Glycosome Heterogeneity, Dynamics, and ER Association in Trypanosoma brucei

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GLYCOSOME HETEROGENEITY, DYNAMICS, AND ER ASSOCIATION IN
Trypanosoma brucei

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Biochemistry

by
Sarah Twitty Bauer
December 2016

Accepted by:
Meredith Morris, Committee Chair
Kimberly Paul
Julia Frugoli
Terri Bruce
ABSTRACT

*Trypanosoma brucei* is the causative agent of human African trypanosomiasis, a disease affecting over 15,000 people annually. During its life cycle, *T. brucei* encounters a number of changes in environmental conditions as it is passed between the mammalian host and tsetse fly vector. These parasites are unique in that they compartmentalize glycolysis in highly specialized, essential organelles called glycosomes. While it has been demonstrated that glycosomal composition changes in response to life cycle differentiation, environmentally dependent changes in glycosome composition had not previously been demonstrated. Here I present data demonstrating that glycosome composition changes in response to glucose, a nutrient whose availability fluctuates throughout *T. brucei*'s life cycle. We used a fluorescent organelle reporter system to demonstrate that glycosomes are remodeled in response to changes in environmental conditions in the insect stage, procyclic form (PF) parasite. We also provide evidence that a membrane protein in the peroxin family is involved in glycosome matrix protein import, TbPEX13.1, localizes to the ER in PF *T. brucei* cultured in low glucose conditions. Furthermore, we describe the glucose dependent heterogeneity of glycosomes within parasite populations and show preliminary investigations focused on determining the mechanisms that regulate glycosome protein expression. Such dynamic nature and heterogeneity has not previously been demonstrated in trypanosome glycosomes. Together the studies presented in this dissertation, in particular the dual-localization of TbPEX13.1 to glycosomes and the ER, provide support for the existences
of an ER-derived de novo mechanism of glycosome biogenesis. Such a pathway has been proposed to occur but has never been demonstrated experimentally.
DEDICATION

For Marty and Norah.
ACKNOWLEDGMENTS

The work presented in this dissertation would not have been possible without the guidance and support of my advisor, Dr. Meredith Morris. Having the opportunity to be her first graduate student is an honor and privilege that I will always be grateful for. She has truly shaped me into the confident scientist that I am today. I would also like to thank my committee, Dr. Kim Paul, Dr. Julia Frugoli and Dr. Terri Bruce. The wisdom and support they have shared over the course of my work at Clemson University has been invaluable. In addition, I would like to thank Dr. James Morris, who has served as a mentor and challenged me every day. I am forever grateful to have had the privilege of working with an all-star crew in the Morris lab. In particular, Terral Patel without whom much of this work would not have been possible. Elizabeth Kahney, Michael Harris, Yijian Qiu, Logan Crowe, Walker Blanding, Stephen Patrick, Alex Beaver, Jillian Milanes and an army of amazing undergraduate students have provided friendship and support throughout all of the ups and downs of graduate school. I would also like to acknowledge the faculty, staff and students of the Eukaryotic Pathogens Innovation Center as well as the Department of Genetics and Biochemistry. I have learned and grown so much over the past few years and it has been an honor to do so with all of you.

Last, I would like to thank my family and friends for their love and support. I would not be who or where I am today without them.
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CHAPTER ONE

GLYCOSOME BIOGENESIS IN TRYPANOSOMES AND THE DE NOVO DILEMMA

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Abstract

Trypanosomatid parasites, including *Trypanosoma* and *Leishmania*, are the causative agents of lethal diseases threatening millions of people around the world. These organisms compartmentalize glycolysis in essential, specialized peroxisomes called glycosomes. Peroxisome proliferation can occur through growth and division of existing organelles and *de novo* biogenesis from the endoplasmic reticulum. The level that each pathway contributes is debated. Current evidence supports the concerted contribution of both mechanisms in an equilibrium that can vary depending on environmental conditions and metabolic requirements of the cell. Homologs of a number of peroxins, the proteins involved in peroxisome biogenesis and matrix protein import, have been identified in *Trypanosoma brucei*. Based on these findings, it is widely accepted that glycosomes proliferate through growth and division of existing organelles; however, to our knowledge a *de novo* mechanism of biogenesis has not been directly demonstrated. Here we review recent findings that provide support for the existence of an ER derived *de novo* pathway of glycosome biogenesis in *T. brucei*. Two studies recently identified PEX13.1, a peroxin involved in matrix protein import, in the ER of *T. brucei*. In other eukaryotes, peroxins including PEX13 have been found in the ER in cells undergoing *de novo* biogenesis of peroxisomes. In addition, PEX16 and PEX19 have been characterized in *T. brucei*, both of which are important for *de novo* biogenesis in other eukaryotes. Because glycosomes are rapidly remodeled via autophagy during life cycle differentiation, *de novo* biogenesis could provide a rapid method of restoring glycosome populations following turnover. Together, the findings we summarize provide support for the
hypothesis that glycosome proliferation occurs through growth and division of pre-existing organelles and *de novo* biogenesis from the ER and that the level each mechanism contributes is influenced by glucose availability.
Glycosomes enable parasite survival in multiple environments

*Trypanosoma brucei* is the protozoan parasite responsible for human African trypanosomiasis (HAT), a lethal disease affecting over 15,000 people living in sub-Saharan Africa (1), and nagana, a wasting disease in cattle (2). *T. brucei* belongs to Kinetoplastida, a class of protozoan parasites named for a mass of mitochondrial DNA, called the kinetoplast, situated at the base of the flagellum in a single, intricate mitochondrion (2, 3). This class also includes a number of other disease causing parasites including *Trypanosoma cruzi*, the parasite responsible for American trypanosomiasis, and the genus *Leishmania*, encompassing a number of species responsible for leishmaniasis (2–5). Combined, these diseases threaten millions of people around the world and have serious social and economic impact (1). Due to the cost burden, harsh side effects, and rise in resistance to currently available drugs, identifying new drug targets is crucial (1, 3).

*T. brucei* parasites are transmitted by tsetse flies. Bloodstream form (BSF) *T. brucei* dwell in the bloodstream and lymphatic system of their mammalian hosts. When a tsetse fly takes in a blood meal, ingested parasites differentiate into procyclic forms (PF) in the midgut of the fly. PFs can then migrate to the salivary glands of the fly and differentiate into metacyclic forms and be transmitted to the next mammalian host (2, 6, 7). Throughout the lifecycle, *T. brucei* encounters a number of environments with different available nutrients. In the mammalian bloodstream, glucose is abundant, being maintained at 5 mM and BSF parasites rely solely on glycolysis for the generation of
ATP. In the tsetse fly midgut glucose levels fall after the bloodmeal to undetectable levels within 15 minutes (7).

Unlike other eukaryotes in which glycolysis is a cytosolic process, *T. brucei* compartmentalizes enzymes involved in glycolysis in single-membrane bounded organelles called glycosomes (5, 8, 9). These organelles are evolutionarily related to peroxisomes and harbor a number of proteins involved in import and biogenesis that are homologous to peroxisome proteins in higher eukaryotes. These proteins are called peroxins (discussed in further detail in section II) (3, 5).

Glycosomes are essential to parasite survival, likely due to compartmentalization of key metabolic enzymes. For example, the glycolytic enzymes hexokinase and phosphofructokinase are not inhibited by their products and compartmentalization of glycolysis likely prevents these enzymes from depleting ATP within the cell (9, 10). Disruption of glycosome biogenesis or glycosome protein import results in mislocalization of these enzymes to the cytoplasm and cell death (11, 12).

Compartmentalization of glycolysis enables parasite survival in the various environments encountered during its lifecycle. In BSF parasites, ATP is generated exclusively through glycolysis, with up to 90% of glycosomal protein content involved in glycolysis (5, 13). PF parasites generate ATP via glycolysis when glucose is present but switch to amino acid metabolism when it is absent (8, 14). In this stage, less than 50% of the total glycosome protein repertoire is involved in glycolysis (14). PFs have a fully functional mitochondrion and protein components of the TCA cycle, electron transport
chain, and proline and threonine metabolism located in the mitochondrion are upregulated (16).

The essential nature of glycosomes in the pathogenic BSF lifecycle stage and the uniqueness of the organelles make them ideal targets for drug development (17). While much about glycosomal matrix protein import and organelle function in *T. brucei* has been resolved, understanding about glycosome biogenesis is lacking. Specifically, it is unclear the extent to which proliferation by multiplication of existing organelles or *de novo* processes involving the ER contribute to glycosome replication. Here, we discuss our current understanding of glycosome biogenesis and summarize data supporting the existence of a *de novo* mechanism of glycosome biogenesis, heretofore unappreciated in *T. brucei*.

**Glycosomes are related to peroxisomes, which proliferate by both growth and division of existing organelles and *de novo* biogenesis from the ER**

Glycosomes are considered specialized peroxisomes because they share similar structure, function, and homologous peroxins (PEX) involved in protein import and biogenesis. Both organelles are membrane bounded microbodies with electron dense matrices. They lack DNA and import fully folded proteins post-translationally (9, 18, 19). Peroxisomes are diverse organelles and activities vary with organism, cell type, and environment (20). While glycosomes share a number of pathways with peroxisomes,
such as beta-oxidation of fatty acids and reduction of reactive oxygen species, they are unique in that they harbor enzymes involved in glycolysis (3, 9, 19, 21).

Glycosomes harbor homologs to yeast, mammal, and plant peroxin proteins that participate in matrix protein import, membrane protein insertion, and organelle fission (3, 5). At least 13 peroxin homologs have been identified in kinetoplastids and although the overall sequence identity of these peroxins ranges between 15-35% when compared to homologs in other organisms, their activities are well conserved (5). Like peroxisomes, glycosomal matrix proteins are targeted for import by the peroxisomal targeting signals, PTS1 or PTS2. PTS1s are motifs consisting of three amino acid residues ((S/A/C)-(K/R/H)-L) that are located on the C-terminus of glycosomal proteins. PTS2s consist of a nine amino acid sequence ((R/K)(L/V/I)X5-(Q/H)(L/A)) located on the N-terminus of glycosomal matrix proteins. These PTS1 and PTS2 signals are recognized by cytosolic receptor peroxins, PEX5 and PEX7, for PTS1 and PTS2 respectively. PEX5 and PEX7 associate with a docking complex in the glycosomal membrane that contains PEX13 and PEX14, where the cytosolic receptor and cargo are translocated into the glycosomal matrix (3, 5). While recycling of PEX7 after cargo delivery is not well understood, PEX5 is mono-ubiquitinated by the ubiquitin conjugating enzyme, PEX4, and recycled back into the cytoplasm (5, 20).

Peroxins also regulate the abundance and size of peroxisomes and glycosomes. Growth and division of peroxisomes is regulated by the integral membrane peroxin, PEX11, which directs elongation of peroxisomes that then undergo constriction by dynamin-GTPase (20, 22). Silencing of PEX11 in *Saccharomyces cerevisiae* blocks
fission resulting in fewer, larger peroxisomes (22, 23). Similarly silencing of TbPEX11 in *T. brucei* results in fewer, larger glycosomes (24).

Mechanisms of *de novo* biogenesis have been characterized in yeasts and mammalian cells. PEX3, PEX16, and PEX19 are hallmark peroxins for this pathway. PEX19 is a cytosolic receptor that recognizes membrane peroxisomal targeting signals (mPTS) on newly synthesized peroxisomal membrane proteins (PMP). Like matrix proteins, most PMPs are synthesized on free ribosomes in the cytosol. The PEX19-PMP docks with PEX3, an integral peroxisomal membrane peroxin involved in the insertion of PMPs (3, 20, 22, 25). Although the function of PEX16 differs slightly among different species, in mammalian cells it has been demonstrated to recruit PEX3 and other PMPs to the ER (25, 26). Mutation of PEX3 or PEX19 in yeast and PEX3, PEX16, or PEX19 in mammalian cells results in cells devoid of peroxisomes, which are restored upon complementation with functional gene copies, demonstrating the importance of these peroxins in *de novo* peroxisome biogenesis (27–39).

Several mechanisms of ER-dependent peroxisome proliferation have been proposed (reviewed in 20,23). In the heterotypic fusion model, yeast PEX3 forms pre-peroxisomal domains within the ER. Other PMPs, including docking complex peroxins (PEX13 and PEX14), and the RING (really interesting novel gene) finger peroxins involved in PEX5 receptor recycling (PEX2, PEX10, and PEX12), are trafficked to the ER. Pre-peroxisomal vesicles (ppV) containing either docking complex PMPs or RING finger peroxins bud from the ER independently. These individual, import-incompetent
ppVs each containing a portion of the import machinery can then fuse and form import-competent peroxisomes (40).

In the vesicle maturation model, PEX3 localizes to the ER in a PEX16-dependent process. These ppVs, containing PEX3 and PEX16, bud from the ER and then are capable of importing PMPs. The ppVs consecutively mature into organelles that support peroxisome protein import. These ppVs can fuse with currently existing peroxisomes, contributing new lipids and PMPs to the growth and division mechanism of biogenesis (41).

While we have gained significant understanding of how proteins are targeted to and imported into glycosomes, our understanding of how these organelles are formed and multiply (ie. glycosome biogenesis) is lacking. Although PEX16 and PEX19 have both been characterized (42, 43), the key peroxin in de novo biogenesis of peroxisomes in other eukaryotes, PEX3, has yet to be identified in any kinetoplastid genome database. With the low sequence identity maintained between homologous peroxins in different species, this is not unexpected (5). However, without knowing the identity of PEX3, characterizing a de novo process of glycosomes biogenesis in T. brucei remains a challenge. In addition, many studies of de novo biogenesis rely on silencing genes yielding cells devoid of peroxisomes and successive complementation to restore peroxisomes (44). Such studies are challenging in T. brucei because glycosomes are essential (17). Nevertheless, a number of recent findings provide support for the existence of a de novo process of glycosome biogenesis in T. brucei.
Recent studies suggest glycosomes can proliferate de novo in trypanosomes

In yeast and mammals, several peroxins localize to the ER during de novo biogenesis of peroxisomes (20, 22, 25, 44). In pulse-chase experiments PEX3 fused to yellow fluorescent protein localized to ER foci before being trafficked to peroxisomes (45). Other PEXs including PEX2, PEX3, PEX8, PEX10, PEX11, PEX13, PEX14, PEX15, PEX16, PEX17, PEX30, and PEX31 have been localized to ER concentrated in areas where ppVs are generated (25, 40, 45–51). Furthermore, in mouse dendritic cells PEX13 localized to lamellar structures that bud from the ER, giving rise to mature peroxisomes (52).

In a recent proteomic study, Güther et al used affinity purification to isolate glycosomes from cells expressing GFP-tagged TbPEX13.1, and characterized the glycosomes of PF T. brucei by SILAC quantitative proteomics. Notably the GFP.TbPEX13.1 fusion was detected in vesicles containing ER proteins (53). We have also noted association of TbPEX13.1 with the ER. We found that TbPEX13.1 localized to foci within the ER in PF T. brucei when the parasites were grown under low glucose conditions (54). Much like ER localized peroxins in other organisms, it is possible that localization of TbPEX13.1 to foci in the ER may be occurring at sites where new glycosomes or pre-glycosomal vesicles bud.

