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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.

Keywords

Trypanosoma brucei; *Trypanosoma cruzi*; *Leishmania major*; Kinetoplastida; peroxisomes; glycosomes; RING; peroxins; PEX10; PEX12

1. Introduction

Trypanosoma brucei, *Trypanosoma cruzi*, and *Leishmania major* are disease causing trypanosomatid parasites collectively referred to as the Trityps. *T. 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 Trityp genomes (Berriman et al., 2005; El Sayed et al., 2005; El-Sayed et al., 2005; 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

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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 (Oppendoes 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 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; Okumoto et al., 1998b; 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; Kiel et al., 2005a; Kragt et al., 2005; Platta et al., 2004b; Platta et al., 2004a; 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; Flaspohler et al., 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 μ g/ml, respectively. For inductions, Tet was used at a final concentration of 2 μ g/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 construction

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 CTTAAGATGCAGCCGGCGACGGCACC and CTCGAGTGCAGACGTGTCATGAAC for TcPEX10, CTTAAGATGCAACCGGCCACTGC and CTCGAGTGACGCACCGCTGTCTCG for TbPEX10, CTTAAGATGTTTCGAGAGCAACCTCCTC and CTCGAGGCACCTCGAAACTCGGCGGATC for LmPEX12 and CTTAAGATGATAGAGTAACCTTGCTCTCG and CTCGAGGCACTCATAAATACGTCTGAAC 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 *AflIII*-*HpaI*-*NruI*-*XhoI* 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 *SalI*/*SpeI* fragment and placed into pBluescript. The BLE gene was excised with *NcoI* and *StuI*, and replaced with a PAC gene (Flaspohler et al., 1999) flanked by the same same sites (a partial digestion with *StuI* was required). The *SalI*/*SpeI* 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 crista* red fluorescent protein (HcRed, BD Biosciences) was amplified by PCR, adding *XhoI*/*BamHI* cloning sites, using the forward and reverse primers, respectively, CTCGAGATGGTGAGCGGCCTGCTGAAG and GGATCCTCAGTTGGCGTTCTCGGGCAG. The HcRed coding region was used to replace GFP⁺ in the pLEW79(PAC)5'mcs-GFP⁺ plasmid, leaving the multiple cloning site intact. The aldolase gene was amplified from *T. brucei* genomic DNA with *AflIII*/*XhoI* sites flanking the ORF and cloned into pLEW79(PAC)5'mcs-HcRed as an *AflIII* and *XhoI* partial digest, creating pLEW79(PAC)TbAldolase-HcRed.

The TrypanoFAN: RNAiT computer program was used to determine a suitable portion of TbPEX10 for RNAi (<http://trypanofan.path.cam.ac.uk/software/RNAit.html>). We amplified nt 267 to 792 of the TbPEX10 ORF, from, and cloned the resulting fragment into pZJM (Wang et al., 2000), such that it is flanked by opposing Tet-regulated T7 promoters. Forward and reverse primers used in this reaction were, respectively, CCGCTCGAGAGAAAGCTGCTTCTCGCAAC and GGGAAAGCTTAAATGGCTCCCTGTGTGTTTCGAC.

2.3. Immunofluorescence and immunoblot analysis

Immunofluorescence analysis was performed as previously described (Furuya et al., 2002) 20–36 hours after induction of fusion proteins with Tet. Cells were fixed on glass slides or coverslips with 2 – 4% formaldehyde and permeabilized with 0.1% Triton X-100. Rabbit anti-glycosome antiserum (Parker et al., 1995), primarily targeting the glycosomal matrix proteins aldolase, glyceraldehyde phosphate dehydrogenase, and pyruvate phosphate dikinase was applied, and followed by Texas Red or FITC-conjugated goat anti-rabbit IgG (Southern Biotechnologies). DNA was stained with DAPI (4',6-diamidino-2-phenylindole dihydrochloride, ICN Biomedicals). After mounting with Prolong Antifade (Invitrogen), slides were viewed using a Nikon Eclipse E300 microscope equipped with Metamorph software. Intrinsic fluorescence of GFP and HcRed was still evident under these conditions.

Immunoblots were performed as described (Jensen et al., 2003) using rabbit anti-*T. brucei* phosphoglycerate kinase (Parker et al., 1995) or rabbit anti-GFP antisera, followed by protein A coupled to horseradish peroxidase (Bio-Rad). The bound protein A was detected using ECL plus (Amersham Biosciences).

