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# Characterization of glycosomal RING finger proteins of trypanosomatids

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**Abstract**

The glycosomes of trypanosomatids are essential organelles that are evolutionarily related to peroxisomes of other eukaryotes. The peroxisomal RING proteins—PEX2, PEX10 and PEX12—comprise a network of integral membrane proteins that function in the matrix protein import cycle. Here, we describe PEX10 and PEX12 in *Trypanosoma brucei*, *Leishmania major*, and *Trypanosoma cruzi*. We expressed GFP fusions of each *T. brucei* coding region in procyclic form *T. brucei*, where they localized to glycosomes and behaved as integral membrane proteins. Despite the weak transmembrane predictions for TbPEX12, protease protection assays demonstrated that both the N and C termini are cytosolic, similar to mammalian PEX12. GFP fusions of *T. cruzi* PEX10 and *L. major* PEX12 also localized to glycosomes in *T. brucei* indicating that glycosomal membrane protein targeting is conserved across trypanosomatids.


*Index Descriptors and Abbreviations:* *Trypanosoma brucei*; *Trypanosoma cruzi*; *Leishmania major*; Kinetoplastida; Peroxisomes; Glycosomes; RING; Peroxins; PEX10; PEX12; DAPI, 4′6-diamidino-2-phenylindole dihydrochloride; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; HcRed, *Hereractis crispa* red florescent protein; IFA, immunofluorescence assay; PEX, peroxin; PGK, phosphoglycerate kinase; Tet, tetracycline

## 1. Introduction

*Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania major* are disease causing trypanosomatid parasites collectively referred to as the Tritryps. *Trypanosoma brucei gambiense* and *T. brucei rhodesiense*, the etiologic agents of human sleeping sickness, threaten an estimated 60 million people living in sub-Saharan Africa (http://www.who.int/en/). Infection is fatal without treatment, yet toxicity of and resistance to existing agents remain significant problems. Likewise, Chagas' disease and leishmaniasis, caused by *T. cruzi* and *Leishmania spp.* respectively, are debilitating and potentially lethal diseases for which few effective, non-toxic treatments exist. Exploring novel aspects of trypanosomatid biology is thus a promising avenue for discovering new drug targets that may lead to effective, less problematic drugs. The recent publication of the Tritryp genomes (Berriman et al., 2005; El Sayed et al., 2005a,b; Ivens et al., 2005) has enhanced the feasibility of this goal.

The glycosome is an organelle unique to kinetoplastids (including the trypanosomatids) that is related to the peroxisome of other eukaryotes. Many proteins involved in biogenesis of these organelles and import of proteins into their matrices are conserved across species and are collectively called peroxins (PEX). Like peroxisomes, glycosomes house many pathways such as β-oxidation of fatty acids and ether–lipid synthesis. However, unlike peroxisomes, glycosomes sequester glycolysis (Opperdoes and Borst, 1977) and are essential to cell survival under normal conditions (Furuya et al., 2002; Guerra-Giraldez et al., 2002).

Many steps in the import of peroxisome matrix proteins have been described for both yeast and mammalian cells (Holroyd and Erdmann, 2001). Briefly, proteins destined for the matrix of the organelle are synthesized on cytosolic ribosomes and bind to one of two cytosolic receptors, PEX5 or PEX7. PEX5 and/or PEX7 (along with accessory

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proteins in certain species) shuttle the protein cargo to the docking complex on the membrane of the peroxisome. This docking complex, comprised of PEX13, PEX14 and, at least in yeast, PEX17, is involved in transporting the cargo across the membrane, though the process by which this occurs is not well understood. The RING proteins, peroxins 2, 10 and 12, form a complex of peroxisome membrane proteins that acts downstream of the docking complex and associates with PEX5 (Chang et al., 1999; Okumoto et al., 2000). The RING peroxins are necessary for ongoing matrix protein import (Chang et al., 1997; Okumoto et al., 1998a,b; Tsukamoto et al., 1991).

The RING peroxins each contain a cytosolic C-terminal RING or RING-like motif (RING is an acronym for "really interesting new gene"), as first noted in PEX2 (Patarca and Fletcher, 1992). RING domains are zinc-binding regions involved in protein–protein interactions and are characteristic of a subset of E3 enzymes, mediators of ubiquitin ligase activity (Joazeiro and Weissman, 2000). It has been suggested that one or more of these peroxins acts as an ubiquitin ligase in the recycling of the PEX5 import receptor after it has delivered its cargo (Kiel et al., 2005b,a; Kragt et al., 2005; Platta et al., 2004b,a; van der Klei et al., 1998). Because glycosomes are essential organelles in trypanosomatids, further characterization of the proteins involved in matrix protein import is warranted.

