Conservation of PEX19-Binding Motifs Required for Protein Targeting to Mammalian Peroxisomal and Trypanosomal Glycosomal Membranes

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Glycosomes are divergent peroxisomes found in trypanosomatid protozoa, including those that cause severe human diseases throughout much of the world. While peroxisomes are dispensable for both yeast (Saccharomyces cerevisiae and others) and mammalian cells in vitro, glycosomes are essential for trypanosomes and hence are viewed as a potential drug target. The import of proteins into the matrix of peroxisomes utilizes multiple peroxisomal membrane proteins which require the peroxin PEX19 for insertion into the peroxisomal membrane. In this report, we show that the specificity of peroxisomal membrane protein binding for Trypanosoma brucei PEX19 is very similar to those previously identified for human and yeast PEX19. Our studies show that trafficking is conserved across these distant phyla and that both a PEX19 binding site and a transmembrane domain are required for the insertion of two test proteins into the glycosomal membrane. However, in contrast to T. brucei PEX10 and PEX12, T. brucei PEX14 does not traffic to human peroxisomes, indicating that it is not recognized by the human PEX14 import mechanism.

Trypanosomatid parasites are responsible for several debilitating, and often lethal, human diseases, including African sleeping sickness, leishmaniasis, and Chagas’ disease, caused by Trypanosoma brucei, Leishmania spp., and Trypanosoma cruzi, respectively. In addition to the human scourges caused by this group of organisms, trypanosomatid diseases in livestock add to health and economic burdens in many developing countries. At present, there are no vaccines available and current drug treatments are complicated by emerging resistance or high toxicity, including sometimes lethal side effects (6, 53). Studies delineating the unique biology of trypanosomatids may hold promise for future treatments that are effective yet have minimal side effects.

The single-celled trypanosomatids contain divergent peroxisomes called glycosomes, so named because these organelles house the glycolytic pathway (36). The sequestering of glycolysis within a membrane-bound organelle is unique to trypanosomatids. Previously it was shown that glycosomes, unlike peroxisomes, are essential to cell survival (19, 23). Since trypanosomatid glycolytic enzymes have lost the typical feedback regulation seen in other organisms, loss of compartmentation likely leads to runaway ATP consumption and overproduction of phosphorylated intermediates, resulting in cell death (1, 4, 19). Despite this major difference, glycosomes and peroxisomes have maintained many similarities over the course of evolution (34, 39). For example, both house ether-lipid biosynthesis and utilize similar trafficking routes for the import of matrix proteins. However, the evolutionary divergence between mammals and trypanosomatids is great and differences in glycosomal biogenesis and function may prove useful in discovering drugs that target the parasite organelle while leaving the host unharmed.

The processes of peroxisome biogenesis and matrix protein import both involve a large set of proteins known as peroxins (PEX). The trafficking of proteins destined to the peroxisome matrix has been well studied (11). These proteins contain peroxisomal targeting sequences that mediate association with their respective cytosolic receptors, which then transport the cargo to the organelle (22, 52). Completion of the matrix protein import process involves several peroxisomal membrane proteins (PMPs), including the RING peroxin PEX10 (3, 33, 42). Several peroxin orthologs have been identified in trypanosomatids, including peroxins 2, 5, 6, 7, 10, 11, 12, 14, and 19 (5, 10, 13, 19, 23, 29, 30), generally showing 20 to 25% sequence identity to the human peroxins. Others, such as PEX3, which are essential for the formation and/or function of peroxisomes in mammals and most yeast species (20, 25, 28, 54), have not been identified in the completed genomes.

PMPs are targeted to the peroxisome via a process entirely different from that for matrix proteins. The mechanistic details are still being sorted out, but it is already clear that the integral membrane protein PEX3 and the largely cytosolic protein PEX19 are important in this process. Trafficking of most PMPs appears to be PEX19 dependent. PEX3 is a notable exception (27). The broad binding specificity of PEX19 for multiple PMPs (17, 43, 44, 48), combined with its ability to prevent the aggregation of these proteins in cell-free translation systems (27, 31, 46), has led to the working hypothesis that PEX19 acts as a PMP chaperone and import receptor (27). In this model, PEX19 binds its PMP cargo in the cytosol and carries it to the...
peroxisome membrane, where PEX19 binds PEX3 for subsequent insertion of the cargo into the membrane. This model is supported by recent work which has shown that PEX19 loaded with PMP cargo has a higher affinity for PEX3 than does unloaded PEX19 and that the import competence of PMPs is determined by a step prior to docking at the peroxisomal membrane (41). However, the finding that for some PMPs, the peroxisome targeting region is separate from the PEX19-binding site suggests that PEX19 may not be a general receptor for all PMPs (17, 48, 55). Rather, it has been postulated that PEX19 may promote assembly or disassembly of some PMP complexes at the peroxisome membrane itself (15, 48). Regardless of current disagreements as to the exact function of PEX19, which may vary in detail between different species, there is little doubt that this protein plays a pivotal role in the life of PMPs.