2.4. Extraction by sodium carbonate and urea

T. brucei procyclic forms were washed twice in PBS, and resuspended in HEDSK buffer (25 mM Hepes, 1 mM EDTA disodium salt, 1 mM DTT, 250 mM sucrose, and 50 mM potassium acetate; pH 7.8) supplemented with leupeptin (100 μ M), aprotinin (50K IU/ml), orthovanadate (100 μ M), and phenylmethylsulfonyl fluoride (1 mM). Samples (10^7 cells) were treated with digitonin at 200 μ g/ml final concentration for 3 min. at 37°C, and then centrifuged to separate supernatant from organelle-enriched pellet. Supernatants were prepared for SDS-PAGE. Pellets were resuspended by douncing in 1 ml 0.1 M sodium carbonate or 0.1 M sodium carbonate/2 M urea. After one hour on ice, samples were centrifuged for one hour at 100,000 $\times$ g at 4°C. The carbonate pellets were resuspended in 75 μ l HEDSK buffer supplemented with protease inhibitors followed by addition of 75 μ l of 2X SDS-PAGE sample buffer, boiled and stored at –70°C. Supernatants were brought to 10% trichloroacetic acid and placed on ice for 15 minutes. These were then centrifuged for 15 minutes at 4°C. Pellets were then washed in 1 ml 80% acetone/20% 10 mM Tris, pH 8.3 and prepared for SDS-PAGE as above. Urea extraction alone was performed as previously described (Jardim et al., 2002) using 4 M or 8 M urea in 25 mM Tris HCl, pH 8.0. Samples (5×10^6 cell equivalents) were subjected to immunoblot analysis.

2.5. Protease Protection Assays

T. brucei procyclic cells (10^7) were treated with digitonin as described above. After centrifugation, the organelle-enriched pellets were resuspended in 100 μ l HEDSK buffer in the presence or absence of 0.1% Triton X-100 and incubated with increasing amounts of proteinase K at 30°C for 30 minutes. Reactions were terminated by the addition of phenylmethylsulfonyl fluoride to 5 mM. All samples were analyzed by immunoblotting.

2.6. RNA analysis

For Northern analysis, cells were collected from induced and uninduced cells on day 3 and day 6 of growth analysis. Cells were washed, resuspended in Trizol and stored at –70°C. RNA preparation and Northern analysis were carried out as previously described (Jensen et al., 2005).

2.7 Cell viability analysis

Cell viability was assessed using the LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes). This assay measures fluorescence of calcein (generated by esterase acting on calcein

AM) and/or ethidium homodimer (which is not taken up by live cells) in a given cell population to determine live or dead status, respectively.

3. Results

3.1. Bioinformatic Analysis of PEX10 and PEX12 in the Tritryps

Using the amino acid sequence of the human PEX10 and PEX12 as queries, we performed a database search in GeneDB (www.genedb.org) to identify the trypanosomatid orthologues. We identified candidate PEX10 genes for *T. brucei* (Tb927.3.2410, 897 bp), *L. major* (LmjF25.2290, 891 bp), and *T. cruzi* (Tc00.1047053508479.190, 903 bp). We also identified candidate PEX12 genes in *T. brucei* (Tb10.61.0440, 1188 bp), *L. major* (LmjF19.1250, 1836 bp) and *T. cruzi* (Tc00.1047053503809.20, 1233 bp). Additionally, a second *T. cruzi* PEX12 candidate (Tc00.1047053503641.19) was identified in the database. This gene is positioned at a contig break and appears as a fragment with 94% amino acid identity over the shared region with the other *T. cruzi* orthologue. Only the sequence derived from the full length ORF was used in the current study.

