et al. 2011). In contrast to the wide distribution of BiP (fractions 18–30) TbPex13.1, is found in fewer fractions (24–28) and we propose two explanations for this difference. First, our TbPex13.1 antibodies are not sensitive as those for BiP and are therefore likely

detecting protein only in fractions in which the concentration is high. Furthermore, in yeast and mammalian cells, peroxisomal proteins localize to subdomains of the ER from which preperoxisomal vesicles bud (van der Zand et al. 2010). It is possible that the tight distribution of TbPex13.1 in the gradient is caused by localization within specific regions of the ER.

Our effort to localize TbPex13.1 in PCF cells was complicated by our lack of TbPex13.1 antibodies that work in IFA. The antibodies we generated against recombinant TbPex13.1 work in western analysis, but not in IFA. Because of this limitation, we expressed epitope-tagged TbPex13.1 (HATbPex13.1) in PCF and used indirect immunofluorescence assays to follow localization. Because initial characterization of TbPex13.1 was done using green fluorescent protein fused to the N-terminus of TbPex13.1, we reason that our smaller HA tag has minimal influence on the localization and function of the protein. In agreement with our biochemical data and the published literature (Verplaetse et al. 2009), HATbPex13.1 localized to glycosomes of cells grown in high glucose. In low glucose, however, the HATbPex13.1 expressing cells had altered ER and glycosome morphology.

Our fractionation experiments were performed with 3×10^{10} cells (Fig. 1). We have performed such fractionations multiples times with different cultures and are confident in our ability to resolve the different subcellular compartments under these purification protocols. However, our transgenic cell lines grow slowly and we are limited in the number of cells we can harvest. We have tried to scale our fractionation protocol to accommodate a smaller amount of cells but have, to date, not been able to resolve ER from glycosomes. This work is ongoing.

In other systems, the PMP exit from the ER through ER exit sites (ERES) (Tani et al. 2011; Yonekawa et al. 2011). If this occurs also in trypanosomes, it may be that the tagged protein is correctly targeted to the ER, but unable to exit either because it cannot be correctly processed or because its overexpression has exceeded the cells ability to process it. There are other examples where overexpression of a glycosome protein resulted in alteration of its resident compartment. Overexpression of TbPex11 in *T. brucei* resulted in cells that had clustered and elongated glycosomes (Lorenz et al. 1998).

This extraglycosomal localization is likely not due to overexpression of the fusion protein in low glucose. Western analysis showed that HATbPex13.1 is expressed at lower levels in low glucose in comparison to high glucose (supplementary). This low glucose repression is observed for all TbPex13.1 variants as well as other glycosome proteins. Furthermore, it is unlikely that these localization changes are not solely a result of ER and/or glycosome stress under low glucose conditions as the organelle morphology is maintained under both conditions (Fig. 2).

The structure of TbPex13.1 has been enigmatic. It has two consensus sequences that are typically involved in localization of glycosome proteins. The Pex19 binding sequence is present in many peroxisome membrane proteins (Kim and Hettema 2015). The chaperone Pex19 binds to the protein in the cytoplasm and then delivers it to peroxisomes. A putative Pex19 binding domain has been identified in TbPex13.1 but, to the best of our knowledge, has not be verified experimentally. In addition to the predicted Pex19 binding domain, there is a C-terminal tripeptide PTS1 sequence. PTS1 sequences bind the soluble receptor Pex5, which then delivers the cargo to the glycosome membrane. Upon deletion of the PTS1 sequence, the protein is localized to glycosomes in glucose-rich or glucose-poor media. This finding suggests that PTS1 sequence is not required for glycosome targeting.

Src homology 3 domains (SH3) domains are ~ 60 amino acids long and form structural domains that usually mediate protein–protein interactions (Musacchio et al. 1992, 1994). The SH3 domains of Pex13 in yeast (Elgersma et al. 1996) and humans (Fransen et al. 1998) bind directly to Pex5p. Mutations in the *Saccharomyces cerevisiae* Pex13 SH3 domain blocked function, but Pex13 was still targeted to the peroxisome membrane (Elgersma et al. 1996). In yeast two hybrid, the TbPex13.1 SH3 domain interacted with TbPex13.2 (Brennand et al. 2012). In our experiments, deletion of the SH3 domain also had no impact on the localization of HATbPex13.1.

Our findings demonstrate that TbPex13.1 localization changes with extracellular glucose levels and suggest that glycosome biogenesis differs between these two conditions. While we have only tested glucose levels, there may be other factors such as temperature and pH that affect glycosome composition and biogenesis. The glucose-dependent phenotype provides further evidence that cellular biology and metabolism of PCF parasites is profoundly influenced by extracellular glucose levels and that ER-glycosome trafficking does occur in trypanosomes. TbPex13.1 may serve as a marker for following such trafficking in trypanosomes. Elucidation of the ER-glycosome maturation pathway may lead to the identification of new drug targets.

ACKNOWLEDGMENTS

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. HATbPEX13.1 expression is down regulated under low glucose conditions.