The long C-terminal domain of PEX19 binds numerous PMPs (16, 32, 46). The C terminus also contains a CAAAX box, but trypanosomatid PEX19s lack this farnesylmyl motif (5). This motif seems to be required for efficient targeting of PMPs in humans and Saccharomyces cerevisiae (21, 31), suggesting that some aspects of PMP targeting might differ in trypanosomatids. Previously, based on studies of S. cerevisiae, we developed a mathematical matrix for the prediction of PEX19-binding sites in PMPs (43). These regions generally consist of approximately 11 hydrophobic or positively charged residues and occur adjacent to or in close proximity to transmembrane domains (43). Although not sufficiently restrictive to identify PMPs out of a predicted proteome of an organism (43), this algorithm has proven useful for predicting PEX19 binding sites in both yeast and human PMPs (24). Its applicability to trypanosomatids has not been extensively studied. Here, we examine PMP targeting by PEX19 via comparative analysis of this process in T. brucei and human cells. We have previously shown that cross-species targeting of PEX10 and PEX12 is conserved among trypanosomatids (45), and therefore we focus the work here on T. brucei as the representative organism for this group. We demonstrate here that although many aspects of PMP trafficking have remained conserved over the course of evolution, subtle differences that may prove useful in fighting trypanosomatid diseases exist.

MATERIALS AND METHODS

Cell strains, media, and growth conditions. T. brucei procyclic strain 29-13 (57) was maintained in JRH Biosciences SDM-79 medium supplemented with 10% fetal calf serum. This strain has integrated copies of the tetracycline (Tet) repressor and T7 polymerase, which allows regulated expression of introduced genes expressed via a T7 promoter with adjacent Tet repressor binding sites. This strain was maintained in JRH Biosciences SDM-79 medium supplemented with 10% fetal calf serum. G418 and phleomycin were maintained in HMI-9 medium with 10% fetal calf serum and 2.5 g/ml. Transfections were carried out as previously described (24). Expression of these HsALDP gene regions in T. brucei, each was amplified from its template by PCR using primers containing 5' AflII and 3' BamHI sites for cloning into the pEGFP-C1 vector. The vector pGST-TbPEX19 designed to express a glutathione S-transferase (GST) fusion protein of T. brucei PEX19 was generated by cloning PEX19 (amplified from genomic DNA using primers containing a 5' EcoRI and 3' NotI site) into pGEX-4T-1 (GE Healthcare). The corresponding vector for the bacterial expression of human PEX19 has been described previously by Halbach et al. (24). Expression of the GST-TbPEX19 fusion proteins was carried out in the BL21 (DE3) derivative C41 strain according to the manufacturer's protocol (GE Healthcare).

RNA analysis. T. brucei bloodstream forms, uninduced or induced (day 2) for RNAi, were collected, washed, and resuspended in TRIzol reagent (Invitrogen). RNA was extracted using the manufacturer's protocol. Northern analysis was carried out as previously described (26).

Immunofluorescence microscopy, cell extraction, and immunoblot analysis. Immunofluorescence microscopy analysis (IFA) of T. brucei cells was performed as described previously (19). Blood form T. brucei cells were fixed in suspension with 4% paraformaldehyde, washed, and applied to poly-L-lysine–coated slides. Procyclic cells were fixed on glass slides with 4% paraformaldehyde. Cells were then permeabilized with 0.1% Triton X-100 and blocked with 8% nonfat milk. Rabbit anti-glycosome antiserum (38), primarily recognizing the glycosomal matrix proteins aldolase, glyceraldehyde phosphate dehydrogenase, and pyruvate phosphate dikinase, was applied and followed with Texas Red-conjugated goat anti-rabbit immunoglobin G (Southern Biotechnologies). DNA was stained with 4',6-diamidino-2-phenylindole dihydrochloride (ICN Biomedica). After Prolong Antifade (Invitrogen) was used as mounting medium, slides were viewed using a Nikon Eclipse E300 microscope equipped with MetaMorph software. Intrinsic fluorescence of GFP was still evident under these conditions. IFA of human fibroblasts that had been transfected with pEGFP-C1-derived plasmids was carried out essentially as described previously by Halbach et al. (24), with the exception that polyclonal rabbit anti-PMP70 antibodies (Invitrogen, Carlsbad, CA) were used to label peroxisomes in cells expressing GFP-TPeX14.

For fractionation experiments, cells expressing the proteins of interest were first treated with digitonin at a final concentration of 200 μg/ml for 3 min at 37°C. Following centrifugation, the organelar pellet was treated with 0.2 M sodium carbonate, pH 11, and the integral and peripheral membrane protein fractions were separated by centrifugation as described previously (45). Cell equivalents of the digitonin supernatant, carbonate supernatant (matrix and peripheral membrane proteins), and pellets (integral membrane proteins) were subjected to immunoblot analysis. Immunoblot analyses were performed using rabbit anti-GFP antisera (Molecular Probes), mouse anti-GFP B2 (Santa Cruz Biotechnology), rabbit anti-phosphoglycerate kinase (38), or, for loading controls in determining expression levels, mouse anti-Nopp44/46 monoclonal antibody 1D2 (40), followed by goat anti-rabbit IRDye 680 (LI-COR Biosciences) and goat anti-mouse IRDye 800CW (LI-COR Biosciences) for simultaneous detection of antibodies. Blots were analyzed using the Odyssey infrared imaging system.

Peptide arrays. Peptide blots were generated as previously described (43). The blots were probed with purified GST-PEX19 fusion proteins (both human and T. brucei procyclic cells or human fibroblasts).