PEX16 plays a pivotal role in the de novo biogenesis of peroxisomes in some yeast and mammalian cells (20, 22, 25, 26, 44). In Yarrowia lipolytica, PEX16 negatively regulates peroxisome fission (55). Y. lipolytica PEX16s are N-glycosylated in the ER, and mutation to ER exit machinery negatively impacts ER exit of PEX16 (26,
In mammalian cells, PEX16 ER exit depends on Sec16B in ER exit sites (43, 56, 57). TbPEX16 was recently characterized in *T. brucei*. Silencing of TbPEX16 resulted in a decrease in glycosome number. Remaining glycosomes were no longer symmetrically distributed throughout the cell but instead sequestered to the posterior half of the parasite (43). *T. brucei* expresses a single Sec16 homolog of human Sec16B (43, 58). The TbSec16 ER exit site is located between the kinetoplast and nucleus (58) in the anterior end of the trypanosome opposite of where glycosomes are sequestered in cells lacking TbPEX16 (43). Kalel *et al.* propose and we agree that TbPEX16 may be targeted to the ER in *T. brucei* and that TbPEX16 silencing may interfere with the budding of glycosomes through the TbSec16ER exit site. This would limit the contribution of *de novo* biogenesis, yielding a decreased number of organelles in this area of the cell (43).

PEX19 also plays an important role in the *de novo* biogenesis of peroxisomes. Silencing of PEX19 in *S. cerevisiae* and *Hansenula polymorpha* results in cells devoid of peroxisomes while complementation with functional variants of PEX19 restores peroxisome populations (30, 38, 39). Analogous results have been observed in mammalian cells (33). In a similar manner, TbPEX19 silencing in *T. brucei* slowed parasite growth in media containing glucose and resulted in a decrease in the total number of glycosomes per cell. When cultured in the absence of glucose, TbPEX19 silencing was less detrimental to cell growth rate likely because mislocalization of glycosomal matrix proteins is not lethal (42). Because complete disruption of glycosome biogenesis is toxic to *T. brucei* when cells are cultured in the presence of glucose (11, 12), it is possible that a more complete knock down of TbPEX19 could be generated in
the absence of glucose (42). Such complementation experiments in *T. brucei* could demonstrate the existence of an ER-dependent mechanism of glycosome biogenesis.

Peroxisomes are degraded through an autophagic process called pexophagy, which is often triggered by environmental factors (59). In *S. cerevisiae*, fatty acids trigger the proliferation of peroxisomes that are degraded when cells are moved to glucose media (60). Environmentally-induced changes in peroxisome composition also occur in the methylotrophic yeast, *H. polymorpha*. In these cells peroxisomes that are induced in methanol are then degraded when the cells are transferred to glucose (61).

*T. brucei* parasites encounter a number of environmental changes in which nutrient availability varies. Extracellular glucose levels fluctuate between ~5 mM in the mammalian bloodstream to undetectable levels in the tsetse fly vector (7). Concomitant with these changes, glycosome composition is altered (21, 62–64). Like peroxisomes, glycosomes associate with lysosomes and get degraded through autophagy during BSF-PF differentiation (62). Glucose dependent changes in glycosome composition that occur in PF parasites occur within three hours, a time frame consistent with that of autophagy (63, 64). Generation of new glycosomes could provide a way to restore the glycosome populations after autophagy with organelles containing a protein repertoire best suited for the cell’s new environment (Fig 1.1).
Figure 1.1. *De novo* biogenesis may facilitate production of new glycosomes after autophagy of old organelles.

(A) Autophagy enables turnover of existing glycosomes. Glucose availability changes through the life cycle of *T. brucei*, ranging from undetectable levels to ~5 mM. In response to an increase or decrease in glucose availability, “old” glycosomes with compositions best suited for prior environmental conditions can be degraded via autophagy. (B) “New” organelles with protein repertoires better suited to the new levels of glucose in the environment can be generated from the ER. (C) Once these new glycosomes are generated, they can be maintained through growth and division. This process enables cells to remodel glycosome composition and adapt to changing glucose levels.
Glycosomes biogenesis is likely influenced by extracellular glucose levels

Mechanisms of de novo peroxisome biogenesis are well established in yeast and mammals, however, a similar mechanism of biogenesis in kinetoplastids has not been directly demonstrated. Because of the similarities in glycosome and peroxisome mechanisms of matrix protein import, PMP insertion, and organelle remodeling, it seems logical that glycosome proliferation could also occur through an ER dependent mechanism. Localization of peroxins to the ER, analogous phenotypes obtained from silencing key de novo biogenesis peroxins, and similarities in organelle maintenance and turnover provide support that like peroxisomes, de novo biogenesis of glycosomes likely occurs in T. brucei.

There is contradicting evidence providing support for the primary mechanism of peroxisome biogenesis. The current consensus is that growth and division of existing organelles and ER derived de novo mechanisms occur synergistically and that the level that each mechanism contributes varies depending on the organism, cell type, and environmental condition. For example, yeast peroxisomes are produced primarily through growth and division of existing organelles when cells are grown on oleic acid, but there is a switch to de novo biogenesis when the organism is transferred to media containing glucose (25, 65).

We hypothesize that de novo glycosome biogenesis and growth and division of existing organelles both occur in PF T. brucei and that environmental triggers, particularly glucose availability, influences the level at which each contributes. We propose that in high glucose, glycosomes proliferate primarily through the growth and
division of existing organelles while in low glucose conditions glycosomes multiply through an ER derived *de novo* mechanism (Fig 1.2).

Generation of peroxisomes through growth and division is kinetically faster than generation from the ER (20). We hypothesize that in glucose rich media glycosomes proliferate rapidly through growth and division, allowing maintenance of metabolic flux, while preventing glucose toxicity (Fig 1.3). Although *de novo* mechanisms of biogenesis are kinetically less favorable, peroxisome composition can be altered through autophagy (20). Degrading “old” glycosomes via autophagy could yield a temporary source of substrates to satisfy metabolic needs (in the form of glycosome resident proteins) while clearing the way for the generation of “new” glycosomes from the ER, which are better suited for growth under glucose poor conditions.
Figure 1.2. Proposed model for influence of extracellular glucose levels on glycosome biogenesis

(A) When cultured in media containing glucose, glycosomes proliferate rapidly through TbPEX11 dependent growth and division of pre-existing organelles to facilitate rapid import of glycosome proteins that are toxic if mislocalized. Under low glucose conditions, the slower de novo biogenesis mechanism plays a larger role in glycosome proliferation. Under glucose poor conditions, the need for rapid import is not essential. In this pathway, TbPEX13.1 and TbPEX16 are first inserted into the ER membrane via TbPEX19 and get incorporated into budding pre-glycosomal vesicles. (B) Growth and division and de novo mechanisms of glycosome biogenesis occur synergistically and the level at which each mechanism contributes is glucose dependent. –Glc (glucose poor, < 0.05 mM), +Glu (glucose rich ~5 mM)
Figure 1.3. Glycosomal matrix protein import is more tightly regulated when cultured in glucose rich media.

(A) Digitonin fractionations of cells grown in high glucose (+Glc) and low glucose (-Glc) reveal glycosomal proteins are detected outside of glycosomes under low glucose conditions. Digitonin is a detergent that permeabilizes cell membranes. Increasing digitonin concentration (mg dig/mg PF) added to cells consecutively permeabilizes membrane bound compartments within the cell. Samples are analyzed by western blot, probing with antibodies raised against an ER marker (BiP), a glycosomal matrix protein
involved in glycolysis (aldolase), and a glycosomal fluorescent reporter system consisting of a peroxisomal targeting signal (PTS2) fused to an eYFP (PTSeYFP). While BiP is released at the same concentration of digitonin under both conditions, both aldolase and PTSeYFP are released at lower concentrations of digitonin under low glucose conditions when compared to high glucose conditions. These experiments suggest that mislocalized glycosomal proteins can be detected extraglycosomally under low glucose conditions. (B) In high glucose, glycosomal proteins localize exclusively to glycosomes and any mislocalization is lethal to cells. Under low glucose conditions, mislocalization of glycosomal matrix proteins is more tolerated and matrix proteins can be detected in the cytoplasm prior to import. Glycosomal proteins depicted in green.

In our current model under conditions where glycosomes are generated through \textit{de novo} biogenesis, PEX13, PEX16, and potentially other membrane peroxins localize to the ER and aggregate in foci where new glycosomes bud. This would explain the localization of TbPEX13.1 to the ER in the absence of glucose, conditions under which \textit{de novo} biogenesis may be the primary mechanism of organelle formation. If TbPEX16 does play a role in \textit{de novo} biogenesis, silencing in glucose rich media would disrupt the contribution of \textit{de novo} biogenesis, resulting in the absence of glycosomes in the ER exit area of the cell observed by Kalel \textit{et al} (43) but not from other regions of the cell where glycosomes are primarily proliferating through growth and division. In glucose poor conditions, TbPEX16 silencing may result in a more dramatic decrease in the number of glycosomes if the parasites are relying on a \textit{de novo} mechanisms of biogenesis in the
absence of glucose. Similarly, silencing TbPEX19 in low glucose conditions may result in glycosome deficient parasites as mislocalization of glycolytic enzymes is anticipated to be more tolerated in *T. brucei* under these conditions (12).

In the future, characterization of peroxin localization and silencing peroxins in low glucose conditions may provide insight into the existence of a *de novo* mechanism of biogenesis and whether mechanisms of glycosome biogenesis differ with changes in extracellular conditions. Complementing silenced peroxins and restoring glycosome populations would also provide evidence of *de novo* glycosome formation. Better understanding of the mechanism of *de novo* biogenesis in glycosomes would allow us to gain knowledge of how glycosome populations are maintained and remodeled through a balance of growth and division, *de novo* biogenesis, and autophagy.

References


CHAPTER TWO

ENVIRONMENTALLY-REGULATED GLYCOSOME PROTEIN COMPOSITION IN THE AFRICAN TRYPANOSOME

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Abstract

Trypanosomes compartmentalize many metabolic enzymes in glycosomes, peroxisome-related microbodies that are essential to parasite survival. While it is understood that these dynamic organelles undergo profound changes in protein composition throughout life cycle differentiation, the adaptations that occur in response to changes in environmental conditions are less appreciated. We have adopted a fluorescent-organelle reporter system in procyclic *T. brucei* by expressing a fluorescent protein (FP) fused to a glycosomal targeting sequence (PTS2). In these cell lines, PTS2-FP is localized within import competent glycosomes and organelle composition can be analyzed by microscopy and flow cytometry. Using this reporter system, we have characterized parasite populations that differ in their glycosome composition. In glucose-rich media two parasite populations are observed; one population harbors glycosomes bearing the full repertoire of glycosome proteins while the other parasite population contains glycosomes that lack the usual glycosome resident proteins but do contain the glycosome membrane protein TbPEX11. Interestingly, these cells lack TbPEX13, a protein essential for the import of proteins into the glycosome. This bimodal distribution is lost in low-glucose media. Furthermore, we have demonstrated that changes in environmental conditions trigger changes in glycosome protein composition. These findings demonstrate a level of procyclic glycosome diversity heretofore unappreciated and offers a system by which glycosome dynamics can be studied in live cells. This work adds to our growing understanding of how the regulation of glycosome composition relates to environmental sensing.
**Introduction**

*Tryptosoma brucei*, the causative agent of human African trypanosomiasis, has a complex life cycle, with developmental stages in the bloodstream of the mammalian host and the tsetse fly vector. Each host provides a distinct environment in which the parasites must survive. Bloodstream form (BSF) parasites are bathed in glucose and generate ATP exclusively by glycolysis. While in the tsetse fly, the procyclic form (PF) parasites experience a drop in glucose levels with a concomitant increase in the availability of amino acids (namely proline). Under these conditions, the parasite adapts its metabolism, generating ATP from both glycolysis and amino acid metabolism (1).

In trypanosomes, many of the enzymes involved in glycolysis are contained within membrane-bounded organelles called glycosomes (reviewed in (2-4)). Similarities between the metabolic activity and the matrix protein import machinery of glycosomes and peroxisomes indicate an evolutionary relationship between the two organelles. In contrast to peroxisomes, however, glycosomes are essential, making mechanisms of glycosome biogenesis and maintenance attractive drug targets.

Glycosome dynamics are governed by a number of processes including organelle biogenesis, protein import, and changes in protein composition. In trypanosomes, a number of proteins involved in import have been characterized and recent studies have begun to identify processes involved in glycosome turnover and remodeling (5-7). However, very little is known about organelle biogenesis.

Proper regulation of glycosome number and composition is essential to *T. brucei*. Silencing of trypanosome PEX proteins involved in glycosome matrix protein import
(TbPEX5, 7, 10, 6, 12, 14) causes the mislocalization of glycosome proteins to the cytosol and compromised growth (8-12). Reduction in the expression of PEX genes involved in glycosome biogenesis (TbPEX11 and TbPEX19) through RNA interference (13, 14), results in parasites harboring fewer, larger glycosomes, while overexpression of TbPEX11 results in cells with many smaller glycosomes (14). As observed after silencing of other PEX genes, reduction in levels of TbPEX11 and TbPEX19 also resulted in growth arrest.

*T. brucei* glycosomes are extensively remodeled during differentiation between BSF and PF parasites. In a recent study, 159 proteins from the glycosomes of BSF and PF were identified by proteomics (15). Of these proteins, approximately 35% were found in both stages of the life cycle. These constitutively expressed proteins included enzymes involved in glycolysis, purine salvage, pyrimidine biosynthesis, phospholipid degradation and glycerol-ether lipid biosynthesis. Forty-two percent of the proteins were PF-specific and included proteins involved in the pentose phosphate pathway, the Calvin-Benson cycle and oxygen radical and peroxide detoxifying enzymes.

While it is understood that the metabolic repertoire of the glycosome is variable, the details of glycosome biogenesis and changes in protein composition are unknown. Recent work suggests that glycosomal proteins can be turned over during the differentiation process in a pathway that is mechanistically analogous to autophagy (16). Glycosomes colocalize with lysosomes during differentiation when there is a change in glycosome protein expression. Autophagy of glycosomes may also play a role in the parasites’ response to the environment, as this pathway appears to be induced under
starvation conditions when parasites are moved from nutrient-rich culture to PBS (16, 17). PBS-induced autophagy is not restricted to *T. brucei*, but has also been reported in the related kinetoplastid, *Leishmania major* (18). In contrast to our knowledge of the changes that occur during the differentiation process, less is known about how the protein composition of PF glycosomes changes in response to different environmental conditions.