Synteny among the trypanosomatid PEX10 and PEX12 ORFs is readily observed. The flanking genes are conserved across species, although several encode hypothetical proteins. For example, PEX2 is found upstream of PEX10, while a gene encoding an AGC type protein kinase is found downstream in each of the Tritryps. For PEX12, both *T. brucei* and *L. major* orthologues have genes encoding an actin-related protein upstream and an RNA binding protein downstream. The genome sequence information available for *T. cruzi* is more fragmented; however, downstream of the ORF an RNA binding protein is also found. Reciprocal BLAST analysis shows that the candidates for each gene are predicted orthologues among the trypanosomatids. The trypanosomatid PEX10 predicted proteins show 51–62% amino acid sequence identity while the PEX12 predicted proteins show 39–57% identity. Alignment of each of these proteins with their respective human and *S. cerevisiae* homologues reveals 20–30% identity among the PEX10 orthologues, and 19–23% identity among the PEX12 orthologues, with greatest similarity in both cases lying in the C-terminal region (Fig. 1). Although these values are somewhat low, they are commensurate with the low level of sequence identity observed between the human and yeast homologues: 21% and 22% for PEX10 and PEX12, respectively.

Each protein possesses a C-terminal RING or RING-like motif (Pfam 00097). The RING finger is a cysteine-rich domain that coordinates the binding of zinc ions (Borden and Freemont, 1996). Like the mammalian, *S. cerevisiae*, and *Arabidopsis thaliana* PEX10 homologues, the trypanosomatid PEX10s contain the C3HC4 type of RING domain with the consensus sequence C-X2-C-X(9–39)-C-X(1–3)-H-X(2–3)-C-X2-C-X(4–48)-C-X2-C. The trypanosomatid PEX10s also contain a version of the conserved TLGEEYV motif in the N terminal region of the proteins (underlined in Fig. 1) (Warren et al., 1998). Mammalian and *S. cerevisiae* PEX12 proteins retain only five of the RING motif cysteine residues, and these are conserved in the trypanosomatids. The PEX12 proteins, including those described here, also contain a proline-rich region upstream of the RING-like domain. Hydrophobicity plots predict two transmembrane domains for all putative Tritryp PEX10 predicted proteins, and for the *T. cruzi* and *L. major* putative PEX12 predicted proteins. Hydrophobicity plots of TbPEX12 reveal one or possibly two very weak hydrophobic regions (see below). This informatic analysis supports these candidates as the Tritryp orthologues for PEX10 and PEX12.

3.2. TbPEX10 and TbPEX12 GFP fusion proteins localize to glycosomes

The *T. brucei* PEX10 and PEX12 ORFs were amplified from genomic DNA and cloned into Tet-regulated expression vectors that specify a fusion protein with GFP on either the C terminus

or N terminus. Stable transfectants were induced and the expression and localization of the fusion proteins examined. As shown in Fig. 2a, all proteins were expressed to similar levels with the exception of GFP-TbPEX12, which was much less abundant, as was TbPEX12-GFP when lower levels of Tet were used for induction (lane marked “low”). Others have shown GFP is cytosolic when expressed in *T. brucei* (Bingle et al., 2001; Hill et al., 1999). In contrast, induction of TbPEX10-GFP (not shown), GFP-TbPEX10 (Fig. 2b), and GFP-TbPEX12 (Fig. 2b) resulted in the characteristic punctate staining pattern of glycosomal proteins when viewed under fluorescence microscopy. The GFP fusion proteins colocalized with glycosomal proteins as revealed with an anti-glycosome antiserum (Fig. 2b). All inductions were performed with serial dilutions of Tet to confirm that glycosomal localization was not a result of overexpression. Unlike the other fusion proteins, induction of the TbPEX12 bearing a C-terminal GFP (TbPEX12-GFP) at our standard Tet concentration (2 µg/ml) resulted in a pattern in which the glycosomes appeared enlarged or clustered. Lowering the amount of Tet to 20 ng/ml reduced expression of the protein (Fig. 2a) and the fluorescence pattern then resembled that of normal glycosomal staining (Fig. 2b).

3.3. *T. cruzi* PEX10 and *L. major* PEX12 are targeted to the glycosome in *T. brucei*

We also evaluated the targeting of *T. cruzi* PEX10 and *L. major* PEX12 in *T. brucei*. To facilitate these studies, we developed a *T. brucei* cell line with Tet-regulated expression of a red fluorescent fusion protein which localized to the glycosome. *H. crista* red fluorescent protein (HcRed) was fused to the C-terminus of *T. brucei* aldolase, which bears a PTS2 glycosomal targeting signal at the N-terminus (Marchand et al., 1988). Immunofluorescence analysis of stable transfectants showed that this fusion protein co-localizes with glycosomal proteins as revealed with anti-glycosome antiserum, demonstrating its utility as a glycosomal marker (data not shown). We then created TcPEX10 and LmPEX12 fusions to the C-terminus of GFP and expressed those in *T. brucei*. In simple wet mounts of live cells (Fig. 2c) the red marker protein colocalized with GFP fusions to both TcPEX10 and LmPEX12. Thus, the heterologously expressed proteins localized to the *T. brucei* glycosome.