CONCLUSIONS

Multicellular eukaryotic organisms employ complex and sophisticated mechanisms to localize proteins to specific subcellular locations. The peroxisome is a compartment that contains a number of proteins important for the cellular metabolism of carbohydrates, fatty acids, and xenobiotics. This organelle is highly dynamic, being the site of biosynthesis and degradation of peroxisomal membrane proteins (PMPs). The import of PMPs into the peroxisome requires the concerted action of several receptor proteins and Pex19p, a member of the Pex10p/12p/14p family of receptors.

The long C-terminal domain of PEX19 binds numerous PMPs (16, 32, 46). The C terminus also contains a CAAAX box, but trypanosomatid PEX19s lack this farnesylmyl motif (5). This motif seems to be required for efficient targeting of PMPs in humans and Saccharomyces cerevisiae (21, 31), suggesting that some aspects of PMP targeting might differ in trypanosomatids. Previously, based on studies of S. cerevisiae, we developed a mathematical matrix for the prediction of PEX19-binding sites in PMPs (43). These regions generally consist of approximately 11 hydrophobic or positively charged residues and occur adjacent to or in close proximity to transmembrane domains (43). Although not sufficiently restrictive to identify PMPs out of a predicted proteome of an organism (43), this algorithm has proven useful for predicting PEX19 binding sites in both yeast and human PMPs (24). Its applicability to trypanosomatids has not been extensively studied. Here, we examine PMP targeting by PEX19 via comparative analysis of this process in T. brucei and human cells. We have previously shown that cross-species targeting of PEX10 and PEX12 is conserved among trypanosomatids (45), and therefore we focus the work here on T. brucei as the representative organism for this group. We demonstrate here that although many aspects of PMP trafficking have remained conserved over the course of evolution, subtle differences that may prove useful in fighting trypanosomatid diseases exist.
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were very reproducible, some time points appear to have fewer replicates. The inset shows results from Northern analysis of the RNAi

line with ( + ) or without ( − ) Tet to induce RNAi. (B) IFA of cells

induced ( + ) for RNAi after 48 h or uninduced controls ( − ). Cells were

stained with anti-glycosome antibody which detects several matrix pro-

tins. The induced cells were heterogeneous (see the text). A cell

exhibiting punctate staining and a cell from the same culture exhibiting

diffuse staining are shown. Bar, 2 μm.

FIG. 1. PEX19 is essential to blood form T. brucei. (A) Growth

analysis of the TbPEX19 RNAi cell line. Bloodstream stage cells con-
taining an RNAi construct targeting PEX19 RNA were induced with

Tet in triplicate and counted by Coulter counter daily. The cumulative

number of particles (cells plus any dead cells of similar size) is indi-
cated on the y axis. Individual measurements are shown. Because the data

were very reproducible, some time points appear to have fewer replicates. The inset shows results from Northern analysis of the RNAi

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diffuse staining are shown. Bar, 2 μm.

bronc (PEX19), and specifically bound protein was revealed with a combination of

monoclonal anti-GST antibodies and secondary horseradish peroxidase-con-

jugated antibodies. As a control, GST was added to replicate blots.

RESULTS

PEX19 is essential in bloodstream stage T. brucei. Previously,

we identified the trypanosomatid PEX19 orthologues and demonstrated that partial depletion of the mRNA

(~70%) resulted in compromised growth of the insect midgut

stage (procyclic form) of T. brucei (5). Mammalian infective

stages, unlike the procyclic stage, require glycolysis for survival.

To assess the importance of PEX19 to the mammalian infective

stage, we analyzed the result of RNAi-mediated destruc-
tion of PEX19 RNA in bloodstream forms. After the isolation

of stable bloodstream form transfectants containing the

PEX19 RNAi plasmid, cultures were induced with Tet. As seen in

Fig. 1A, by day 2 of this assay, growth of the induced

population slowed compared to that of their uninduced coun-
terparts. Although particles were still detected at day 4 using

the Coulter Counter, these represented dead cells; no live cells

could be found by microscopic visualization. Northern analysis

and phosphorimaging showed that PEX19 RNA was reduced

by about 70% compared to uninduced controls (Fig. 1A, inset).

By day 7, viable parasites were again detected. This result is

commonly seen with RNAi of essential genes in T. brucei,

where genetic changes in the engineered strain lead to loss of

RNAi (8). These results demonstrate that PEX19 is an essen-
tial protein in the bloodstream stage of the trypanosome life

cycle.

It can be difficult to detect bloodstream form cells showing

mislocalization of glycosomal proteins via IFA, since the par-
asites become fragile and die rapidly (23). Nonetheless, we

attempted to assess glycosomal protein localization to func-
tionally tie the PEX19 phenotype to glycosome biogenesis.

IFA was carried out 48 h after induction by using an anti-
glycosome antibody that detects three glycosomal matrix en-

zymes (Fig. 1B). Uninduced cells showed the characteristic

punctate glycosomal staining with this antisem. In the in-

duced population, 25% showed diffuse cytosolic staining. The

remaining cells exhibited a punctate staining pattern, although

some of these contained enlarged glycosomes, a phenotype we

observed when PEX19 abundance was reduced in procyclic

cells (5). It is likely that this phenotype represents an interme-

diate state with residual PEX19.