In yeast and mammalian cells, peroxisomes can proliferate through the growth and division of existing organelles as well as through *de novo* synthesis from the endoplasmic reticulum. Aside from the observations that the silencing and overexpression of TbPEX11 leads to defects in glycosome number and morphology, nothing is known about how glycosomes proliferate through the growth and division of existing organelles and, to our knowledge, a *de novo* pathway of glycosome biogenesis in *T. brucei* has not been examined. Searches of the trypanosome genome have failed to identify any homologs of genes involved in *de novo* biogenesis, however, this is not surprising as these proteins are not well conserved and trypanosome sequences are highly divergent from higher eukaryotes.

To study glycosome dynamics, we have adopted a fluorescent-organelle reporter system used to study peroxisome biogenesis in yeasts and mammals for use in procyclic form *T. brucei*. In this system, mature glycosomes import glycosomally-targeted fluorescent protein while immature organelles do not. Using flow cytometry and electron microscopy, we have identified and characterized two parasite populations that differ in their glycosome composition. One population contains “mature” glycosomes harboring
the expected repertoire of glycosomal proteins including TbPEX13, a protein essential for import of proteins into glycosomes, while the other population contains glycosomes, as demonstrated by EM, but expressed little or no glycosome proteins including TbPEX13. The relative proportions of each parasite population varied in different media conditions. In the presence of glucose, two distinct populations were present; one “dim” population harboring immature glycosomes and one “bright” population harboring mature glycosomes, with very few cells of intermediate fluorescence observed. Interestingly, in low-glucose media this bimodal population structure was lost and a range of cells with different fluorescence intensity was observed. When parasites were moved from low-glucose media into media containing 5 mM glucose, cells of intermediate fluorescence were lost within 24 h. In addition to changes in steady state glycosome composition under different media conditions, changes in glycosome expression as monitored by PTS2-FP expression was observed in live cells during changes in environmental conditions. This change was initiated within 3 h of cell passage from log-phase culture to fresh media and was complete within 24 h.

The results presented here demonstrate a level of glycosome diversity previously unrecognized. Furthermore, this is the first demonstration that PF parasites harbor immature glycosomes and provides the first suggestion that *T. brucei* glycosomes may proliferate via a *de novo* pathway. The glycosome reporter system utilized in these studies provides a rapid, high-throughput, real-time protocol to monitor processes such as autophagy, which likely regulate glycosome remodeling, in live cells.
Materials and Methods

Reagents

All reagents were purchased from Fisher Scientific unless specified.

Growth and transfection of parasites

*T. brucei* 29-13 procyclic form parasites, which express T7 RNA polymerase as well as the tetracycline repressor, were maintained in SDM-79 as described (19). PF-PTS2-FP previously named pXS2-Aldo-PTS-eYFP (20) and PF-FP were maintained in SDM-79 containing G418 (15µg/ml), hygromycin (50µg/ml) and blasticidin (15µg/ml). Clonal cell lines were obtained by limiting dilution (~0.33 parasites/well) into 96 well plates.

Generation of fluorescent reporter strains

To generate PF-FP parasites, the open reading frame of green fluorescent protein (GFP) was cloned using the *HindIII* site into the pXS2 (21) (kind gift provided by J. Bangs, University of Wisconsin) expression vector in which the neomycin resistance gene was replaced with the blasticidin resistance gene (pXS2bla). This plasmid integrates into the tubulin locus and constitutive expression is driven by the PARP promoter. Orientation was confirmed by sequencing. PF parasites were stably transfected as described (22) with 10 µg of the *MluI* linearized pXS2-FP construct and selected by supplementing the growth media with 15 µg/mL blasticidin.
**Live cell microscopy**

For analysis of fluorescence in live cells, parasites were collected by centrifugation (800 x g, 10 min.) and washed once with PBS. Cell pellets were then resuspended in vectashield mounting media (Vector laboratories, Burlingame, CA) and visualized on a Zeiss Axiovert 200M using Axiovision software version 4.6.3 for image analysis.

**Transmission electron microscopy**

For ultrastructural analysis, PF parasites were fixed in 2% paraformaldehyde/2.5% glutaraldehyde in 100 mM phosphate buffer for 1 hr at room temperature. Samples were washed in phosphate buffer and postfixed in 1% osmium tetroxide (Polysciences Inc., Warrington, PA) for 1 hr. Samples were then rinsed extensively in dH2O prior to en bloc staining with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, CA) for 1 hr. Following several rinses in dH2O, samples were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella Inc.). Ultrathin sections of 90 nm were obtained with a Leica Ultracut UCT ultramicrotome (Leica Microsystems Inc., Bannockburn, IL), stained with uranyl acetate and lead citrate, and viewed on a JEOL 1200 EX transmission electron microscope (JEOL USA Inc., Peabody, MA) at the Molecular Microbiology Imaging Facility, Washington University School of Medicine in St. Louis.
**Glycosome measurements**

ImageJ software (http://rsb.info.nih.gov/ij/) was used to analyze electron microscopy images of both bright and dim parasite populations. The area of cell visible and individual glycosomes were measured for each image. To calculate glycosome density, the total number of glycosomes was divided by the area of cell visible. This value was then converted to glycosomes/100µm². To calculate the percent of cell area occupied by glycosomes, the area of all glycosomes was summed and divided by the area of cell visible, for each image analyzed. These values were averaged for each population. From the dim population, 13 images, containing 24 cells and 87 glycosomes were analyzed. From the bright population, 13 images, 26 cells and 119 glycosomes were analyzed.

**Cytometric analysis, cell sorting and western blot analysis**

Enhanced YFP fluorescence in live cells was monitored using either a BD FACSCan flow cytometer, Accuri C6, or BD Influx cell sorter. Fluorescence emission at 530/540 nm (FITC channel) was collected after excitation with a 488 nm laser and data were analyzed using FLOWJO software (TreeStar Inc., Ashland, OR). Cells were sorted directly into SDM-79 using the BD Influx with a 100 micron tip at a sheath pressure of 12 psi and a drop frequency of 28.7 kHz and samples were processed for EM and western analysis immediately. Cell viability after sorting was confirmed by microscopy and estimated (by counting of live cells) to be > 90%. For western blotting, cell lysates (5x 10⁶-10⁷ cells) were resolved by 12% SDS-PAGE and transferred to Protran
nitrocellulose. Blots were processed as described in (23) and probed with the following antibodies: TbHK (1:10,000), PFK (1:10,000), FPBase (1:10,000), triosephosphate isomerase (TIM) (1:10,000), G6PDH (1:10,000), GK (1:10,000), PEX 13 (1:10,000), which were provided by Paul Michels (de Duve Institute and Université catholique de Louvain, Brussels (9)). Rabbit anti-glycosome antibodies (1:1000) 2841D were provided by Marilyn Parsons ((24) SBRI). TbPEX11 (1:4,000) antibodies were provided by Christine Clayton (14) (Universität Heidelberg, Heidelberg, Germany). Mouse anti-GFP antibodies (1:1,000, Molecular Probes, Eugene, OR), were used to detect eYFP.

Analysis of environmentally-dependent changes in glycosome composition

Cells cultured for extended periods of time are diminished in their ability to respond to environmental changes. Therefore, immediately after transformants emerge from drug selection, stable cell lines are stored in freezing media (24mM KCl, 0.03 mM CaCl$_2$, 2 mM K$_2$HPO$_4$, 5 mM HEPES, 0.4 mM EDTA, 1 mM MgCl$_2$ in 50% glycerol) in LN$_2$. Before use, cells were thawed and seeded at a density of 1x10$^5$/ml. When cells reached log-phase, ~6 x 10$^6$/ml, they were passed into fresh media to a final concentration of 1 x 10$^5$/ml or 5 x 10$^5$/ml and analyzed by flow cytometry over 24 h. Prior to each remodeling assay, cells were seeded to a density 1x10$^5$/ml and grown to log-phase. After transformation, cells were passaged no more than twice after which they were decontaminated and discarded and new cells are transformed.
Results

*Cytometric analysis reveals two cell populations that differ in their relative fluorescence.*

We have previously generated a fluorescent glycosomal reporter system for use in live cells by fusing the peroxisome targeting sequence 2 (PTS2) of aldolase, which targets proteins to the glycosome (25), to the open reading frame of eYFP (FP). In this cell line, PF-PTS2-FP (previously named 29-13-pXS2-Aldo-PTS-eYFP), constitutively expressed PTS2-FP is targeted to glycosomes as indicated by fluorescence microscopy (Fig. 2.1A and (20)).

Flow cytometry of PF-PTS2-FP cultures revealed two distinct populations (one “bright” and one “dim”) that differed by almost two orders of magnitude in relative fluorescence (Fig. 2.1B). This was in contrast to PF-FP cells expressing GFP lacking a PTS2 signal sequence (PF-FP, Fig. 2.1B), which yielded a single peak. Both bright and dim cell populations in the PF-PTS2-FP cultures excluded propidium iodide equally, suggesting that viability was not compromised in the cells with reduced fluorescence (data not shown). Four independent clonal cell lines obtained by two separate transformations were analyzed and all contained both bright and dim populations, further suggesting that the differences in expression were not due to unexpected integration events or artifacts introduced via selection.
Figure 2.1. Heterogeneous distribution of PF-PTS2-FP.

(A) PTS2-eYFP and GFP were cloned into the expression vector pXS2bla, a derivative of pXS2 (2) in which the neomycin resistance gene has been replaced with the gene encoding blasticidin resistance. The expression plasmid (image modified from reference 2) contains the procyclin promoter (gray box), the PARPβα intergenic region (black box), the Tubβα intergenic region (white box), and the blasticidin resistance gene (hatched box). Fluorescent PF-PTS2-FP cells were incubated with Lysotracker and imaged by fluorescence microscopy. (B) Histograms of PF-PTS2-FP and PF-FP cultures. Fluorescence at 530/540nm (FL1-FITC) was measured. A total of 10,000 events of each population were analyzed.
Cells of different fluorescent intensities differ in glycosome protein composition but have similar glycosome morphologies.

To explore the absence of PTS2-FP in the dim cells and directly assess the expression of other glycosomal proteins, both bright and dim populations were analyzed by western blotting. Cells were sorted according to their relative fluorescence intensities and sorting efficiencies were confirmed by cytometric analysis of the sorted populations (Fig. 2.2A). Lysates from equal cell equivalents (5 x 10^5) of each population were then analyzed by western blotting using antibodies that recognize eYFP, and antisera (2841D, (24) that recognize the two glycosomal matrix proteins, aldolase and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Notably, the cells from the dim population lacked detectable levels of PTS2-FP, aldolase and GAPDH but expressed equivalent levels of the surface protein, procyclin (Fig. 2.2B). Further western analysis was used to assess the expression of other glycosomal proteins in bright and dim cells (Fig. 2.2C). Dim cells expressed low or undetectable levels of TbHK, FPBase, TIM, G6PDH, and glycerol kinase. Interestingly, TbPEX11, an integral glycosome protein was detected in dim cells, while a protein essential to glycosome protein import, TbPEX13, is not detectable. These findings suggest that dim cells harbor glycosomes but that they are not competent to import matrix proteins.
Figure 2.2. Western blot analysis of dim and bright cells.

PT-PTS2-FP cultures at $10^7$/ml were analyzed by flow cytometry and sorted into two populations based on their fluorescence in the FITC channel (530/540 nm). (A) Histograms of initial presort population (top panel) and postsort analysis of 10,000 events from the dim population (middle panel) and bright population (bottom panel) sorts. (B) A total of $5 \times 10^5$ cell equivalents of each population were resolved by SDS-PAGE and analyzed by Western blotting. Procyclin and a cross-reactive protein (control) were used as loading controls. (C) Western analysis of lysates probed with antibodies against glycosome resident proteins. Coomassie blue-stained protein is shown as a loading control. HK, hexokinase; PFK, phosphofructokinase; FBPase, fructose 1,6-bisphosphatase; TIM, triosephosphate isomerase; GK, glycerol kinase.
We next used transmission electron microscopy to confirm the presence of glycosomes in dim cells as well as analyze morphology of these organelles in the different cell populations (Dim, Fig. 2.3A; Bright, Fig. 2.3B). In both bright and dim cells, electron dense glycosomes were observed. There was no dramatic difference in size, number or location of glycosomes in each of the populations. Bright cells had an average of 112 glycosomes/100μm² cell area (Fig. 2.3E), with an average area of 0.02μm² (Fig. 2.3C) and made up 2% of the cell area (Fig. 2.3D). Average glycosome number in dim cells was 119 cells/100μm² (Fig. 2.3E), with an average area/glycosome of 0.027μm² (Fig. 2.3C) and comprised 2.43% of the area of the cell (Fig. 2.3D).

Cells of intermediate fluorescence are observed in low glucose media.

Dim cells have glycosomes, as demonstrated by EM. However, the lack of TbPEX13 suggested that these glycosomes were not able to import glycosomal proteins. It is known that the mislocalization of glycosomal proteins is lethal when cells are grown in standard SMD-79 with glucose. We therefore hypothesized that cells harboring a large number of immature glycosomes must repress glycosome protein expression under standard culturing conditions. In the absence of glucose, the mislocalization of glycosome proteins is less detrimental to the parasites and we reasoned that under these conditions, cells of intermediate fluorescence would be detected. To test this, we grew cells in low-glucose media (SDM-80), which was used previously to study how PF metabolism changes in response to extracellular levels of glucose and proline (26), and monitored fluorescence by flow cytometry (Fig. 2.4). Indeed, when cultures were grown
in low-glucose conditions, cells of intermediate fluorescence were observed, resulting in a single broad peak consisting of cells exhibiting a continuous range of fluorescence intensities. Furthermore, addition of glucose (5mM) to SDM-80 restored the bimodal population distribution indicating that glucose is the media component responsible for these differences in glycosome composition (Fig. 2.4).

**Figure 2.3. Transmission electron microscopy.**

Fluorescence-activated cell sorting (FACS) was used to sort cultures based on their fluorescence intensities. (A and B) Dim (A) and bright (B) cells were fixed and processed for TEM as described in Materials and Methods. Arrows indicate glycosomes and acidocalcisomes. Mitochondria (M) and kinetoplasts (K) are indicated. Scale bar=0.5µm. The average area/glycosome (C), number of glycosomes/cell area (D), and percent cell area comprised of glycosomes were calculated as described in Materials and Methods.
Figure 2.4. Glycosome composition is regulated by extracellular glucose levels.

Cells were grown in SDM-79 (A), SDM-80 (B), and SDM-80 plus glucose (C) and analyzed by flow cytometry. Side scatter (SSC) and fluorescence at 530/540 nm (FL1-FITC) of 10,000 events were plotted.
We next measured the rate at which this intermediate population changed in response to different media conditions. Cells grown in SDM-80 were passed into SDM-79 and analyzed by flow cytometry. A decrease in the number of intermediate cells was observed at one hour and by 24h two distinct populations were observed. In contrast, when cells were moved from SDM-79 into SDM-80, it took longer for the intermediate population to appear (Fig. 2.5). By day 5 a continuous range of cells with varying levels of fluorescence intensities was observed. Because cells harboring immature glycosomes are so well resolved in SDM-79, we have chosen to use these conditions to study changes in glycosome composition that occur in response to changing environmental conditions.