3.4. TbPEX10 and TbPEX12 behave like integral membrane proteins

PEX10 and PEX12 yeast and human homologues are integral peroxisomal membrane proteins whose C terminal RING or RING-like domains extend into the cytosol (Okumoto et al., 1998a; Okumoto et al., 1998b; Okumoto et al., 2000). Hydrophobicity plots using TMHMM (Krogh et al., 2001) or Tmpred (Hoffman and Stoffel, 1993) predict at least two transmembrane domains for each of the trypanosomatid proteins with the exception of TbPEX12, which shows, at best, two weak hydrophobic regions (both with a probability of less than 0.2 in TMHMM). To verify that the putative *T. brucei* orthologues for these proteins, especially the TbPEX12 candidate, are integral to the glycosomal membrane, we performed extraction with sodium carbonate at pH 11, urea, or both together. These treatments strip membranes of peripherally associated proteins, which remain in the supernatant following centrifugation. Organelle-enriched fractions were prepared by digitonin treatment of each of the four aforementioned TbPEX10 and TbPEX12 GFP fusion cell lines. These fractions were then treated with sodium carbonate, sodium carbonate plus 2 M urea, 4 M urea, 8 M urea, or HEDSK buffer followed by ultracentrifugation. Pellets and supernatants from each group were analyzed by Western blot using anti-GFP. The detected proteins corresponded well to the predicted molecular masses of GFP fusions to PEX10 (62 kDa) or PEX12 (72 kDa). In some experiments background bands were seen at 60 kDa and 48 kDa, regardless of the cell line being tested. As shown in Fig. 3a, both GFP-PEX10 and the GFP-PEX12 were seen in the pellet fractions after treatment with carbonate or carbonate plus urea. Likewise, PEX10-GFP and PEX12-GFP were also in the pellet fractions (data not shown). Similar results were seen after treatment with 4M or 8M urea (data not shown). As a control, we probed with antiserum directed against TbPEX14, an integral membrane protein that comprises part of the docking complex for matrix

protein import (Furuya et al., 2002). PEX14 was found in the pellet fraction (Fig. 3a). As a glycosomal matrix marker, we examined the 56 kDa isozyme of PGK. This protein was enriched in the digitonin pellet as expected, and subsequently in the carbonate supernatant, although some was present in the carbonate pellet. That some amount of PGK is present in the carbonate pellet may be due to the tendency of the glycosomal matrix proteins to remain in large “cores” under low salt conditions, even in the presence of non-ionic detergent (Sommer et al., 1990). As a control we determined that all four of the fusion proteins (and PEX14) were readily solubilized with non-ionic detergent (data not shown). Taken together the data indicate that the GFP fusion proteins are integral to the glycosome membrane.

3.5. Protease protection assays demonstrate that both the TbPEX12 N and C termini are exposed to the cytosol

Mammalian PEX12 contains two membrane spanning regions, with both termini extending into the cytosol (Okumoto et al., 1998b). In contrast, ScPEX12 contains one membrane spanning region, extending the N terminus into the peroxisome and the C-terminal RING-like domain into the cytosol (Albertini et al., 2001). For TbPEX12, TMHMM and Tmpred predict one weak hydrophobic domain at approximately aa 278 to 300, and an additional even weaker hydrophobic domain at approximately aa 228 to 250 of the 397 aa protein. To determine if the TbPEX12 was more similar in topology to the yeast or to the mammalian PEX12, we performed protease protection assays on the TbPEX12-GFP and the GFP-TbPEX12 cell lines. After induction, the organelle-enriched fractions were prepared as above and incubated with increasing amounts of proteinase K in the presence or absence of Triton X-100. The GFP tags for both fusion proteins were sensitive to protease, even in the absence of detergent. The cleavage was not due to permeabilization of the organellar fraction during the digitonin step, since the 56 kDa glycosomal PGK was protected from proteinase K, while the 45 kDa cytosolic form was not (Fig. 3b, lower panels). Once detergent was added, all molecules were cleaved by proteinase K (Fig. 3b). These results indicate that both termini of TbPEX12 are cytosolic.