PEX19 binding site-dependent targeting of human ALDP in

T. brucei. Since the RNAi knockdown indicated that PEX19 is

essential for peroxisome/glycosome biogenesis in trypano-
somes, as it is in humans and yeast, we asked how closely the

function of TbPEX19 resembles those of the corresponding

human and yeast orthologues. To this end, the targeting of

fragments of the human adrenoleukodystrophy protein

(ALDP) was inspected in trypanosomes. ALDP is a PMP of

the ATP-binding cassette (ABC) transporter protein family

that is believed to be involved in the transport of very-long-

chain fatty acids into the peroxisome for degradation (56).

Peroxosomal targeting of ALDP in human cells and in S. cer-

visiae was previously shown to require the presence of both a

transmembrane domain (TMD) and a PEX19-binding site

(24). The same regions of ALDP were assessed here for their

ability to route GFP to the glycosomes of T. brucei. Amino

acids (aa) 66 to 87, containing the PEX19-binding site of

ALDP, aa 87 to 164, containing two TMDs, and aa 66 to 164,

containing both the PEX19-binding site and the TMDs,

were each cloned into a T. brucei GFP expression vector for the

creation of fusion proteins containing an N-terminal GFP.

These constructs were transfected into procyclic form T. brucei

for the generation of stable cell lines. After induction with Tet,

each cell line expressed an appropriately sized GFP fusion

protein (Fig. 2A). An IFA using an antiserum directed against

glycosomal matrix proteins was carried out for each of these

cell lines; intrinsic GFP fluorescence marked the localization

of the fusion proteins. Of these GFP fusion proteins, only the

one containing both the PEX19-binding site and the TMDs,

ALDP(66-164), showed specific glycosomal localization (Fig.

2B, left panels). The cells expressing GFP fused to the PEX19-

binding site, ALDP(66-87), showed diffuse fluorescence, indi-
cating likely cytosolic and nuclear localization of the fusion

protein. Cells expressing GFP fused to the TMDs, HsALDP(87-164), showed diffuse, reticular, and punctate pat-
terns, with some colocalization to glycosomes but significant

localization elsewhere in the cell, including a variety of intra-
cellular membranes and perhaps the cytosol.
To further characterize the subcellular distribution of these GFP fusion proteins in *T. brucei*, we analyzed cell fractions by immunoblot analysis. Digitonin treatment and centrifugation yielded the cytosolic supernatant and organellar pellet. The latter was extracted with sodium carbonate to release matrix and peripheral membrane proteins (18). Integral membrane proteins remain membrane associated and partition to the pellet fraction upon ultracentrifugation. The samples were subjected to immunoblot analysis using anti-GFP and anti-phosphoglycerate kinase. The latter antiserum detects both the 45-kDa cytosolic isozyme and the 56-kDa glycosomal matrix isozyme. The cytosolic phosphoglycerate kinase was found in the digitonin supernatant, whereas the glycosomal form partitioned to the digitonin pellet. From there, it associated predominantly to the carbonate supernatant fraction, although some fractionated with the carbonate pellet as observed previously (45). As shown in Fig. 2B (right panels), the GFP-HsALDP(87-164) fusion protein was exclusively present in the integral membrane protein fraction. In contrast, GFP-HsALDP(66-87) was predominantly in the digitonin soluble fraction, although a small amount was associated with the integral membrane fraction. GFP-HsALDP(87-164) was predominantly in the integral membrane protein fraction, although some was seen in the cytosolic fraction.

Interestingly, the expression of glycosomally targeted GFP-ALDP(66-164) was toxic, as these cells rounded up and began to die within 24 h of induction, indicating that the correctly targeted fragment may adversely affect the structure or metabolic function of the glycosome. Despite this effect, matrix proteins continued to be properly localized, as shown both by the IFA and by digitonin fractionation (Fig. 2B). Immunoblot analysis showed that this toxic fusion protein was not overexpressed relative to the other HsALDP fusion proteins (Fig. 2A). This interference notwithstanding, the data are clear in that the HsALDP fragments showed a subcellular distribution in *T. brucei* similar to that observed in human fibroblasts and *S. cerevisiae* and that targeting to the glycosome requires both the regions containing the TMDs and the previously described PEX19-binding site.

The apparent requirement for the human PEX19-binding site further suggested that TbPEX19 is able to recognize this site, even though the two PEX19 orthologues exhibit only 21% amino acid sequence identity. To experimentally address this possibility, peptide arrays were synthesized to contain the
previous studies, the central leucines (but not the tryptophan) appear to be important features for binding. Thus, TbPEX19 does recognize sequences similar to those detected by human PEX19.