*Changes in media conditions trigger changes in glycosome protein expression.*

In yeast and mammalian cells, peroxisome turnover is triggered by changes in media conditions (27). To test the effect that environmental changes have on glycosome composition, we passed cells from a log-phase culture into fresh SDM-79 at a concentration of $10^5$/ml bright and dim measured populations by flow cytometry. When cells were passed from log-phase culture to fresh media at a concentration of $1 \times 10^5$/ml, the number of cells falling within the left gate (includes dim cells exhibiting background fluorescence as well as cells of intermediate fluorescence) increased 3-fold with 30.1% dim cells present at 3h post-passage (Fig. 2.6B) as opposed to 10.8% of cells in the left gate in log-phase culture (Fig. 2.6A). By 24 h., the original population distribution was restored (Fig. 2.6C). When cells were subjected to a less dramatic change in cell number
and media conditions by passing them to a final concentration of $5 \times 10^5$/ml there was no change in population distribution (Fig. 2.6D and E).

**Figure 2.5. Kinetics of medium-dependent glycosome remodeling.**

(A) Cells grown in SDM-80 were passed into SDM-79 and analyzed by flow cytometry.

(B) Cells grown in SDM-79 were passed into SDM-80. Side scatter (SSC) and fluorescence at 530/540 nm (FL1-FITC) of 10,000 events were plotted.
Figure 2.6. Passage into fresh medium triggers changes in glycosome composition.

Cells were passed from log-phase culture (A) to 1x10^5/ml (B and C) or 5x10^5/ml (D and E) and analyzed by flow cytometry at 3h (B and D) and 24h (C and E). All cells were passaged and grown in SDM-79. (F) Experiments were performed in triplicate and averages and standard deviations of percent dim cells calculated.
Discussion

We have generated a fluorescent glycosome reporter system in *T. brucei* that allows us to quickly visualize glycosomes in large numbers of live cells under a variety of different conditions. Using this system, we have identified two populations of PF parasites in culture that harbor different glycosomes. This work reveals that PF glycosome composition varies in an environmentally-dependent manner (Fig. 2.7).

![Changes in glycosome protein composition](image)

**Figure 2.7. Proposed model for glycosome dynamics in *T. brucei*.**

In SDM-79, two parasite populations, one bright and one dim, are present. Dim cells harbor a majority of immature glycosomes that are import incompetent and repress glycosome protein expression. Bright cells harbor enough mature glycosomes to support glycosome protein import. In low-glucose (SDM-80) medium, cells of intermediate fluorescence are observed, presumably because under these conditions any potential
cytosolic localization of glycosome proteins is tolerated. Dilution of log-phase cultures into fresh medium triggers changes in glycosome composition, resulting in a transient increase in dim parasites.

Dim cells express very little or undetectable levels of a number of glycosome matrix proteins. TEM showed that dim cells do harbor glycosomes; a finding that is consistent with the expression of the integral glycosome membrane protein, TbPEX11, in these cells. These results, along with the absence of TbPEX13, a protein involved in glycosome protein import, suggested that dim parasites harbor immature glycosomes that are unable to import glycosome proteins. We propose that because these organelles are import incompetent and because mislocalization of glycosomal proteins is lethal under most conditions (12, 28), the expression of glycosomal proteins must be repressed until enough mature organelles are present to correctly localize glycosomal matrix protein. We hypothesize that this tight regulation of glycosome protein is responsible for the bimodal population distribution observed in standard SDM-79 media. Consistent with this hypothesis is the observation that when cells are grown in low-glucose conditions in which the mislocalization of glycosome proteins is tolerated, cells with intermediate fluorescence intensities are observed.

Several lines of evidence argue against the supposition that these observations are a consequence of misregulated expression of recombinant fluorescent protein in a transgenic system. First, it should be noted that the expression of cytosolic GFP from the same expression vector (pXS2 derivative) yielded a single homogenous population,
indicating that the bimodal population is likely not a consequence of unidentified regulation signals in the expression constructs or integration into unanticipated sites in the genome. Furthermore, the dim cells lack not only the PTS2-eYFP reporter, but also aldolase and GAPDH as well as a number of other glycosome proteins. It is unlikely that transformation and expression artifacts would affect expression of proteins other than PTS2-FP.

*De novo* biogenesis of peroxisomes has been demonstrated in yeast and mammalian cells ((29, 30). In this process, the sequential maturation of peroxisomes is observed whereby pre-peroxisomal vesicles bud from the ER. In *S. cerevisiae*, the peroxin, PEX3, was fused to yellow fluorescent protein and found to localize to immature pre-peroxisomal vesicles which bud from the ER. The protein first localized to the ER with another peroxin, PEX19. PEX3-PEX19 containing foci were then found to bud off from the ER and newly formed peroxisomes capable of importing matrix proteins, developed into mature glycosomes or fused with existing organelles (31). Another example of the temporal regulation of peroxisome protein import is seen in the yeast *Hansenula polymorpha*. In this model system, peroxisomes first grow by the incorporation of lipids and proteins. After reaching a threshold size, the organelles give rise to new ones through fission. The progenitor organelle is metabolically active, but no longer capable of importing new protein (32, 33). To our knowledge the presence of a *de novo* biogenesis process or sequential glycosome maturation in *T. brucei* has never been examined and to date, database analysis has failed to reveal a PEX3 homolog.
We found that changes in glycosome composition occur rapidly in response to changes in environmental conditions. Passage of cells from a log-phase culture to fresh media triggered a transient increase in the percentage of “dim” cells that fell within the left gate. This change was initiated within 3h and the original population distribution was restored within 24h. We find that the manner in which the cells are cultured dramatically affect their ability to respond to changes in the environment. In some cases, this increase in the “dim” population approached 100%, while cells cultured for long periods of time become insensitive to changes in the environment (data not shown). To control for this, cells used in remodeling assays are thawed from stabilates and seeded at 1 x10^5/ml and grown to a density of 6x10^6/ml before being passed into fresh media. Cells used in the assays are not passed more than twice. We have found that these conditions provide the most reproducible, although not always the most dramatic, results. Changes in cellular metabolism as well as changes in media composition that occur during growth in culture likely contribute to the variability of the cells response to changes in environment. We are currently in the process of identifying and characterizing the effect of different variables on changes in glycosome composition as we believe this is important to understanding the biological relevance of such responses.

The time-frame of these changes in glycosome composition is consistent with the process of autophagy. To date, we have not been able to demonstrate any effect of autophagy inhibitors such as 3-methyladenine and wortmannin on remodeling. However, it is difficult to determine if these compounds work to inhibit autophagy under our experimental conditions. We are in the process of assessing the efficacy of such
inhibitors in blocking autophagy under our conditions. Furthermore, we are using TEM to assess the extent to which autophagic structures are formed during this remodeling process.

Dilution of cells from a log-phase culture into fresh media changes the carbon source availability and triggers a change in glycosome composition. Peroxisome remodeling in response to environmental conditions is documented in a number of organisms. For example, in *S. cerevisiae*, changes in peroxisome composition are observed when cells are moved from an oleic acid based media to one in which glucose is included as a carbon source (34). Under these conditions, the peroxisome matrix protein, Fox3p, is degraded. In methanol media, the yeast *Pichia pastoris* has large, clustered peroxisomes whereas small, diffused organelles are present when grown in oleic acid (27). In another yeast, *Hanensula polymorpha* pexophagy is induced when cells are moved from methanol to glucose media.

We observed a change in glycosome composition when cells were passed back to $1 \times 10^5$/ml but not when they were passed to $5 \times 10^5$/ml. At this point we do not know if this change in glycosome composition is the result of changes in cell density or media composition as the cells were not washed between passages. It is possible that the residual media carried with cells through passage affected the cellular response. Separating these two variable has been difficult as washing the cells before diluting them to $1 \times 10^5$/ml greatly reduces cell viability.

In *T. brucei*, it is possible that the two parasite populations have different metabolic capacities. Parasites containing dim glycosomes lacking aldolase and GAPDH
may rely heavily on amino acid metabolism and therefore do not require the large number of glycolytic enzymes normally housed in the glycosome.

Additionally, *de novo* synthesized glycosomes may be primarily populated with proteins carried from the ER, likely the source of origin. The lack of PEX13 may block the import of metabolically important proteins and serve as a means to regulate maturity. Further work on the protein composition and metabolic status of each population will be required to address this model.

We do not know if glycosomes of BSF parasites exhibit the type of plasticity observed in PF parasites. We predict that the obligate dependence on glucose metabolism may prevent or repress dramatic changes in glycosome matrix protein composition.

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**References**


CHAPTER 3

USING FLUORESCENT PROTEINS TO MONITOR GLYCOSOME DYNAMICS IN THE AFRICAN TRYPANOSOME

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Short Abstract
Glycosome dynamics in African trypanosomes are difficult to study by traditional cell biology techniques such as electron and fluorescence microscopy. As a means of observing dynamic organelle behavior, a fluorescent-organelle reporter system has been used in conjunction with flow cytometry to monitor real-time glycosome dynamics in live parasites.

Long Abstract
Trypanosoma brucei is a kinetoplastid parasite that causes human African trypanosomiasis (HAT), or sleeping sickness, and a wasting disease, nagana, in cattle (1). The parasite alternates between the bloodstream of the mammalian host and the tsetse fly vector. The composition of many cellular organelles changes in response to these different extracellular conditions (2-5).

Glycosomes are highly-specialized peroxisomes in which many of the enzymes involved in glycolysis are compartmentalized. Glycosome composition changes in a developmental and environmentally-regulated manner (4-11). Currently, the most common techniques used to study glycosome dynamics are electron and fluorescence microscopy; techniques that are expensive, time and labor intensive, and not easily adapted to high-throughput analyses.
To overcome these limitations, a fluorescent-glycosome reporter system in which enhanced yellow fluorescent protein (eYFP) is fused to a peroxisome targeting sequence (PTS2), which directs the fusion protein to glycosomes (12), has been established. Upon import of the PTS2eYFP fusion protein, glycosomes become fluorescent. Organelle degradation and recycling results in the loss of fluorescence that can be measured by flow cytometry. Large numbers of cells (5,000 cells/sec) can be analyzed in real-time without extensive sample preparation such as fixation and mounting. This method offers a rapid way of detecting changes in organelle composition in response to fluctuating environmental conditions.
Introduction

Trypanosoma brucei causes African sleeping sickness in humans and a wasting disease, nagana, in cattle. Drugs used in the treatment of these diseases are antiquated and extremely toxic, vaccines are not available, and the potential for the development of drug resistance necessitates the search for new drug targets (1).

During its life cycle, T. brucei, alternates between an insect vector and mammalian host; two hosts that present very different environments in which the parasite must survive. A number of metabolic and morphological changes occur as the parasite is exposed to different environmental conditions. Some of the most dramatic changes are observed in single-membrane-bounded parasite-specific microbodies, termed glycosomes (13).

Glucose levels are relatively high (~5 mM) in the bloodstream and bloodstream parasites (BSF) generate ATP exclusively through glycolysis while mitochondrial metabolism is repressed (14). Unlike other eukaryotes in which glycolysis occurs in the cytoplasm, T. brucei compartmentalizes most of the glycolytic enzymes in glycosomes (14, 15). The parasites are taken up by the tsetse fly during a bloodmeal and experience a drop in glucose, which falls to undetectable levels within 15 min of being ingested by the fly. The metabolism of insect, procyclic form (PCF), parasites is more flexible and glucose, as well as amino acids such as proline, can be used in the synthesis of ATP (16-18). Comparative proteomic studies reveal life cycle dependent changes in glycosomal and mitochondrial proteins with glycolytic proteins increased in bloodstream parasites and mitochondrial proteins involved in TCA cycle and respiratory chain (13, 19). While
many studies have focused on the differences between BSF and PCF glycosomes, little is
known about the changes in PCF glycosomes that occur in response to environmental
changes.

In the hindgut of the fly, glucose levels are low with transient increases during a
feeding (20). In most in vitro studies, PCF parasites are grown in media containing
-glucose. However, recent studies have demonstrated that PCF metabolism changes
significantly in response to glucose availability (17). In the absence of glucose, proline
uptake and proline dehydrogenase activity increase (18). This change in mitochondrial
metabolism is likely accompanied by a change in glycosome composition and
morphology, however, this has not been directly assessed.

Electron and fluorescence microscopy are common techniques used to study
glycosome dynamics in T. brucei (2, 22-24). These protocols are time and labor-
-intensive, expensive, and difficult to adapt to real-time studies and high-throughput
protocols. To overcome this limitation, a fluorescent-organelle reporter system used to
study organelles in mammalian and yeast systems has been modified for use in T. brucei
(12).

Fluorescent-organelle reporter systems have been extensively used in higher
eukaryotes such as yeast, plant, and mammalian cells (25-27). In such systems, a
fluorescent protein is fused to an amino acid sequence that targets the protein to specific
organelles. The degradation or synthesis of the targeted proteins is measured via
fluorescence and changes in organelle composition are reflected by changes in cell
fluorescence.
When the open reading frame of enhanced yellow fluorescent protein (eYFP) is fused to a type II peroxisomal targeting sequence (PTS2) (12) (Fig 3.1A), the PTS2eYFP protein is imported into mature, import-competent glycosomes and fluorescence can be monitored via flow cytometry (Fig 3.1B). Variations in glycosome composition are reflected by changes in cellular fluorescence. This system can aid in resolving the mechanisms that regulate environmentally-induced changes in glycosome composition.

This manuscript describes the generation of a glycosome reporter system in PCF parasites in conjunction with flow cytometry to monitor real-time glycosome dynamics in live parasites and provides an example of how it has been used to follow changes in glycosome composition in response to different environments. In summary, glycosome composition is influenced by extracellular glucose concentrations and passage of log-phase cultures into fresh media triggers changes in glycosome composition. This system can be modified to study the dynamic behavior of other organelles in trypanosomes and other parasites.
Figure 3.1: Fluorescent glycosome reporter system.  

A. PTS2eYFP expression construct integration. PCF trypanosomes were transformed with the pXS2PTS2eYFP plasmid linearized with the restriction enzyme MluI. This construct integrates via homologous recombination into the tubulin locus (Tub) and PARP sequences (PARP) includes the promoter that drives the constitutive expression of PTS2eYFP and the sequences required for RNA processing. 

B. PTS2eYFP import. PTS2eYFP is synthesized in the cytoplasm where it binds the soluble receptor, PEX7, which delivers the reporter protein to the glycosomes. Once delivered to the glycosome membrane, the protein is imported. Mature organelles containing import machinery import PTS2eYFP and fluorescence while those that do not contain functional import machinery remain dim.
Protocol

1. General trypanosome husbandry.

1.1) Weigh solids for SDM79 media preparation (Table 1).