3.6. An unusual RNAi integration event

RNAi is a commonly used tool to study gene function in *T. brucei*. To further examine the role of PEX10, we cloned a portion of the ORF between the two Tet-regulated T7 promoters of the pZJM vector. This vector is designed to integrate into the rDNA spacer region, where it remains transcriptionally silent until the addition of Tet. We isolated a line of procyclic form transfectants, designated P10-1. These transfectants grew somewhat more slowly than the parental line (doubling time ~17 hr as compared to 14 hr, not shown). When T7 transcription was induced by the addition of Tet, the growth rate of the population slowed dramatically after four days (Fig. 4a), although at this time viability in the induced population remained high as determined by live/dead assay. By day ten, the same assay determined that approximately 32% of the morphologically recognizable cells in the induced population were dead, and significant debris was present. Another 20% of the cells, though still alive, had a rounded or very irregular cell morphology. Eventually the population resumed growth as has been previously observed in this RNAi system (Drozdz et al., 2002; Furuya et al., 2002; Wilkinson et al., 2003). Surprisingly, Northern analysis showed that the abundance of the 1.2 kB PEX10 transcript was decreased in uninduced P10-1 as compared to the parental 29-13 line (Fig. 4b). It was further decreased in P10-1 upon addition of Tet. In the transfectant, an additional transcript was detected by the probe, likely corresponding to leaky transcription of the PEX10 fragment from the RNAi vector. The abundance of this transcript also increased after addition of Tet.

Interestingly, genomic analysis by Southern and PCR showed that the plasmid did not integrate into the rDNA locus. Southern analysis pointed to integration at the PEX10 locus. We are aware of one other case in which integration did not occur at the rDNA locus, but rather it occurred at the RNAi target locus (Motyka et al., 2004). In that case, the authors found that induction

via Tet led to knockdown of the targeted mRNA and overexpression of downstream mRNAs. It had no effect on upstream mRNAs. Because of the anomalous integration, without further study we cannot directly attribute the lethal effect of induction to an effect on PEX10. However, in the meantime, another group has reported that a PEX10 RNAi was lethal to the parasite (Krazy and Michels, 2006) answering that question. This second report of an unusual integration of the RNAi vector advises caution in interpretation of knockdown studies.

4. Discussion

Here we report the characterization of the products of trypanosomatid *PEX10* and *PEX12* genes, as expressed in *T. brucei*. While this manuscript was in preparation, another report identified trypanosomatid PEX10 and PEX12 by sequence similarity and demonstrated by RNAi that the *T. brucei* proteins are essential proteins (Krazy and Michels, 2006). Our work is in accord with that report and extends those studies with analysis of topology and cross-species targeting. The PEX RING proteins play an essential role in the peroxisome matrix protein import cycle. The identification of these peroxins trypanosomatids adds to the evidence of a common origin for peroxisomes and glycosomes. Nonetheless the level of PEX sequence identity across phyla is low. While the presence of specific motifs can provide greater support for a putative function, experimental verification is still a key endeavor when dealing with such divergent proteins. In addition, the specifics of each peroxin's function may differ among organisms. Such variations include the presence of different numbers of transmembrane domains among PEX12s and the integral versus peripheral membrane association of PEX14 in different trypanosomatids (Furuya et al., 2002; Jardim et al., 2002). Perhaps more dramatic is the phenotype of *PEX19* knockouts, which in most organisms lead to absence of peroxisomes, but in *Yarrowia* leads to the generation of peroxisome-like organelles with modified components (Lambkin and Rachubinski, 2001).