**Prediction and identification of PEX19-binding sites in *T. brucei* PMPs.** To identify potential PEX19-binding sites in trypanosomatid PMPs, we used an algorithm that had been developed for the prediction of PEX19-binding sites in *S. cerevisiae* PMPs (43) and which had proven valuable for the prediction of such sites in human PMPs (24). This in silico method generates predictions in 15-residue segments, with the central 9 aa representing the primary sequence-specific entity. We submitted known PMPs from three trypanosomatid species (*T. brucei, T. cruzi, and Leishmania major*) to the algorithm, resulting in predictions of binding for most proteins (Table 1). However, for a few proteins, namely PEX14 from all three species and *T. cruzi* Gim5a, no high-scoring peptide (>3,000) was identified. To determine whether this algorithm is able to correctly predict PEX19-binding sites in trypanosomatid PMPs, proteins with at least one site that scored above 3,000 were further analyzed using peptide arrays. All peptides spanning the high-scoring regions and some additional minor scoring regions were synthesized on arrays, which were then probed with GST-TbPEX19. Each tested region was represented by nine 15-mer peptides covering 31 aa in total, with the central residue on the array representing the peak scoring peptide. The results of this PEX19 binding assay are shown in Fig. 4A (left panel). Many trypanosomatid PMPs bound Tb-PEX19 in the regions predicted by the pattern search. More precisely, of the 32 binding sites analyzed, 21 exhibited significant binding. Of these, 16 showed little or no binding to GST and five showed some binding to GST, albeit at a lower level (Fig. 4A, right panel). These results compare well with those of a study of predicted sites in yeast PMPs, where 38% of the predicted peptides bound to GST-ScPEX19 (43). The results are summarized in Table 1. In some cases, the peptides which bound GST-PEX19 were in homologous positions in multiple proteins, while in other cases they were unique to a given protein. For all of these PMPs, except TbPEX12 and LmGIM5a, peptides corresponding to at least one predicted site bound to GST-TbPEX19. As an example, Fig. 4B shows the prediction scores for the RING finger peroxin TbPEX10, with a peak at aa 175 to 189. Several peptides centered around aa 175 to 189 bound to GST-TbPEX19 (Fig. 4A and C), although a second predicted region (aa 101 to 115) failed to do so (Fig. 4A).

To otherwise test whether TbPEX19 recognizes sites in TbPEX10 that are not foreseen by the algorithm, full-length TbPEX10 was subjected to peptide array scanning (Fig. 4C). GST-TbPEX19 bound to the highest scoring region (aa 172 to 196), named hereafter as BS2, which was consistent with the result of the binding-site screen (Fig. 4), but also to a second region in PEX10 (BS1; aa 82 to 102) that was not classified as a PEX19-binding site by the prediction program. To see whether the latter site is trypanosome specific, a replicate blot was incubated with purified GST-HsPEX19. Human PEX19 bound strongly to the predicted BS2 but only marginally to BS1 in this assay. This result suggests that the latter site significantly deviates from the typical conserved PEX19-binding site.

![FIG. 3. Substitution analysis of the PEX19 binding site in ALDP. Replicate peptide arrays harboring the 13-amino-acid PEX19 binding peptide of HsALDP (FLQRLLWLLRLLF) as well as peptides with single amino acid substitutions thereof were tested for interaction with GST fused to human PEX19 (upper panel) or *T. brucei* PEX19 (middle panel) or with GST alone (bottom panel). The first column has replicate peptides with no substitutions. Subsequent columns have peptides in which each residue is replaced individually by the amino acid indicated at the top of the column (e.g., column 2 has peptides with Ala substitutions). Bound PEX19 was visualized by monoclonal anti-GST antibodies in combination with the enhanced chemiluminescence reaction system. Spots of reduced intensity reflect peptides with a decreased binding affinity for PEX19. Similar binding patterns were observed for both PEX19 proteins.](image-url)
The presence of two apparently nonequivalent PEX19 binding sites in TbPEX10 prompted us to test for their individual roles in targeting this PMP in vivo. We generated constructs encoding TbPEX10 deletion mutants fused to GFP (as well as a full-length GFP-TbPEX10 fusion) for expression in both T. brucei and human cells. Immunoblot analysis of lysates from induced T. brucei transfectants showed that all proteins were expressed and that