<table>
<thead>
<tr>
<th>SDM79 solids</th>
<th>Weight (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Graces insect cell media powder</td>
<td>2</td>
</tr>
<tr>
<td>Glucose</td>
<td>1</td>
</tr>
<tr>
<td>HEPES</td>
<td>8</td>
</tr>
<tr>
<td>MOPS</td>
<td>5</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>2</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>0.1</td>
</tr>
<tr>
<td>L-alanine</td>
<td>0.2</td>
</tr>
<tr>
<td>L-arginine</td>
<td>0.1</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>0.3</td>
</tr>
<tr>
<td>L-methionine</td>
<td>0.07</td>
</tr>
<tr>
<td>L-phenylalanine</td>
<td>0.08</td>
</tr>
<tr>
<td>L-proline</td>
<td>0.6</td>
</tr>
<tr>
<td>L-serine</td>
<td>0.06</td>
</tr>
<tr>
<td>L-taurine</td>
<td>0.16</td>
</tr>
<tr>
<td>L-threonine</td>
<td>0.35</td>
</tr>
<tr>
<td>L-tyrosine</td>
<td>0.1</td>
</tr>
<tr>
<td>Adenosine</td>
<td>0.01</td>
</tr>
<tr>
<td>Guanosine</td>
<td>0.01</td>
</tr>
<tr>
<td>Glucosamine HCl</td>
<td>0.05</td>
</tr>
<tr>
<td>Folic acid</td>
<td>0.004</td>
</tr>
<tr>
<td>ρ-aminobenzoic acid</td>
<td>0.002</td>
</tr>
<tr>
<td>biotin</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

*Table 3.1:* SDM79 solid components. Solid media components and amount (g/L) are provided.

1.2) Store at 4°C in 50 ml conical or a ziplock bag. Note: Reagents are stable for at least 6 months.
1.3) Thaw fetal bovine serum (FBS) in a 37°C waterbath, and mix periodically by inversion. Note: FBS is received from the supplier as a sterile solution. Filter sterilizing FBS reduces its ability to support parasite growth.

1.4) Once the entire bottle is thawed, heat inactivate serum for 30 min in a 56°C waterbath, mixing periodically to minimize precipitation of serum components.

1.5) Thaw penicillin/streptomycin solution (pen/strep), hemin (2 mg/ml in 50 mM NaOH) and basal Medium eagle vitamin solution at room temperature.

1.6) To a 1000 ml graduated cylinder, add 20.2 g of SDM79 solids (Table 3.1) to the liquid components (Table 3.2) and mix well on a stir plate.

<table>
<thead>
<tr>
<th>SDM79</th>
<th>Volume (ml/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM with glutamine</td>
<td>600 ml</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>10 ml</td>
</tr>
<tr>
<td>BME vitamin solution</td>
<td>10 ml</td>
</tr>
<tr>
<td>MEM amino acids solution</td>
<td>8 ml</td>
</tr>
<tr>
<td>MEM non-essential amino acid solution</td>
<td>6 ml</td>
</tr>
<tr>
<td>hemin</td>
<td>3.75</td>
</tr>
<tr>
<td>water</td>
<td>162.25 ml</td>
</tr>
</tbody>
</table>

Table 3.2: SDM79 liquid components. Volumes ml/L are given.

1.7) Adjust pH to 7.35, and bring volume to 850 ml with water.
1.8) Filter-sterilize media by attaching a vacuum hose to the bottom of a filter bottle. Apply vacuum until the solution is filtered.

1.9) Remove filter top in the biosafety cabinet and add 150 ml of heat-inactivated FBS. Remove plastic covering from sterile cap and seal media bottle.

1.10) Prepare SDM80 media in the same way as SDM79 with the following exceptions; do not add glucose or glucosamine and use MEM without glutamine. In place of 150 ml of heat inactivated FBS, add 135 ml of dialyzed, heat inactivated FBS and 15 ml of heat inactivated FBS.

1.11) Culture PCF parasites in 25cm² flask with 10 ml appropriate medium at 27°C and 5% CO₂. Note: Cells should be counted daily using a hemocytometer or a flow cytometer (see section 6) and cultures should be maintained at densities between 1 x 10⁵ and 5 x 10⁶ cells/ml.

2. Transfection of PCF parasites with the fluorescent reporter construct.

Note: To follow glycosome dynamics, a reporter protein containing the peroxisomal targeting sequence (PTS2) of aldolase fused to enhanced yellow fluorescent protein is expressed in the parasites. The sequence encoding the fusion protein is cloned into
pXS2bla (12), which contains the procyclin promoter, the tubulin βα intergenic regions, which direct homologous recombination into the genome and the blasticidin resistance gene for selection. Procyclic cell lines harboring the genes encoding the T7 polymerase and tetracycline repressor (PF29-13) are transformed with the targeting construct, pXS2:PTS2eYFP.

2.1) Prepare cytomix by mixing the components listed in Table 3.3. Filter sterilize and store at RT.

<table>
<thead>
<tr>
<th>cytomix</th>
<th>For 20 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>120 mM KCl</td>
<td>1.4 ml (1M)</td>
</tr>
<tr>
<td>0.15 mM CaCl(_2)</td>
<td>3 µl (1M)</td>
</tr>
<tr>
<td>10 mM K(_2)HPO(_4)</td>
<td>400 µl (0.5M)</td>
</tr>
<tr>
<td>25 mM HEPES</td>
<td>500 µl (1 M)</td>
</tr>
<tr>
<td>2 mM EDTA</td>
<td>80 µl (0.5M)</td>
</tr>
<tr>
<td>5 mM MgCl(_2)</td>
<td>100 µl (1M)</td>
</tr>
<tr>
<td>water</td>
<td>16.52</td>
</tr>
</tbody>
</table>

**Table 3.3:** Cytomix recipe.

2.2) Linearize plasmid DNA with MluI by pipetting 10 µl of DNA (1 µg/µl), 5 µl restriction enzyme buffer, 33 µl water and 2 µl of MluI enzyme into a microcentrifuge tube. Incubate at 37°C for 1 hr.
2.3) Purify the restriction digest by adding 1 volume of binding buffer to the restriction enzyme digest. Mix binding buffer and digested DNA by vortexing. Briefly centrifuge to collect the sample at the bottom of the tube.

2.4) Insert a DNA binding column into a 2 ml collection tube, and transfer digested DNA/binding buffer solution to column.

2.5) Centrifuge for 10,000 x g for 1 min. After centrifugation, discard filtrate from collection tube and return the column to the collection tube.

2.6) Add 700µl of SPW wash buffer and centrifuge at 10,000 x g for 1 min.

2.7) Discard filtrate from collection tube, return the column to the collection tube and repeat SPW wash step and centrifugation.

2.8) Discard filtrate, return column to collection tube, and centrifuge empty column for 3 min at 10,000 x g to remove residual ethanol.

2.9) In biosafety cabinet, throw away collection tube and place column in a sterile microcentrifuge tube.
2.10) To elute the DNA, add 25µl of sterile water to column and incubate at room temperature for 2 min. Centrifuge at 10,000 x g for 1 min.

2.11) Add another 25µl of sterile water in biosafety hood to column and incubate at room temperature for 2 min.

2.12) Centrifuge at 10,000 x g speed for 1 min.

2.13) In a biosafety cabinet transfer entire volume of DNA to a new sterile microcentrifuge tube. Note: This step prevents contamination from the open lid during centrifugation.

2.14) Add the sterile, purified, linearized DNA (10µg) to 400µl of filter-sterilized cytomix.

2.15) Count cells as described in section 6 and harvest 5 x10^7-10^8 cells by centrifugation at 800 x g for 15 min. at RT.

2.16) Pour off supernatant and resuspend the cell pellet in 450µl of DNA + cytomix using a 1ml serological pipette.
2.17) Transfer solution containing cells, DNA and cytomix to a sterile 4 mm gap cuvette and place cuvette in the electroporation chamber.

2.18) Select “Exponential decay” and manually enter the following settings: Voltage: 1.5kV, Capacitance: 25mF, Resistance: Ω, and Cuvette: 4 mm. Press pulse. Once the pulse is complete, remove cuvette from the electroporation chamber and return to the biosafety cabinet.

2.19) Into a new sterile flask, pipette 10 ml of SDM79. Transfer the transformed cells to the flask with 10 ml of SDM79.

2.20) Incubate WITHOUT drug selection for 24 hr at 27°C, 5% CO₂. After 24 hr, add G418 (15μg/ml), hygromycin (50μg/ml) and blasticidin (10μg/ml). Pass 1ml of transformed cells with drug into 9ml of SDM79 with drug.

2.21) Analyze cells daily as described in sections 6 and 7. When cells are fluorescent, and have reached a cell density of 1 x 10⁷/ml, make stabilates for long-term storage (3.1-3.3).

3. Making stabilates
3.1) To make freezing media, add an equal volume of 100% glycerol to cytomix and filter sterilize.

3.2) Add 200µl of freezing media to 800µl of cells (1 x 10^7) in a cryovial, mix gently by inversion and place between two styrofoam racks and freeze at -80°C.

3.3) Once frozen (~ 24 h), transfer cells to liquid nitrogen. Note: It is important to move cells into LN2 after 24 h, as longer storage at -80°C leads to a decrease in cell viability.

4. Thawing frozen stocks

4.1) Remove frozen vial from -80°C and thaw at RT for approximately 10 min.

4.2) Pipette 9 ml of SDM79 with drug into a new sterile flask. Add thawed cells to this flask. Pass cells 1:10 by adding 1ml of this culture to 9ml of SDM79 in a new flask (final concentration of 1 x 10^5/ml).

4.3) Before the cells reach a density of 10^7/ml, begin cytometer setup and dilution assays.
5. *Cytometer setup*

5.1) Turn on the flow cytometer and open CFlow Plus program. Note: Before running any samples, the fluidics should be backflushed and rinsed according to steps 5.1-5.9.

5.3) Replace the tube of nanopure water in which the sip is stored with an empty tube.

5.4) Select the “backflush” button.

5.5) Once backflush is complete, remove microcentrifuge tube from sip and discard.

5.6) Place a new microcentrifuge tube containing 1ml of new nanopure water on the sip.

5.7) Select a new well in CFlow program. Under “Run Limits” set time for 2 minutes. Under “Fluidics” select “fast” and “Run”.

5.8) Once the 2 min time limit is complete, click the “Delete Sample Data” button.

6. *Cell Counting using the flow cytometer*

6.1) Replace water with the sample to be counted. Set “Run Limits” to 30 seconds, “Fluidics” to “fast” and then select “Run”.

70
6.2) After the 30 second time limit is complete, CFlow plus will provide the events/µl under “Last Run.” Repeat count for each culture. Note: Before cells reach $1 \times 10^7$ cells/ml, proceed with dilution assay. Cells should be discontinued after one dilution assay is completed. Cells cultured for longer periods lose their ability to respond to environmental conditions.

7. **Measuring fluorescence using the flow cytometer**

7.1) To assess fluorescence, set “Run Limits” to 10,000 events, and “Fluidics” to “slow”. Select “Set threshold”. Under “Primary Threshold”, select “Permanently eliminate events on”, “FSC-H” (in drop down box) and enter less than 30,000. Click “apply”, then “close”.

7.2) Select the “Histogram” button in one of the new plot windows and select “FSC-A” From the drop down list, select “FL1-A”. Note: FL1-A measures fluorescence in the 530 nm wavelength.

7.3) Select “Run” and repeat for all cultures.

5.14) Run cleaning solution through the fluidics line for 2 min and water for 2 min.
6. Dilution assays

6.1) Pass cells to a density of $1 \times 10^5$ cells/ml in 3 ml of SDM79 in a 25 cm$^2$ culture flask and measure florescence immediately after passage and then at 3 h and 24 h.

7. Data analysis

7.1) Select “Analyze” tab on CFlow Plus.

7.2) Click “Histogram” button under “Make a new plot.” Select “FSC-A” and a drop down list will appear. Select channel “FL1-A.”

6.5) Highlight a well to analyze. Select “Gate” button, and manually draw a gate for the population of interest.

6.7) CFlow Plus will provide cell “Count” within this gate and “% of This Plot”.

Representative results

In this system, a glucose-dependent change in glycosome composition was observed. When cells are grown in glucose containing media, two populations are observed; one bright and one dim (Fig. 3.2A). Dim cells harbor immature glycosomes,
which have not imported the PTS2eYFP while bright cells harbor a mixture of mature and immature glycosomes (12). When glucose is present in the media, mislocalization of glycosome proteins is lethal (15, 28) and glycosome protein expression is likely coupled closely with import. This tight regulation is responsible for the appearance of dim cells in which glycosome protein expression is suppressed when the cells lack sufficient import capability. Once the glycosome import machinery is fully assembled and functional, the cells express glycosome proteins, which are then imported into glycosomes, yielding fluorescent cells. In glucose-deplete media, the mislocalization of glycosome proteins is tolerated (15), and the bimodal population distribution is replaced by a single peak with a broader range of fluorescence (Fig. 3.2B).

This system has been used to identify conditions that trigger changes in glycosome composition. When high density cultures containing ~10% dim cells (Fig. 3.3A) are passed into fresh media, there is a transient increase in the percentage of dim cells (Fig. 3.3B). By 24 hr. the original population distribution is re-established (Fig. 3.3C).
**Figure 3.2. Glucose-dependent glycosome composition.** PCF cells were grown in SDM79 (+Glc) or SDM80 (-Glc) and analyzed by flow cytometry. Histograms of 10,000 events. **A.** Analysis of cells grown in SDM79 consistently reveals two peaks. Fluorescent cells harbor a mixture of mature and immature organelles with cells of higher fluorescence intensities having more mature glycosomes. In SDM79, mislocalization of glycosome proteins is lethal and glycosome protein expression and import must be tightly controlled. This is reflected in the absence of cells with intermediate fluorescence intensities. **B.** In SDM80, the mislocalization of glycosome proteins is tolerated, the bimodal population distribution is lost, and cells of intermediate fluorescence are observed.
Figure 3.3. Glycosome remodeling. Remodeling of glycosomes is triggered by passage into fresh media. **A.** Histogram of 10,000 events in the starting culture (~4 x 10⁶/ml). **B.** Three hours after dilution into fresh SDM79 there is an increase in the proportion of dim cells with immature glycosomes falling within the left gate. **C.** Histogram of the diluted culture after 24 hr. After 24 hr, the population distribution has returned to normal, as the immature glycosomes now contain the peroxisome proteins necessary to import the fluorescent protein.

**Discussion**

Glycosomes are essential, dynamic, parasite-specific organelles. The processes that regulate the biogenesis, maintenance, proliferation and remodeling of these organelles likely include drug targets that could be exploited for therapeutic purposes. Despite the potentially high abundance of such drug targets, the field of glycosome biogenesis has lagged behind the study of similar processes in other organisms,
predominately due to the lack of a tractable, high-throughput system by which to monitor rapid, dynamic, organelle responses in live cells.