Informatic analysis on the PEX2 (Flaspohler et al., 1997) and PEX10 orthologues reveals that the trypanosomatid proteins, like their mammalian, yeast and plant counterparts, contain the C3HC4 type RING domain, a cysteine-rich region that coordinates the binding of two zinc ions (Borden and Freemont, 1996). On the other hand, PEX12 in all species studied thus far has retained only five of these cysteine residues (Okumoto et al., 1998b). The residues for binding the first zinc ion are conserved: C1, C2, C5, and C6, but only one of the four residues responsible for binding the second zinc ion (C7) is conserved. The remaining residues have been replaced with other amino acids that vary across species. These RING and RING-like domains, whether coordinating one or two zinc ions, are essential for the function of their respective proteins (Okumoto et al., 2000), and facilitate their interactions with each other as well as PEX5 (Chang et al., 1999; Okumoto et al., 2000). As possession of the RING domain implicates PEX10 and PEX2 (and possibly PEX12), in E3 ubiquitin ligase activity, it will be interesting to see how future studies delineate the specific role(s) these proteins have in ubiquitylation. Many E1 (ubiquitin-activating), E2 (ubiquitin-conjugating) and E3 (ubiquitin-ligating) genes are present in *T. brucei*, as seen by scanning GeneDB. However, it is not clear which E2 corresponds to PEX4, the peroxisomal ubiquitin conjugating enzyme. Similarly, the PEX22 homologue which anchors PEX4 to the peroxisomal membrane has not been identified.

GFP fusions to Tritryp PEX10 and PEX12 proteins revealed colocalization with glycosomal markers. It is interesting to note that when GFP was appended to the C-terminus of TbPEX12, we observed larger foci of glycosomal staining compared to when the other fusion proteins were expressed. These findings suggested clustering of glycosomes, as was seen upon overexpression of *S. cerevisiae* PEX12 in that organism (Albertini et al., 2001). Based on this observation, Albertini et al. suggested that PEX12 may be under tight regulation. TbPEX12-GFP was indeed more highly expressed than its GFP-PEX12 counterpart (which demonstrated a normal distribution of glycosomes). It may be that the interaction of PEX12 with other RING

proteins drives inter-organellar association upon overexpression, resulting in the “clumpy” phenotype. Another potential explanation is the formation of enlarged glycosomes such as those we observed upon knockdown of TbPEX19 (Banerjee et al., 2005).

Peroxisomal RING proteins studied thus far in other organisms contain at least one transmembrane domain, extending the RING motif into the cytosol. Transmembrane domain prediction programs suggest that most of the trypanosomatid orthologues are similar in topology. However, some of the predictions are less than clear. For example, three transmembrane domains are strongly predicted for *L. donovani* PEX2, while only two are functional (unpublished results). Likewise, the two hydrophobic regions for TbPEX12 scored very weakly in various programs, yet the protein is integral to the membrane, with both termini extending into the cytosol. TbPEX12 may have two membrane-spanning regions or may be embedded via membrane-dipping sequences that do not span the bilayer (Claypool et al., 2006; Garavito et al., 1995; Gupta et al., 2004). To our knowledge, *S. cerevisiae* PEX12 is the only RING protein in the peroxisome with a single transmembrane domain, but it is the only fungal PEX12 orthologue to have its topology determined experimentally.

PEX proteins are essential in trypanosomatids (Flaspohler et al., 1999; Furuya et al., 2002; Guerra-Giraldez et al., 2002; Krazy and Michels, 2006; Moyersoen et al., 2003) in contrast with other organisms, in which peroxins are not essential for cellular survival in standard media. Although many processes are shared among mammalian, yeast, plant, and trypanosomatid peroxisomes, the latter houses purine salvage and the glycolytic pathway. Compartmentation of glycolysis is one reason why this organelle is essential to parasite survival (Furuya et al., 2002; Kessler and Parsons, 2005). This profound difference between glycosomes and peroxisomes may prove useful in the development of drugs against these parasites. However, of the approximately 30 peroxins identified to date in yeast and mammals, only a small fraction of these have been identified in trypanosomatids — and all but one (Flaspohler et al., 1997) was identified through homology-based approaches. We are rapidly approaching the limit for the identification of peroxin homologues among the kinetoplastid protozoa by homology searching. New methods for the continuing evaluation of this elaborate and essential machinery must be developed. Recent proteomic analysis provides promise in this endeavor

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Abbreviations

DAPI	4', 6-diamidino-2-phenylindole dihydrochloride
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
HcRed	<i>Hereractis crista</i> red florescent protein
IFA	immunofluorescence assay
PEX	peroxin
PGK	phosphoglycerate kinase
Tet	tetracycline

Fig 1.