### Table 1. Prediction of PEX19-binding sites

| Gene ID     | PMP        | Position no. of central 15-mer | Sequence of central 15-mer | Score   | Binding |
|-------------|------------|--------------------------------|---------------------------|---------|---------|
| Tb03.4808.580 | TbPEX2     | 191–205                        | svswmiliSLHTLFATLTAQY      | 3,613   | –       |
|             | TbPEX2     | 172–186                        | gagsVKRLKLKLKAVS          | 2,674   | ND      |
| TbPEX2      | 76–90      | 123–137                        | VYQDEFSFLLDVFIFgst         | 2,080   | ±       |
| TbPEX2      | 135–149    | gqrpDAVLHVSIVLPSR             |                          | 2,022   | +       |
| LmjF25.2230 | LmPEX2     | 77–91                          | aYQEEILVLDALLYRlwswrmg     | 10,796  | ±       |
| Tb03.4808.510 | TbPEX10    | 175–189                        | lklgFVLMLELLIRLWRA         | 5,271   | +       |
| Tb03.4808.510 | TbPEX10    | 101–115                        | G81G12                  | 3,894   | –       |
| LmjF25.2290 | LmPEX10    | 174–188                        | pgsvylVLVLVVELVRLWRYs      | 6,469   | +       |
| LmjF25.2290 | LmPEX10    | 87–101                         | prGPARVLLAVLQLVe          | 4,674   | –       |
| Tb00.1047053058479.230 | TbPEX10    | 85–99                          | MMIGTlKLlLALLAlcpw        | 23,923  | –       |
| Tb00.1047053058479.230 | TbPEX10    | 51–65                          | LAKGLYVLVLRLQG           | 10,262  | +       |
| Tb00.1047053058479.230 | TbPEX10    | 174–188                        | GILVLEELIVRLWRYm         | 6,975   | +       |
| Tb10.61.0440 | TbPEX12    | 56–70                          | pYNSEIWLVHLALEHrll        | 5,342   | –       |
| Tb10.61.0440 | TbPEX12    | 283–297                        | aamilGRVLVLVLLGFLMe       | 5,267   | –       |
| LmjF19.1250 | LmPEX12    | 228–242                        | RRNKVISLLLTLKPy           | 7,679   | +       |
| LmjF19.1250 | LmPEX12    | 341–355                        | raGPALLVARVLVLVLPgfrild   | 5,453   | –       |
| Tb00.1047053058479.230 | TbPEX12    | 287–301                        | RALMLARVLVLFLgfrlle       | 20,558  | +       |
| Tb00.1047053058479.230 | TbPEX12    | 240–254                        | RSLRFYYQLFLLEl             | 12,928  | +       |
| Tb00.1047053058479.230 | TbPEX14    | 253–267                        | GKAELStiGLKLRQG           | 1,683   | ND      |
| Tb00.1047053058479.230 | TbPEX14    | 71–85                          | GQPRKTLNEIKRLSeprpv       | 1,263   | ND      |
| LmjF21.1840 | LmPEX14    | 29–43                          | DADTTVQSAIRFLQDp          | 2,715   | ND      |
| LmjF21.1840 | LmPEX14    | 242–256                        | SPAALTEEVKRLQTeideakea    | 2,226   | ND      |
| Tb00.1047053058479.230 | TbPEX14    | 282–296                        | TNDNLESRIKLQEEvdk         | 2,941   | ND      |
| Tb00.1047053058479.230 | TbPEX14    | 133–147                        | LALGIVQRLYLFVKL           | 5,882   | –       |
| Tb00.1047053058479.230 | TbPEX14    | 175–189                        | elkrAFVNLLKCVYFLAltcipe   | 4,722   | +       |
| LmjF35.3700 | LmGIM5a    | 90–104                         | QLSHFFHVCFYFFEn          | 5,968   | –       |
| Tb00.1047053058479.230 | TbPEX5a    | 190–204                        | PLVPLHELARIAPN            | 2,468   | ND      |
| Tb00.1047053058479.230 | TbPEX5a    | 108–122                        | ggvaVVCWLYTLLLIGARQayll   | 1,710   | ND      |
| Tb00.1047053058479.230 | TbGAT1b    | 132–146                        | gPNATVVRVGLLLLWfa         | 12,114  | –       |
| Tb00.1047053058479.230 | TbGAT1    | 98–112                         | resGMLMIISSLLISRTF         | 6,530   | +       |
| LmjF31.0540 | LmGAT1     | 440–454                        | VGGYTRRLAQLLAAAltra        | 8,460   | +       |
| LmjF31.0540 | LmGAT1     | 179–193                        | legnLRHAIRALALFAVSCVpa    | 7,314   | ±       |
| Tb00.1047053058479.230 | TbGAT1     | 79–113                         | resGMLMIISSLLILQTSTI      | 12,476  | ±       |
| Tb00.1047053058479.230 | TbGAT1     | 75–89                          | RPSVYRFLFLRLIPpss         | 7,920   | +       |

*Shown are the position and the sequence of each peak scoring peptide (in bold capital letters) within a predicted binding site. Adjoining amino acids in lowercase letters reflect additional hits in close proximity to peak scoring peptides. Scores are presented in arbitrary units. Peptide binding to GST-TbPEX19, as shown in Fig. 4, is tabulated under the Binding column. ND, not determined. Gene identification numbers (IDs) are the systematic names of the genes in the GeneDB database (www.genedb.org). GAT1 is one isoform of the PMP70-like proteins of trypanosomatids (58).
each migrated at its expected molecular mass (Fig. 5). Figure 5 shows the targeting of each construct in human cells (left panels) or in T. brucei (right panels). Full-length TbPEX10 fused to GFP was localized to glycosomes in T. brucei and to peroxisomes in human fibroblasts, demonstrating that this trypanosomatid PMP is able to target to the human organelle. The necessity of the individual PEX19-binding sites for appropriate targeting was then analyzed by splitting the protein in two parts, one fragment containing aa 1 to 124, including the first predicted TMD and BS1, and a second fragment containing aa 113 to 299, including TMD2 and BS2. The N-terminal fragment was cytosolic in both T. brucei and human cells, indicating that BS1 and TMD1 are not sufficient for targeting to either the glycosome or the peroxisome (Fig. 5). In contrast, the C-terminal TbPEX10 fragment gave rise to a peroxisomal staining pattern when expressed in human fibroblasts, although some cytosolic staining was also noted. Expression of the same construct in T. brucei resulted in a punctate, glycosomal pattern for the GFP fluorescence. Surprisingly, the IFA showed that in these cells, glycosomal matrix proteins were mislocalized [see anti-glycosome antibody staining for TbPEX10(113-299) in Fig. 5], indicating that the truncated protein, while able to target to the glycosome, caused a dominant-negative defect in matrix protein import. Within 2 days after induction, many of the parasites were swollen or dead. This fusion protein contains the only motif known to be essential for function, the C-terminal RING domain (35). Although not the focus of the current study, this result indicates that the N-terminal half of PEX10 may also play a critical although yet undetermined role. Indeed, a roughly corresponding N-terminal deletion of S. cerevisiae PEX10 is not able to complement a pex10 mutant (S. Daubert and R. Erdmann, unpublished data).