Fluorescent-organelle reporter systems have been used to study organelle dynamics in higher eukaryotes such as yeast, plants, fungi and mammals (23-25). We have generated a transgenic strain of PCF parasites that express PTS2eYFP that is targeted to mature glycosomes, yielding fluorescent organelles. Changes in glycosome composition can be monitored via following cellular fluorescence. Using this system, we have found that environmental conditions, notably glucose, regulate glycosome composition (12).

In contrast to microscopy methods often used to study organelle dynamics in kinetoplastid parasites, this reporter system offers a method by which to rapidly screen compounds and conditions that influence overall glycosome dynamics. However, the changes in fluorescence reflect changes in overall organelle compositions. Further biochemical and microscopic experiments are required to define the specific molecular differences between cell populations exhibiting different fluorescence intensities.

We have identified a number of critical steps in the remodeling assays. Interestingly, we have found that as the cells are cultured for longer periods (more than two passes), their behavior in response to environmental changes is unpredictable. After prolonged culturing, cells passed from high densities into fresh media, exhibit a temporary increase in the dim population, indicating that they are still able to remodel glycosomes, but die after 24 hr. We have also encountered situations where no remodeling is observed. The reason for this behavior is unclear but we have found that
when cells are handled as described here (limiting the number of cell passages to two and maintaining cultures below $1 \times 10^7$/ml), glycosome remodeling is reproducible. When cells are no longer responsive to environmental conditions, re-transforming cells with the reporter-construct plasmid usually remedies this situation.

While this system has been used to study glycosome behavior in African trypanosomes, it can also be adopted to the study of organelles in other parasites by fusing fluorescent proteins to amino acid sequences that direct protein localization to other cellular compartments.

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References


CHAPTER FOUR

LOCALIZATION OF A TRYPANOSOME PEROXIN TO THE ENDOPLASMIC RETICULUM

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Abstract

Trypanosoma brucei is the causative agent of diseases that affect 30,000-50,000 people annually. T. 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.
Introduction

*Trypanosoma brucei* is a flagellated protozoan parasite that causes human African trypanosomiasis (HAT) and a wasting disease in cattle called nagana (1). 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 membrane-bounded organelles called glycosomes that have been identified as good drug candidates (2, 3). Glycosomes lack DNA, import proteins post-translationally, 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 (4, 5). 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 (5–15).

Peroxisomes are ubiquitous organelles found in most eukaryotic organisms (16). 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 (3).
The processes that regulate peroxisome biogenesis and maintenance have been studied in yeast, plants, and mammals and include organelle proliferation, post-translational protein import, and organelle specific degradation via pexophagy (17). Protein import has been relatively well studied in trypanosomes (4) and glycosome turnover via organelle specific autophagy, pexophagy, has also been documented (18, 19). 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) (16, 20, 21). 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 in vesicles also containing ER proteins (22). In other eukaryotes, Pex13 forms part of the membrane channel that facilitates post-translational import of peroxisomal matrix proteins (23). In addition to its role at the peroxisome membrane, this protein is also found in the ER (24). 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, \textit{T. brucei}, has two Pex13 homologs, \textit{TbPex13.1} and \textit{TbPex13.2} (7, 13, 14). \textit{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 (25). Because \textit{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 \textit{Trypanosoma cruzi}, \textit{Leishmania} and \textit{Crithidia}.

\textit{T. brucei} experiences fluctuating extracellular glucose levels during the life cycle as it alternates between the mammalian bloodstream and the tsetse fly vector (1, 26). 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 5mM glucose. In other eukaryotes, peroxisome composition and biogenesis are influenced by environmental conditions such as nutrient availability (27). In trypanosomes, glycosome metabolism and the requirement for glycosomal protein import vary with extracellular glucose levels (8, 28) and
compartmentalization of glycolytic enzymes is essential at high extracellular glucose levels (8, 29, 30). 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 (YPYDVPDYA) was fused to the 5' end of different *TbPex13.1* variants, which included the full-length open reading frame of *TbPex13.1* (*HATbPEX13.1*), the *TbPex13.1* gene in which the nucleotides (973-1150) 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 (31), 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 brucei* procyclic form (PCF) 29-13s (32) were transfected as previously described (33) using a Bio-Rad Gene Pulser Xcell (Exponential, 1500V, 25µF, 4mm 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) (30) as previously described (34). 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 post-nuclear fractions over an Optiprep gradient (Sigma Aldrich, St. Louis, MO) as described previously (35). 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 15,000g 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) (13), TbPex11 (1:4,000) provided by Dr. Christine Clayton (Zentrum für Molekulare Biologie der Universität Heidelberg, Germany) (36), and TbBiP (1:100,000) provided by Dr. Jay Bangs (University at Buffalo, Buffalo, NY) (31).
Immunofluorescence Microscopy

Immunofluorescence assay (IFA) conditions were modified from that previously described by (37). Cells were harvested at 800g 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$_2$PO$_4$, 16 mM Na$_2$HPO$_4$, 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, St. Louis, MO) 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, Grand Island, NY) 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 (35). We utilized this protocol to examine glycosome composition
under high and low glucose conditions. In agreement with published data (7, 11, 13), 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. 4.1A, 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. 4.1A right panel). Instead, the protein was found in higher fractions (26-30), which also contained the ER resident protein BiP.

Figure 4.1. TbPex13.1 localizes with ER marker proteins in density gradient and has multiple domains. A. PCF parasites were grown in high glucose SDM79 (5 mM glucose) or low glucose SDM80 (< 0.5 mM glucose). Post-nuclear supernatant was resolved by density centrifugation over an Optiprep gradient. One ml fractions were collected from the bottom of the gradient, and 2.5µg protein from each fraction was resolved by SDS-PAGE and assayed by western blotting with antibodies that recognize,
aldolase (TbAld), 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 (grey oval), an SH3 domain and a PTS1 sequence (black rectangle).

*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* (7). 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. 4.1B). 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 (25). 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. 4.2A) as is typically observed with *T. brucei* glycosomes (13). Additionally, BiP localization did not change with extracellular glucose levels. In both high and low conditions, BiP antibodies labeled tubular structures (Fig. 4.2B) consistent with ER (38).

**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 (13) and our fractionation experiments (Fig. 4.1A), HATbPex13.1 localized to punctate structures distributed throughout the cytoplasm in a pattern that largely overlapped with the glycosome-resident protein aldolase (Fig. 4.3A). Cells expressing HATbPex13.1 were also stained with antibodies against the ER protein BiP, which labeled reticular structures typical of ER staining (Fig. 4.3B). Under these high glucose conditions, HATbPex13.1 staining exhibits more overlap with glycosomal aldolase than with ER localized BiP.
Figure 4.2. 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 4.3. HATbPex13.1 localizes to glycosomes in SDM79 glucose rich media.

PCF 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).
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. 4.4). 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. 4.4A). 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. 4.4B).

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. 4.4 and 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. 4.5A). In low glucose,
however, HATbPex13.1ΔSH3 staining was restricted to foci that also stained with aldolase and BiP (Fig. 4.5B).

**Figure 4.4. 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.
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. 4.6). 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.
Figure 4.5. 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).
Figure 4.6. 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).
Discussion

Glycosomes are highly specialized peroxisomes found only in kinetoplastid parasites (39–42). 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 coordination of peroxisome protein import, organelle biogenesis and organelle degradation (16). 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 (5–15).

The overall mechanism of protein import into glycosomes appears to be conserved with higher eukaryotes (4). 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 (17).

The extent to which each proliferation pathway predominates varies with organism, cell type, life cycle stage, and environmental regulation (17). In yeast, peroxisomes are usually formed by fission of existing organelles (43) except when cells are temporarily depleted of peroxisomes through deletion of PEX genes essential for peroxisome biogenesis (44, 45). 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
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 (22). 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 (5).

T. brucei encounters a number of different environmental conditions as it alternates between the mammalian bloodstream and the insect vector (1). In the mammalian bloodstream, glucose levels are present at relatively constant and high levels (~5mM). After the parasites are taken up during a blood meal, glucose levels fall to undetectable levels within 15 min (47). Glycosome composition of bloodstream form (BSF) parasites differs significantly from insect, procyclic form (PCF) parasites (35). Furthermore, glycosome metabolism (29, 30), glycosome composition (48) and the requirement for glycosome protein import in the PCF parasite (8) 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 (13, 35). 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 (49, 50). 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 as 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 (51). 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 (13), HA-TbPex13.1 localized to glycosomes of cells grown in high glucose. In low glucose, however, the HA-TbPex13.1 expressing cells had altered ER and glycosome morphology.

Our fractionation experiments were performed with 3 x 10^10 cells (Fig. 4.1). 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) (50, 52). 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 (36).

This extraglycosomal localization is likely not due to overexpression of the fusion protein in low glucose. Western analysis showed that HA-TbPex13.1 is expressed at lower levels in low glucose in comparison to high glucose (Appendix, Fig A-1). 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. 4.2).

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 (25). 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 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 (53, 54). The SH3 domains of Pex13 in yeast (55) and humans (56) bind directly to Pex5p. Mutations in the S. cerevisiae Pex13 SH3 domain blocked function, but Pex13 was still targeted to the peroxisome membrane (55). In yeast two hybrid, the TbPex13.1 SH3 domain interacted with TbPex13.2 (7). 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 suggests 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.

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References


CHAPTER FIVE

UNDERSTANDING THE GLUCOSE DEPENDENT REGULATION OF GLYCOosomal PROTEIN EXPRESSION

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**Abstract**

*Trypanosoma brucei* is the causative agent of Human African Trypanosomiasis, a disease affecting approximately 30,000 people per year. *T. brucei* and other kinetoplastids have essential, highly specialized peroxisomes that compartmentalize most of the enzymes involved in glycolysis, called glycosomes. In other eukaryotes, peroxisome composition is influenced by changes in environmental conditions, including nutrient availability. Glycosome composition is influenced by extracellular glucose concentration, a nutrient whose availability changes throughout the life cycle of *T. brucei*. After the tsetse fly host takes in a blood meal, glucose is depleted in the procyclic from (PF) environment within the midgut in 15 minutes. In order to adapt to this change in nutrient availability, PFs can metabolize proline in the absence of glucose. In the lab, PF parasites are often grown in media containing ~ 5mM glucose. We are interested in defining the glycosome composition and behavior under low glucose conditions encountered in the fly. Here, we present fluorescent and electron microscopy images of glycosomes in parasites grown in high and low glucose conditions demonstrating changes in overall organelle composition. Changes in glycosomal protein expression are also seen by western blot analysis. Additionally, we provide preliminary support that glucose dependent regulation of glycosomal protein expression may occur at the level of mRNA stability combined with an overall decrease in translation in cells grown in low glucose conditions compared to those grown in high glucose conditions. This data will provide insight into the dynamic nature of glycosomes and how organelles are remodeled to meet the metabolic needs of cells.
Introduction

Human African trypanosomiasis is a lethal disease caused by the protozoan parasite *Trypanosoma brucei* (1). *T. brucei* has a complex life cycle in which nutrient availability changes as the parasite is passed between mammalian hosts by the tsetse fly vector. Bloodstream form (BSF) parasites in mammalian hosts are exposed to high glucose availability and rely solely on glycolysis for the generation of ATP. Procyclic forms (PF) in the tsetse fly midgut experience constantly fluctuating glucose availability. When the tsetse fly first takes in a blood meal, glucose availability in the fly midgut is similar to that of the mammalian blood stream, approximately 5 mM. Within 15 minutes, glucose concentration has dropped to undetectable levels (2). In the absence of glucose, PF *T. brucei* rely on the metabolism of proline for ATP generation (3, 4).

Unlike other eukaryotes in which glycolysis is a cytosolic process, *T. brucei* sequester most of the enzymes involved in glycolysis in organelles evolutionarily related to peroxisomes called glycosomes. This compartmentalization is essential for *T. brucei* survival (5–8). Mislocalization of the glycolytic proteins to the cytoplasm results in cell death (9, 10). Like peroxisomes, glycosomes are single membrane bound organelles whose biogenesis and protein import are carried out by homologous proteins called peroxins. Glycosomes lack any DNA so fully folded proteins are imported post-translationally from the cytoplasm (7, 11, 12). In addition to glycolysis, glycosomes compartmentalize beta-oxidation of fatty acids, pyrimidine salvage, purine salvage, pentose phosphate pathway, ether lipid synthesis, and gluconeogenesis (13, 14).
Peroxisome composition varies depending on the organism and cell type, and can be influenced by changes in environmental conditions. For example, when methylotrophic yeast *Hansenula polymorpha* are grown in conditions where methanol is the available carbon source, peroxisome proliferation is induced. Transfer back to glucose containing conditions results in degradation of existing peroxisomes (15). Similarly, high fatty acid growth conditions trigger peroxisome proliferation in *Saccharomyces cerevisiae*, which then degrade their peroxisomes upon transfer back to glucose containing media (16).

Changes in peroxisome composition correspond to changes in expression of peroxisomal proteins. Environmentally-dependent transcriptional regulation of peroxisomal proteins has been demonstrated in yeast and mammals (17–20). In *S. cerevisiae*, transcription factors regulate expression of peroxisomal proteins involved in fatty acid metabolism that are upregulated during peroxisome induction in high fatty acid conditions (17–19).

Glycosome composition is altered during life cycle differentiation and in response to environmental changes. During differentiation from BSF to PF, glycosomes are remodeled by autophagy (21) and differences in glycosome composition between the two life cycle stages have previously been demonstrated (13). Using a fluorescent organelle reporter system consisting of a peroxisomal targeting signal (PTS) fused to an enhanced yellow fluorescent protein (eYFP), we demonstrated that glycosome composition is also influenced by glucose availability (22, 23).
Although we have gained understanding of how glycosome composition is dependent on the life cycle stage and environmental conditions of *T. brucei*, our understanding of the mechanism by which they regulate glycosomal protein expression to coordinate these composition changes is lacking. Unlike most eukaryotes, trypanosome transcription is polycistronic (24, 25) and thus gene regulation occurs post-transcriptionally. Gene expression is regulated by changes in mRNA stability, levels of translation, or protein degradation (25). RNA binding proteins regulate mRNA stability by binding to 3’ untranslated regions (UTR) and can either stabilize the mRNA, increase efficiency of its translation, or target the mRNA for degradation thereby determining the half-life of the mRNA (26–28).

PF parasites must adapt to fluctuating glucose availability in the midgut of the tsetse fly in order to survive (2) and changes in overall glycosome composition in response to extracellular glucose have been previously demonstrated (22). Here we begin to characterize the specific changes that occur in glycosome composition in PF *T. brucei* in response to changes in glucose availability and investigate the mechanism by which *T. brucei* regulates changes in glycosomal protein expression that correspond to changes in composition.
Materials and Methods

Growth of parasites.