The characterization of proteins involved in matrix protein import in trypanosomatids has been rapidly advancing in recent years, with the identification of several PEX proteins. These studies have shown that functional glycosomes are essential for trypanosomatid survival (Flaspohler et al., 1999; Furuya et al., 2002; Guerra-Giraldez et al., 2002; Krazy and Michels, 2006; Moyersoen et al., 2003). Previously, we identified the *Leishmania donovani* PEX2 orthologue (Flaspohler et al., 1997, 1999). Here we describe *T. brucei* PEX10 and PEX12. We demonstrate colocalization of each protein with glycosomal proteins and verify that the *T. brucei* proteins behave as integral membrane proteins. Expression of GFP chimeras of *L. major* and *T. cruzi* orthologues in *T. brucei* demonstrates the conservation of glycosomal membrane protein targeting in trypanosomatids.

## 2. Materials and methods

### 2.1. Cell lines and growth conditions

The *T. brucei* procyclic cell line 29–13 (Wirtz et al., 1999) was used as the background line for all *T. brucei* experiments. This strain has been engineered to contain copies of the tetracycline (Tet) repressor and T7 polymerase to allow for inducible expression of genes using a T7 promoter with adjacent Tet repressor binding sites. Cells were cultured in SDM-79 medium (JRH Biosciences) supplemented with 10% fetal calf serum. Transfections were performed as previously described (Anderson et al., 1998). To select for stable transfectants, G418, hygromycin and phleomycin were added to the final concentrations of 15, 50, and 2.5 μg/ml, respectively. For inductions, Tet was used at a final concentration of 2 μg/ml, except where noted. In some experiments, we employed the 29–13 cell line expressing a Tet-regulated *T. brucei* aldolase–HcRed fusion as a glycosomal marker. To select for cells bearing the latter construct, puromycin (PAC) was added to a final concentration of 1 μg/ml.

### 2.2. Plasmid contruction

For creation of GFP fusion constructs, PEX10 and PEX12 were each amplified from genomic DNA from *T. brucei* TREU667, *T. cruzi* CL Brener and *L. major* Friedlin by polymerase chain reaction (PCR) using Taq DNA polymerase (Roche Diagnostics Expand High Fidelity PCR system). Forward and reverse primers (all sequences are 5′ to 3′), respectively, were CTTAAGATGCAGCCGGCGAC GGCACC and CTCGAGTGCAGACGTGTCATGAAC for TcPEX10, CTTAAGATGCAACCGGCCACTGC and CTCGAGTGACGCACCGCTGTCGC for TbPEX10, CT TAAGATGTTCGAGAGCAACCTCCTC and CTCGA GGCACTCGAAAACTCGGCGGATC for LmPEX12 and CTTAAGATGATAGAGAGTAACTTGCTCTCG and CTCGAGGCACTCATAAATACGTCGAAC for TbPEX12. Each PCR product was cloned into pGEM-Teasy (Promega). These plasmids were sequenced, and the genes subsequently cloned into the pLEW79 (BLE) 5′mcsGFP$^+$ and the pLEW79(BLE)GFP$^+$3′mcs expression plasmids which were constructed to facilitate the creation of C-terminal or N-terminal GFP fusion proteins with Tet-regulated expression. These plasmids are derivatives of pLEW79 (Wirtz et al., 1999) and contain common multiple cloning sites (mcs) of *Afl*II-*Hpa*I-*Nru*I-*Xho*I in frame on either the 5′ or 3′ end of GFP$^+$. GFP$^+$ is a modified version of GFP that is GC rich and has an S56T mutation (Ha et al., 1996). The PCR amplified *L. major* PEX12 gene was identical to the sequence available from GeneDB, while the PCR amplified *T. cruzi* PEX10 gene had two predicted amino acid changes, V58A and S205P.

Generation of pLEW79 constructs bearing the PAC gene was accomplished as follows. The BLE gene and flanking regions were removed from pLEW79 as a *Sal*I/*Spe*I fragment and placed into pBluescript. The BLE gene was excised with *Nco*I and *Stu*I, and replaced with a PAC gene (Flaspohler et al., 1999) flanked by the same sites (a partial digestion with *Stu*I was required). The *Sal*I/*Spe*I fragment was then cloned back into both pLEW79 GFP$^+$ cloning vectors, thereby incorporating the PAC gene for selection.

For creation of a glycosomal protein fusion to an inducible fluorescent marker protein, the gene encoding *Hereractis crispa* red florescent protein (HcRed, BD Biosciences) was amplified by PCR, adding *Xho*I/*Bam*HI cloning sites, using the forward and reverse primers, respectively, CTCG AGATGGTGAGCGGCCTGCTGAAG and GGATCCT CAGTTGGCGTTCTCGGGCAG. The HcRed coding region was used to replace GFP$^+$ in the pLEW79 (PAC)5′mcs-GFP$^+$ plasmid, leaving the multiple cloning