To test for the importance of BS2 in the context of the full-length protein, a fusion protein missing just BS2 (aa 173 to 190) was also expressed in these two cell lines (Fig. 5). In both human fibroblasts and T. brucei, this fusion protein was cytosolic, indicating that BS2 is indeed necessary for targeting. The importance of TMDs for trypanosomatid PMP targeting was analyzed with a final GFP fusion protein containing aa 140 to 299, which includes BS2 but lacks either TMD. This truncated protein did not localize to T. brucei glycosomes or to human peroxisomes. Taken together, these results show that the predicted binding site BS2 and a transmembrane domain are required for targeting to either glycosomes or peroxisomes. Overall, these data show that the targeting patterns for TbPEX10 are similar in both human and T. brucei cells and that the nonconserved BS1 plays no role or only an ancillary role in PMP targeting.

Targeting of TbPEX12 and TbPEXP14 in human fibroblasts. The results for TbPEX10 localization in both human
and T. brucei indicate that PEX19-dependent targeting is conserved for at least one trypanosomatid PMP. That this conservation is valid on a more general basis is suggested by the number of correctly predicted PEX19-binding sites shown in Fig. 4A. However, another PMP, the RING finger protein TbPEX12 (29, 45), does not seem to follow this prediction pattern, as it did not show binding of its highest scoring regions to GST-TbPEX19. We therefore assessed whether this protein would target to human peroxisomes. Despite the fact that our algorithm was unable to predict a PEX19-binding site for this protein, TbPEX12 clearly localized to human peroxisomes (Fig. 6A, left panels), as indicated by its punctate staining pattern and colocalization with the peroxisomal marker protein HsPEX14.

PEX14, another PMP, is involved in the docking of PEX5 to the peroxisome membrane (2, 14). Previous studies indicate that HsPEX14 binds to a different region of HsPEX19 than do other human PMPs (16). Unlike in PEX12, for which we did identify a PEX19-binding site in the L. major and T. cruzi orthologues, the prediction algorithm did not detect a significant PEX19-binding site in any of the trypanosomatid PEX14 proteins (Fig. 4A). We therefore expressed GFP-TbPEX14 in human fibroblasts to determine whether it would target appropriately to peroxisomes. Interestingly, TbPEX14 was localized to the cytosol in human peroxisomes (Fig. 6B, left panels), although this same fusion protein clearly targets to glycosomes when expressed in trypanosomes (Fig. 6B, right panels). These results indicate

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**FIG. 5.** Evolutionarily conserved PEX19 binding site-dependent targeting of TbPEX10. Full-length GFP-TbPEX10 and various truncations (cartooned at left of panels) were expressed in human or T. brucei procyclic cells to assess the function of the TbPEX10 targeting signal. Immunoblot analysis of the T. brucei transfectants are shown for each construct, either uninduced (−) or induced (+) for expression of the fusion protein. These blots demonstrate migration to the expected molecular masses of ~59 kDa for GFP-TbPEX10Δ173-190, ~42 kDa for GFP-TbPEX10(1-124), ~48 kDa for GFP-TbPEX10(113-299), and ~45 kDa for GFP-TbPEX10(140-299). The full-length construct in T. brucei was previously verified (45). (Left three panels) GFP fusion proteins were expressed in human fibroblasts by transient transfection with appropriate expression plasmids. Cells were probed for the IFA using polyclonal anti-PEX14 antibodies. Merged images reveal predominant colocalization of the GFP fusion proteins with peroxisomal PEX14 (Px). Bar, 10 μm. (Right three panels) GFP fusion constructs were transfected into T. brucei procyclic cells to create stable cell lines. Induction with Tet was followed by the IFA at 24 h, as described for Fig. 2. Bar, 2 μm.
that targeting of PEX14 represents an exception to conserved PEX19-dependent targeting of PMPs.

**DISCUSSION**

Molecular taxonomy indicates that trypanosomatids are highly divergent organisms related to relatively obscure groups, such as diplomonds, and more well-studied organisms, such as fungi, metazoans, and Apicomplexa (47). The ancient divergence of these organisms is reflected in novel processes, such as mitochondrial RNA editing (51) and polycistronic transcription coupled with obligatory trans splicing (37). Trypanosomatids are the most divergent organisms known to possess peroxisomes, and therefore it is perhaps not surprising that the metabolic capabilities of the modified organelle, the glycosome, have adapted considerably compared to those of other organisms (9). Nonetheless, early studies showed that the import signals on matrix proteins are closely related and predominantly cross-functional with those seen in traditional yeast and human systems (7, 50). Furthermore, several of the peroxins required for peroxisomal protein import are conserved in trypanosomatids, implying conservation of the mechanisms and processes involved.

The mechanisms by which membrane proteins are targeted to and inserted in peroxisomal membranes have come under scrutiny recently. A key player in this process is PEX19, a predominantly cytosolic protein that interacts with multiple PMPs (17, 27, 43, 44, 48) and prevents their aggregation in cell-free translation systems (41, 46). PEX19 is required for the biogenesis of functional peroxisomes in all organisms analyzed so far. Previously, we showed that a modest knockdown of PEX19 in *T. brucei* procyclic cells leads to the generation of enlarged glycosomes and delayed growth (5). Here, we demonstrated by RNAi that PEX19 knockdown in the bloodstream stage is lethal, further validating the essentiality of the glycosome to the mammalian infective stages.