To compare high and low glucose conditions, cells were cultured in SDM79 media (+Glc) containing 5mM glucose or in SDM80 (-Glc) containing less than 0.5mM glucose as previously described (3). To ensure cultures were adapted to each condition, cells were maintained in respective media types for >4 days before being used for downstream analysis. PF 29-13s were maintained with G418 (15 µg/ml) and hygromycin (50 µg/ml). Reporter cells line 29-13 pXS2-Aldo-PTS-eYFP (PTS2-FP) were generated as previously described (22, 23) and were maintained with G418 (15 µg/ml), hygromycin (50 µg/ml), and blastcidin (15 µg/ml).

Live-cell microscopy.

To compare fluorescence in cells cultured in +Glc and –Glc, PTS2-FP parasites were harvested by centrifugation at 800xg for 10 min and resuspended in phosphate buffered saline (PBS). Cells were applied to a clean slide, imaged and analyzed on a Zeiss Axiovert 200M with Axiovisoin software 4.8.2.

Transmission electron microscopy (TEM).

Samples were prepared for TEM as described in Bauer et al. 2013 (22). To compare ultrastructural morphology of glycosomes in parasites cultured in +Glc and –Glc, 29-13s were harvested by centrifugation at 800xg for 10 mins, resuspended in fix solution (2% paraformaldehyde-2.5% glutaraldehyde in 100mM phosphate buffer) and
incubated at room temperature for 1 hour. Fixed cells were washed with PBS, postfixed for 1 hour in 1% osmium tetroxide (Polysciences Inc., Warrington, PA) and washed with distilled water (dH₂O). Cells were en bloc stained for 1 hr with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, CA) and then washed with excessive dH₂O. A graded series of ethanol solutions was used to dehydrate samples. Cells were en bloc stained for 1 hr with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, CA) and then washed with excessive dH₂O. A graded series of ethanol solutions was used to dehydrate samples. Cells were embedded in Eponate 12 resin (Ted Pella Inc.) and 90nm ultrathin sections were obtained using a Leica Ultracut UCT ultramicrotome (Leica Microsystems Inc., Bannockburn, IL). Sections were stained with lead citrate and uranyl acetate. A JEOL 1200 EX transmission electron microscope (JEOL USA Inc., Peabody, MA) was used to image sections at the Molecular Microbiology Imaging Facility, Washington University School of Medicine, St. Louis, MO.

**Glycosome measurements.**

TEM measurements were performed using ImageJ software. Visible glycosomes were counted and the area of over 100 visible cells and 600 individual glycosomes from three separate biological replicates per condition was measured. For each condition, the following calculations were made. Glycosome areas were averaged. Glycosome density was calculated by dividing the total number of glycosomes by the total area of cells visible and converting to glycosomes/100µm². Percentage of total cell area was calculated by dividing the sum of all glycosome areas by the sum of all cell areas and multiplying by 100%.
**Western blot analysis of glycosomal proteins.**

To compare changes in expression of glycosomal proteins, 1x10^6 cells were harvested at 800xg for 10 mins and resolved by 12% SDS-PAGE. After transfer, membranes were probed with antibodies against *T. brucei* PEX13.1 (1:10,000), PEX11 (1:4,000) (provided by Christine Clayton, Universität Heidelberg, Heidelberg, Germany), aldolase (1:50,000), hexokinase (1:100,000), and commercially available tubulin (1:8,000) (Millipore clone YL1/2, Temecula, CA). Signal quantification was performed using ImageJ software.

**Quantitative Real Time PCR.**

Cells were harvested (2x10^6) and RNA was isolated using the Aurum Total RNA Mini Kit (BioRad). Quantitative real time PCR was carried out using the Verso SYBR Green 1-step kit with primers for PEX13.1, PEX11, aldolase, and hexokinase. Relative quantification of mRNA was compared to the housekeeping genes Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and 60s rRNA using the 2^{-ΔΔCt} method.

**Glycosomal protein half-life.**

To determine protein half-life, cells grown under each condition were treated with 100µg/ml of cycloheximide. For western blot analysis, 2x10^6 cells were harvested immediately prior to treatment (P), immediately following treatment (0), at 1 hour (1), 2 hours (2), 4 hours (4), and 6 hours (6). Samples were resolved by SDS-PAGE. Western
blots were probed with anti-aldolase (1:50,000) as a glycosomal representative, and tubulin (1:8,000) (Millipore clone YL1/2, Temecula, CA) as a control.

Ribosomal profile analysis.

To compare overall levels of translation in +Glc and −Glc, polysomes from each condition were isolated and polysome profiles were generated using methods adapted from Brecht et al. 1998 (29). Briefly, 5x10^8 cells were harvested at 800xg for 10 minutes, resuspended in 5mL of media containing 100µg/ml cycloheximide and incubated for 5 min. Cells were centrifuged for 800xg for 10 mins and washed with ice cold PBS containing 100µg/ml cycloheximide, 1µM DTT, and ~5U/µL RNasin. Cells were resuspended in 750µL ice cold buffer A (10mM Tris-HCL, pH 7.4; 10mM MgCl_2; 300mM KCl; 100µg/ml cycloheximide; 1µM DTT; ~U/µL RNasin) and incubated on ice for 3 minutes. 125µL of lysis buffer (buffer A with 1.0% (v/v) Triton X-100 and 0.2M sucrose) was added and cells were passed through a 21G needle 20-30 times. Lysate was centrifuged at 15,000xg for 10 min at 4°C. Supernatant was transferred to a tube with 32µL of 5M NaCl, 100µL heparin, and 1µL of 40U/µL RNasin. For a control, samples were divided in half and 10µg/mL puromycin was added to the control sample for each condition to dissociate polysomes. 400µL of extract from each sample and control sample was added to a 3mL 15-50% sucrose gradient and centrifuged for 2 hours at 230,000xg at 4°C. 25 fractions of 30µL were pipetted from the top of the gradient and loaded directly into a 96 well UV plate. Then 30 fractions of 75µL were pipetted from
the top of the remaining gradient and placed in labeled tubes. 30µL from each tube was loaded into the same 96 well. The OD\textsubscript{254nm} was measured for each plate and data points were plotted in Microsoft Excel to generated a polysome profile curve.

Results

\textit{Glycosome composition is heterogeneous in low glucose conditions.}

We previously adapted a fluorescent organelle reporter system commonly used to study peroxisome dynamics in yeast and mammals to \textit{T. brucei}. Reporter cell lines express the PTS2 of aldolase fused to eYFP, enabling visualization of overall changes in glycosome composition by microscopy (22, 23). We cultured these cells in +Glc media and –Glc media and found that in +Glc media, glycosomes are homogenous and distributed throughout the cell (Fig 5.1A). However, under low glucose conditions glycosomes are heterogeneous with some cells having glycosomes distributed throughout the cell, much like +Glc, and other cells appearing to have fewer PTS-FP positive glycosomes (Fig 5.1B).

We next analyzed cells grown in +Glc and –Glc media by TEM (Fig 5.1C and D). Although average glycosome area remained similar under both conditions being 0.074\(\mu\text{m}^2\) and 0.084\(\mu\text{m}^2\) for +Glc and –Glc respectively, contrary to the fluorescent reporter cell data there were approximately four times the number of glycosomes per 100\(\mu\text{m}^2\) in –Glc compared to +Glc (Fig 5.1E and F). Glycosomes made up only 1.46% of the total cell area in +Glc but 7.21% in –Glc (Fig 5.1G). In addition, in +Glc the
electron density appeared to be similar in all glycosomes, while in –Glc there was a
greater diversity in electron density. These findings suggest that there may not be fewer
glycosomes in PTS-FP cells grown in –Glc media, just fewer PTS-FP positive
glycosomes.

Figure 5.1. Glycosome composition changes in response to glucose availability.
Live cells expressing PTS-FP grown in +Glc (A) and –Glc (B). TEM images of cells
grown in +Glc (C) and –Glc (D) with glycosomes indicated by orange arrows (scale bar =
0.5µm). Average area of glycosomes (E), number of glycosomes/cell area (F), and percent cell area comprised of glycosomes (G).

Glycosomal protein expression is down regulated in low glucose conditions.

Colasante et al used mass spectrometry to compare the proteome of glycosomes isolated from PF and BSF cells. In this study, they demonstrated that only 35% of glycosomal proteins were present in both life cycle stages, while 23% were BSF specific and 42% were PF specific (13). To assess such changes in protein expression in PF cells grown in +Glc and –Glc, we did western blot analysis probing whole cell lysate with a number of known glycosomal antibodies including two membrane bound peroxins involved in glycosome biogenesis, PEX13.1 (30) and PEX11 (31), and two matrix proteins involved in glycolysis, aldolase and hexokinase (32). We quantified expression of each of these proteins under the two conditions and found that PEX13.1 and aldolase were downregulated more than 2 fold in –Glc compared to +Glc (Fig 5.2A and B). PEX11 was slightly downregulated in –Glc and Hexokinase was upregulated in –Glc.

Glycosomal protein mRNA abundance change in low glucose conditions.

Trypanosomes can regulate protein expression by regulating mRNA stability (25). We performed qRT-PCR and analyzed glycosomal protein mRNA levels relative to reference genes, HGPRT and 60s rRNA, using the 2^ΔΔCt method. Corresponding to protein expression, PEX13.1 and PEX11 mRNA levels were reduced while hexokinase
was upregulated in –Glc (Fig 5.2C). Aldolase mRNA abundance was similar under both conditions. This data combined with protein expression data (Fig 5.2) suggests that PEX13.1, PEX11, and HK expression may be regulated at the level of mRNA abundance while aldolase expression could be regulated by a mechanism other than mRNA stability, such as translation or protein degradation. Although changes in mRNA levels were statistically significant, we have yet to confirm their biological significance. In the future, more replicates will help us to better understand the relationship between mRNA abundance and glycosomal protein expression.

Aldolase protein expression is not regulated by degradation.

Protein abundance can be regulated through protein degradation via targeting to the proteasome or lysosome (33). To determine if aldolase protein expression is influenced by changes in protein half-life, we treated cells with cycloheximide to inhibit protein expression and monitored glycosomal protein levels over time via western blot analysis. We found that aldolase was very stable, having a half-life greater than 6 hours in both +Glc and –Glc (Fig 5.3A and B) in agreement with previous findings by Clayton 1987 (34). Tubulin half-life was approximately 2 hours, as previously determined (35). Such stability suggests that aldolase expression regulation occurs by a mechanism other than protein degradation.
Figure 5.2. Glycosome protein expression is down regulated and mRNA abundance is altered in low glucose.

(A) Cells were grown in +Glc and -Glc and protein expression measured by western analysis using antibodies against different glycosome proteins. (B) Bands on the western blots were quantitated using Image J. (C) mRNA was quantified by qRT-PCR, and analyzed by the 2-ΔΔCt method. (+Glc: grey; -Glc: purple)
Figure 5.3. Aldolase stability is not altered in low glucose.

Cells grown in +Glc (A) and -Glc (B) were incubated with cyclohexamid to block translation. Cell lysates from pre-treated cells (P) and time points taken at 0 through 6 hours were analyzed by western blotting with anti-aldolase antibodies.

Association of mRNA with polysomes is decreased in low glucose conditions.

To measure changes in overall protein translation in response to changes in glucose availability, we used density gradients to isolate actively translating ribosomes (polysomes) and the messages associated with them. Preliminary polysome profiles suggest that there is an overall decrease in the amount of polysomes and increase in free ribosomes in –Glc compared to +Glc (Fig 5.4) suggesting that protein translation is downregulated in –Glc. It is possible that the decrease in overall translation could also play a role in the downregulation of glycosomal protein expression, however, this is
unlikely as we have yet to observe a reduction of non-glycosomal proteins under low glucose conditions.

![Free ribosomes and monosomes vs Polysomes](image)

**Figure 5.4. Polysomes decrease in low glucose conditions.**

Cells were grown in +Glc (blue) and –Glc (red). Fractions from each sucrose gradient were collected by hand and measured at OD\textsubscript{254nm}. Polysome profile was generated by plotting the OD\textsubscript{254nm} measurements in Microsoft Excel.

**Discussion**

Previous studies in glycosome composition have primarily been conducted in high glucose conditions (13, 14). Glucose concentration remains at undetectable levels the majority of the time for PF *T. brucei* in the tsetse fly midgut. Only when the fly takes in
a blood meal will the glucose availability spike to that of mammalian blood (~5mM) (2). Glycosome composition changes during BSF and PF life cycle stages to better adapt the parasites to the environmental conditions encountered in hosts and vector (13). In a similar manner, it seems logical that PF glycosomes, as the home of the first seven glycolytic enzymes in PF cells as well as a number of other metabolic pathways (13, 14), would be dynamic and have compositions that fluctuate in response to changes in nutrient availability. Gaining understanding of the changes in glycosome composition that occur in both high and low glucose conditions and the mechanisms by which these regulations occur could provide new understanding about glycosome plasticity and how parasites adapt to changes in nutrient availability.

In this study we examine how glycosome composition is influenced by changes in extracellular glucose. We first utilized PTS-FP cells to observe differences in overall glycosome composition. We find that while in +Glc cells appear to have homogeneous glycosome distribution, in –Glc glycosome distribution in cells is more heterogeneous with some cells appearing to have fewer glycosomes. This is contradictory to the TEM image analysis demonstrating that in –Glc cells have ~4X the number of glycosomes (Fig 5.1). Together this data suggests that in –Glc there may be PTS-FP negative glycosomes not visible by fluorescence microscopy. This would explain why cells that have more glycosomes appear to have less when expressing PTS-FP.

We believe that this could be a reflection of cells possessing more than one population of glycosomes under low glucose conditions. We hypothesize that there is more than one “type” of glycosome and that under different conditions certain types may
predominate. Preliminary results from our lab of mass spectrometry analysis of glycosomes isolated from +Glc and –Glc suggest that in –Glc conditions enzymes involved in gluconeogenesis are enriched more than two fold (Appendix Fig A-2). It is possible that in –Glc conditions cells could be increasing the number of glycosomes that contain gluconeogenic activity and that our reporter PTS-FP may not be imported into these organelles.

Environmentally influenced changes in expression and localization of some peroxisomal proteins have been demonstrated to occur in *S. cerevisiae* when grown on oleate compared to glucose (36). We noted a decrease in expression in glycosomal proteins when cells were grown in –Glc (Fig 5.2). The proteins that we probed against were two matrix proteins involved in glycolysis, hexokinase and aldolase (32), and two membrane bound peroxins involved in glycosome biogenesis, PEX11 (31) and PEX13.1 (30). If our hypothesis that there is more than one kind of glycosome is correct, we may see an increase in expression in certain glycosomal proteins involved in processes other than glycolysis in –Glc compared to +Glc, such a gluconeogenesis. As a protein involved in glycosome biogenesis, we were surprised to see a decrease in PEX13.1 expression. However, *T. brucei* also expresses a second PEX13 that is likely involved in matrix protein import called PEX13.2 (37). It is possible that PEX13.1 and PEX13.2 are present in different types of glycosomes and help to regulate the proteins imported into each population of organelles. It was recently demonstrated in *S. cerevisiae* that a matrix protein receptor peroxin, PEX9, selectively imports specific peroxisome matrix proteins in an environmentally dependent manner (38, 39). If PEX13.1 and PEX13.2 are in
different types of glycosomes, we may see an increase in expression of PEX13.2 in –Glc conditions. We are in the process of generating antibodies against a PEX13.2 peptide. Although we see an increase in expression in the glycolytic enzyme hexokinase, we believe this protein may be moonlighting in a role outside of glycolysis in low glucose conditions.