Alignment of trypanosomatid PEX10 (a) and PEX12 (b) predicted proteins for strain 927. Hs, *Homo sapiens*; Lm, *L. major*; Sc, *S. cerevisiae*; Tb, *T. brucei*; Tc, *T. cruzi*. Residues are marked as follows: * denotes conserved RING finger cysteines (numbered for reference in text), ▲ denotes the histidine in position C4 of the cross brace motif, + denotes conserved prolines of proline-rich region of PEX12. The location of the TLGEEYV motif in PEX10 is underlined. The predicted amino acid sequence of *T. brucei* PEX10 amplified from TREU667 had two differences (Q204E and R271Q) from that of the genome strain 927, but is identical to that of strain 427 (Krazy et al., 2006). Similarly, our PEX12 sequence had three differences from strain

927 (G140R, S313G and A317T), with the last change also being found in strain 427 (Krazy et al., 2006).

Fig 2. Localization of PEX10 and PEX12 GFP fusion proteins expressed in *T. brucei*

2a. Western analysis of *T. brucei* fusion proteins. Expression of the fusion proteins was induced by the addition of 2 μ g/ml Tet, except in the lane marked “low” where 20 ng/ml Tet was used. Whole cell lysates from each cell line were collected and analyzed by immunoblotting using anti-GFP. NOG1, a nucleolar protein, was used as a loading control (Park et al., 2001).

2b. Localization of the indicated GFP fusions was revealed by intrinsic fluorescence. Antibodies to glycosomal matrix proteins were used to show the location of the glycosomes. All samples were induced with 2 μ g/ml Tet, except 20 ng/ml was used in the sample marked “low”. Images are pseudocolored.

2c. Membrane protein targeting is conserved across species. The localization of GFP fusions to *T. cruzi* PEX10 and *L. major* PEX12 in live *T. brucei* procyclic forms was assessed by monitoring intrinsic fluorescence. The glycosomal marker was aldolase-HcRed.

Fig 3. GFP-PEX10 and GFP-PEX12 membrane topology

3a. Integral membrane status. GFP-PEX10 and GFP-PEX12 remain insoluble after treatment with sodium carbonate and urea. Organelle fractions from induced cells from GFP-PEX10 and GFP-PEX12 cell lines were prepared by digitonin treatment. Pellets were then extracted with NaCO₃ (pH 11.5) or NaCO₃ + urea. All samples were subjected to immunoblot analysis using anti-GFP, anti-PEX14 (integral membrane protein), and anti-PGK (soluble protein). For PGK, the glycosomal isozyme (56 kDa) is shown. The band seen in the digitonin supernatant using anti-GFP with GFP-PEX10 cells was occasionally but not reproducibly observed in different cell lines. It likely represents a weakly cross-reacting antigen.

3b. The N and C termini of TbPEX12 extend into the cytosol. TbPEX12 cell lines containing either a C terminal GFP (PEX12-GFP) or an N terminal GFP (GFP-PEX12) were treated with digitonin. The organelle-enriched pellet was then treated with 0, 10, 20 or 60 µg/ml proteinase K in the presence or absence of Triton X-100 (Tx). Samples were separated by SDS-PAGE and probed with anti-GFP and anti-PGK as above. For PGK, both the glycosomal (G, 56 kDa) and cytosolic (C, 45 kDa) isozymes are shown, although most of the latter is removed during preparation of the digitonin pellet. The open arrowhead marks the full-length GFP fusion protein. The bands at approximately 60 kDa and 48 kDa occasionally appeared when using the anti-GFP antibody (see above), regardless of the cell line being tested.

Fig 4. Characterization of a PEX10 RNAi line

4a. Growth phenotype. Transcription of the PEX10 RNAi construct was induced in the stable transfectant P10-1 by the addition of Tet. Cell numbers were monitored by Coulter counting. Cumulative cell number is depicted. The growth of the uninduced population is also shown (-Tet).

4b. Northern analysis. RNA was collected from the 29-13 background cell line and from induced and uninduced P10-1 cells on day 6. Top panel depicts levels of TbPEX10 RNA in each cell line, as revealed by an antisense probe which detects both the full-length PEX10 transcript (solid arrowhead) and PEX10 transcripts encoded by the RNAi construct (lower band). Bottom panel indicates α -tubulin levels.