Several previous studies have shown that many PMPs can target appropriately when introduced into heterologous species. Several yeast PMPs target to peroxisomes in mammals (12), human PMPs target to yeast peroxisomes (24), and recently, it was shown that glycosomal ABC transporter GAT2 fragments are able to target to human peroxisomes (58). Molecular analyses have revealed much of the basis for this cross-targeting. The rigid C-terminal domain of PEX19 binds specifically to PMPs in regions that have been previously analyzed by two-hybrid (17) and mutational (16) studies and verified by in vivo testing (27, 31). Such work enabled the generation of an algorithm that predicts potential PEX19 binding sites on yeast and human PMPs (24, 43). Here we extend the studies to the most divergent case, the trypanosome. When fragments of the human ALDP protein were expressed in *T. brucei*, targeting to glycosomes required the presence of regions containing both the previously identified HsPEX19-binding site and a TMD, similar to the minimal requirements for proper targeting in yeast and human cells. These findings indicate that TbPEX19 can recognize a human PEX19-binding site. Likewise, the HsALDP binding sites for human and *T. brucei* PEX19 were found to be nearly identical when tested by substitution blot. The fact that both HsPEX19 and TbPEX19 can recognize the same region of this human PMP is especially interesting in light of the relatively low homology between the two PEX19 proteins and the lack of the farnesylation motif on the trypanosomatid orthologue (5). We have gathered evidence that farnesylation is required to increase the binding affinity of human PEX19 towards PMPs without changing its binding site specificity (data not shown).

The above results encouraged us to employ the algorithm to search for PEX19-binding sites in all known trypanosomatid PMPs and then to test these sites experimentally for their abilities to bind TbPEX19. Almost all PMPs contained one or more predicted sites, and typically at least one of these bound to TbPEX19. We studied one protein, PEX10, in detail. The algorithm predicted two sites, and peptide scanning confirmed one of these sites. In view of the predictions for the entire TbPEX10, the appearance of the true binding site is striking.
with a number of hits clustering around the peak scoring peptide (FVMLMLELLLIRLWRAV) (Fig. 4B), whereas the erroneously predicted site is represented by a single peak only. Additionally, a PEX19 binding site was predicted in all three trypanosomatid orthologues in this position. In vivo studies then confirmed that this specific binding site is required for the targeting of TbPEX10 to glycosomes and peroxisomes. In studies of the PMP70-like protein GAT1, other researchers have shown that residues 1 to 169 are sufficient to confer glycosomal targeting in GFP fusions, whereas aa 1 to 141 are not (58). Interestingly, the PEX19 binding site identified in our study maps in silico to aa 98 to 112 (Table 1) and in vitro to aa 92 to 120 (Fig. 4A) (TbGAT1). The 1-141 fusion may traffic improperly because it is truncated within the transmembrane domain adjacent to the binding site identified in the current studies. On the other hand, the algorithm fails to correctly predict a PEX19-binding site for TbPEX12, even though this protein clearly is able to target to both trypanosome and human peroxisomes. It is interesting to note that while TbPEX12 is an integral membrane protein, it does not possess strongly predicted transmembrane domains and could be targeted via a distinct interaction, which could either map to a different region of PEX19 or possibly be PEX19 independent. Hence, the algorithm is a useful but imperfect tool for guiding in vivo experiments assessing potential targeting sequences of trypanosomatid PMPs.

PEX3 and PEX14 are the only PMPs known to bind to the N-terminal portion of PEX19. PEX3 binds with high affinity to a 31-aa region at the N terminus of human PEX19 and with much lower affinity to a region slightly overlapping the PMP binding region, while PEX14 binds just downstream of the PEX3 high-affinity binding region (16, 32, 46). The C-terminal region of PEX19 binds to all other PMPs analyzed thus far, and this interaction is the focus of the prediction matrix. Thus, the inability to detect a PEX19-binding site in TbPEX14 is not unexpected, particularly given that the predicted PEX19-binding site in HsPEX14 did not bind HsPEX19 (24). It is interesting to compare the sequence similarity of human and T. brucei PEX19 across these domains. The alpha helix known to convey high-affinity binding to PEX3 is most highly conserved (45% similar or identical residues), and the helices involved in PMP binding are also relatively well conserved (41%). In contrast, the PEX14 binding helix is only 21% conserved. The high level of similarity in the PEX3 binding region is intriguing given that it has not been possible to identify a PEX3 orthologue in trypanosomatids on the basis of sequence analysis. Given its essential role in PMP insertion and peroxisome biogenesis, a functional PEX3 orthologue likely exists, although the sequence has considerably diverged. The low similarity in the PEX14 binding region of PEX19 ties well with our findings that TbPEX14 did not target to peroxisomes. A precedent exists for a higher level of species specificity within this region. Previous work using a chimera of human and yeast PEX19 containing the N-terminal two-thirds of the yeast protein and the C-terminal one-third of the human protein showed that it could complement a yeast PEX19 mutant (21). All other combinations failed to complement. Only the C-terminal domain of PEX19 (the PMP interactor) is exchangeable. The potential of specifically interrupting glycosomal biogenesis as opposed to peroxisomal biogenesis could lie in the PEX14-PEX19 interaction or in the as yet unidentified PEX3.

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