We are currently in the process of conducting mass spectrometry analysis on glycosomes isolated from cells grown in +Glc and –Glc to gain understanding about the detailed molecular differences in glyosome composition under these two conditions. In an effort to determine if there are more than one population of glycosomes, we plan to conduct microscopy experiments comparing the localization of enzymes involved in a number of different glycosomal processes. A partial or total lack of colocalization between glycosomal proteins involved in different processes would provide support that more than one population of glyosome exists. In the future, isolating different glycosomal populations would allow for detailed mass spectrometry analysis of enzyme content, providing information about the division of functions between populations.

*S. cerevisiae* regulates nutrient dependent expression of peroxisomal proteins through the activation of transcription factors (17–19). *T. brucei* transcription is polycistronic and thus most regulation occurs post-transcriptionally (25). To determine the mechanism by which *T. brucei* regulates environmental dependent expression of glycosomal proteins, we first compared overall levels of the mRNA of glycosomal proteins, PEX13.1, PEX11, aldolase, and hexokinase. In most studies conducted to date mRNA half-lives depend on sequences within their 3’ UTR that are recognized by RNA
binding proteins that can stabilize mRNAs or target them for degradation (25–28).

Destabilization of glycosomal protein mRNA in −Glc could provide a way to decrease expression. By western blot analysis we see a decrease in PEX13.1, PEX11 and aldolase and an increase in hexokinase (Fig 5.2A and B). Similarly, we see a statistically significant decrease in mRNA levels for PEX13.1 and PEX11 and an increase in hexokinase mRNA (Fig 5.2C). While aldolase protein expression is decreased in −Glc compared to +Glc, mRNA levels remain unchanged. Based on our findings it is possible that regulation of glycosomal proteins occurs at the level of mRNA stability.

We treated cells with cycloheximide to look for differences in protein half-life under +Glc and −Glc. Under both conditions aldolase was very stable, with a half-life beyond 6 hours suggesting that protein degradation may not play a role in aldolase protein expression regulation (Fig 5.3).

We are in the process of examining the role of translational regulation of glycosomal proteins. Preliminary data comparing polysome profiles from cells grown in +Glc and −Glc demonstrate a decrease in the number of polysomes in low glucose conditions (Fig 5.4). It is possible that regulation of glycosomal proteins could result from an additive effect of down regulation of mRNA message and decrease in overall translation. It is unlikely that this decrease in overall protein translation in −Glc conditions alone results in a decrease in glycosomal protein expression. If this were the case, we would also expect to see reduced expression of non-glycosomal proteins – a phenotype which we have yet to observe.
While mRNA levels of some of the glycosomal proteins that we have investigated so far correlate to amount of protein, this may not be the case for other proteins as it has been demonstrated that in trypanosomes mRNA and protein levels only moderately correlate (40–42). We have yet to identify the mechanism by which aldolase expression is decreased in –Glc. Regulation of glycosomal protein expression may be multifaceted and depend on the kind of protein (ie membrane bound vs. matrix, peroxin vs. metabolic, etc.) or the type of stimulus initiating glycosomal remodeling.

We are in the process of gaining a better understanding of the mechanisms *T. brucei* uses to regulate glycosomal proteins. Northern blot analysis of polysome fractions probing against glycosomal protein mRNA may provide insight as to whether there is a change in translation of glycosomal proteins between +Glc and –Glc. We also plan to conduct RNA-seq on RNA isolated from whole cells and from polysome fractions. This will enable us to compare whole cell level of mRNA messages to what is actively being translated providing insight into the correlation of mRNA abundance and protein translation. If mRNA abundance appears to play a key role in the regulation of glycosomal proteins, detailed comparison of their 3’ UTR sequences may identify specific binding sights for RNA binding proteins that could be stabilizing or destabilizing these messages. In addition, mass spectrometry analysis of whole cells grown in +Glc and -Glc to compare RNA binding protein expression under both conditions may help to identity binding proteins potentially involved.

The findings discussed in this paper demonstrate a level of glycosome heterogeneity heretofore unappreciated. Regulating glycosomal composition is likely
essential for *T. brucei* to adapt metabolic needs to changing environmental conditions. Although standard lab procedure is to culture PF *T. brucei* in the presence of glucose, culturing under low glucose conditions may give a more realistic picture of parasite biochemistry in the tsetse vector host. While we have focused our efforts on characterizing the differences in response to changes in glucose availably, there may be other metabolites or environmental factors that can influence glycosomal protein composition and expression. Better understanding of how *T. brucei* parasites adapt to changing environmental conditions could lead to identification of new drug targets.

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**References**


CHAPTER SIX
SUMMARY AND FUTURE DIRECTIONS

Historically, *Trypanosoma brucei* glycosomes were considered to be static organelles within the mammalian bloodstream form (BSF) and tsetse fly vector procyclic form (PF) life cycle stages. Five years ago, our understanding was that organelles within each stage harbored a defined proteome (1) which was remodeled only during differentiation (2). PF glycosomes sequester a number of metabolic pathways including glycolysis, purine salvage, pyrimidine biosynthesis, peroxide metabolism, beta-oxidation of fatty acids, the pentose phosphate pathway (1, 3), and gluconeogenesis (4–6). *T. brucei* PF parasites preferentially utilize glycolysis for the generation of ATP but in the absence of glucose are capable of metabolizing proline to generate ATP and to produce glucose via gluconeogenesis, feeding the pentose phosphate pathway for NADPH production (4).

Unlike peroxisomes in other eukaryotes whose biogenesis occurs both through growth and division and ER-derived *de novo* mechanisms (7, 8), trypanosome glycosomes were thought to proliferate only through growth and division of existing organelles (9) while evidence for a *de novo* mechanism was scarce. In addition, traditional lab procedures entailed culturing PF parasites in media containing 5mM glucose, in stark contrast to the tsetse fly midgut where glucose remains at undetectable levels with occasional and brief spikes to ~5mM during the 15 minutes immediately following a blood meal (10).
The work presented in this dissertation provides a few examples of how our understanding of glycosomes has changed in the recent half decade. In Chapter One, we review recent findings offering support for the existence of a mechanism of de novo glycosomes biogenesis in *T. brucei*. Chapter Two and Three summarize our methodology using the fluorescent organelle reporter, PTS-FP, to demonstrate how glycosomes are dynamic organelles in PF *T. brucei*. Our results show that glycosome composition changes in response to glucose availability and that changes in extracellular environment can induce remodeling of glycosomes in a time frame consistent with autophagy (11, 12). *De novo* glycosome biogenesis following autophagy could provide a rapid mechanism to restore glycosome populations better suited for new environmental conditions or life cycle stages.

In Chapter Four we present evidence of a peroxin involved in glycosome matrix protein import, TbPEX13.1, exhibiting glucose dependent localization. In media containing glucose TbPEX13.1 localizes to glycosomes as previously published. However in low glucose conditions, TbPEX13.1 appears to localize to the ER (13). Localization of PEX13 to the ER in yeast and mammals occurs during ER-derived de novo biogenesis of peroxisomes (14–17). Such change in localization suggests that glycosomes may be proliferating through different mechanisms under each condition, relying on growth and division in high glucose conditions and de novo processes under low glucose conditions. Different mechanisms of biogenesis could contribute to the heterogeneous glycosome populations exhibited in low glucose conditions discussed in Chapter Five.
We now understand that glycosomes are dynamic, heterogeneous organelles whose composition can be remodeled to adapt PF *T. brucei* to changes in environmental conditions. Contrary to the static model of glycosome composition in which organelles only replicate through growth and division, the findings presented in this dissertation have lead us to a different model. We hypothesize that PF parasites harbor different populations of glycosomes with distinct protein compositions specializing in specific glycosomal functions and that changes in environmental conditions and metabolic needs for cells could result in a redistribution of these populations (Fig 6.1).

In Chapter Five, we show that cells cultured in low glucose conditions have approximately four times the number of glycosomes visible by EM with varying electron densities (Fig 5.1). Fluorescence images of cells expressing PTS-FP show that low glucose cultured parasites appear to have fewer PTS-FP positive glycosomes compared to high glucose conditions suggesting that there are different glycosome populations, with different matrix protein repertoires.

In Chapter Two, we identified two populations of PTS-FP cells in culture: a bright population and a dim population (Fig 2.2). At the time we hypothesized that these may be mature import competent and immature import incompetent glycosomes based on the fact that a number of glycosomal proteins in the dim population were undetectable by western blot analysis, including PEX13.1 (11). The data presented in Chapter Five now leads us to believe that these may be different populations of glycosomes all together.
Figure 6.1. Hypothesized model of environmentally regulated glycosome heterogeneity and dynamic biogenesis.

Green glycosomes represent PTS-FP positive glycosomes that contain enzymes involved in glycolysis. TbPEX13.1 serves as the docking complex in these organelles. Purple glycosomes represent PTS-FP negative glycosomes containing the enzymes involved in gluconeogenesis. We propose that TbPEX13.2 may serve as the docking complex in these organelles. Different proportions of green and purple glycosomes exist in +Glc and –Glc and may be regulated by adjusting the contribution of growth and divisions, which predominates in +Glc, and ER-derived *de novo* biogenesis, which predominates in –Glc. Differences in matrix proteins repertoire between green and purple glycosomes could extend beyond glycolysis and gluconeogenesis which are highlighted here for simplicity and will be investigated in greater detail in the future.
Preliminary mass spectrometry analysis of cells cultured in low glucose conditions (where we see such heterogeneous glycosome populations), show a two-fold increase in the proteins involved in gluconeogenesis (Appendix Fig B-1). In dim populations FBPase, a gluconeogenesis specific enzyme, and triosephosphate isomerase (TIM), a bidirectional enzyme involved in gluconeogenesis and glycolysis, are both detectable by western analysis in while other glycosomal enzymes are not (Fig 2.2). In addition, glycosome densities appear to be similar in TEM images captured of bright and dim populations (Fig 2.3) (11) suggesting that dim cell glycosomes do contain protein and are not empty, immature organelles. It is possible that the cells with fewer PTS-FP positive glycosomes in low glucose conditions discussed in Chapter Five and the dim cells discussed in Chapter Two contain a greater proportion of glycosomes that are PTS-FP negative and harbor gluconeogenesis or other glycosomal metabolic processes and not glycolysis. Gluconeogenesis has been demonstrated to play a role in NADPH homeostasis in *T. brucei* grown under low glucose conditions (4). An increase in the number of glycosomes specializing in gluconeogenesis could reflect a need for NADPH production.

In addition to changes in matrix protein expression, we also noted a decrease in TbPEX13.1 expression in dim cells compared to bright cells in Chapter Two (Fig 2.2) (11) and in cells cultured in low glucose conditions compared to high glucose conditions (Fig 5.2 and Fig A-1). As previously discussed in Chapter Five, it is possible that *T. brucei*'s second PEX13, TbPEX13.2 (18), could be serving as the acting PEX13 in this second type of glycosome. Environmentally induced expression of PEX9 in
Sarcchomyces cerevisiae has been demonstrated, where this peroxin selectively imports certain proteins into peroxisomes serving as a mechanism to remodel these organelles in response changes in carbon source availability (19, 20). In a similar manner TbPEX13.1 and TbPEX13.2 could each serve as the docking complex of specific types of glycosomes, regulating import of only glycosomal matrix proteins involved in specific functions.

A change in the mechanism of organelle biogenesis reviewed in Chapter One could provide a way to adjust proportions of glycosome populations. By altering the level at which growth and division and de novo organelle biogenesis contribute, glycosome composition could be regulated based on the metabolic needs of the cell. Localization of TbPEX13.1 to the ER under low glucose conditions (Fig 4.4) may be a reflection of changes in glycosome biogenesis as the cell adaptsto changes in extracellular glucose (13).

In the future, we plan to test this hypothesis by comparing localization of TbPEX13.1 and TbPEX13.2 as well as different glycosomal metabolic pathways, such as glycolysis, gluconeogenesis, and purine salvage. If different types of glycosomes exist, staining for enzymes involved in different metabolic pathways may show little to no overlap in immunofluorescence colocalization studies. If different organelle populations can be identified, isolation of the different types of glycosomes and mass spectrometry analysis of each could provide a detailed picture of the differences in protein repertoire and function of each population.
Our understanding of glycosomes has drastically changed in the past decade. We now appreciate that these organelles are heterogeneous and dynamic and that regulation of their composition is likely essential for adaptation to changes in extracellular environment for *T. brucei*. Traditional methods of PF culturing limit the amount of knowledge we can gain about glycosome biogenesis and maintenance that is likely exhibited in the tsetse fly midgut as cells are cultured in a consistently higher and more stable concentration of glucose than PF cells normally encounter in the wild. To more accurately study cellular and metabolic processes in PF *T. brucei*, culturing in low to no glucose conditions that are more similar to what the parasite encounters in the wild should be considered. While we have demonstrated that glucose availability influences glycosomes composition (11, 13) and likely biogenesis, we have yet to test other factors that may have similar effects on *T. brucei* cell biology, such as changes in cell density, temperature, pH, or in availability of other nutrients. Gaining understanding of how changes in glycosome composition enable *T. brucei*’s adaptation to changes in environmental conditions could lead to the discovery of new drug targets – a crucial goal in our battle to end the suffering of human African trypanosomiasis.

References


APPENDICES
Appendix A

HATbPEX13.1 Expression

Figure A-1. HATbPEX13.1 expression is down regulated under low glucose conditions. Cells (2 x 10^6) grown in SDM79 and SDM80 were pelleted, boiled in cracking buffer, and resolved by SDS-PAGE. Western blots were probed with anti-HA and anti-BiP antibodies.
Appendix B

Preliminary Mass Spectrometry Analysis
**Figure B-1:** Mass spectrometry analysis was performed on glycosomes isolated from cells grown in high glucose media (+Glc) and low glucose media (–Glc) via sucrose gradient density centrifugation. A total of 450 glycosomal proteins were identified (protein groups plotted in “Glyosome proteins” pie chart). The abundance of 58 proteins was influenced by glucose availability. 22 of those proteins were enriched >2 fold in glycosomes isolated from cells cultured in +Glc (protein groups plotted in “Enriched in glucose containing media” pie chart). 36 proteins were enriched >2 fold in glycosomes isolated from cells cultured in –Glc (protein groups plotted in “Enriched in low glucose media” pie chart).