The human pathogens of the Leishmania and Trypanosoma genera compartmentalize glycolytic and other key metabolic pathways in unique subcellular microbodies called glycosomes, organelles related to the peroxisomes of mammals and yeast. The molecular machinery that carries out the specific targeting of glycosomal proteins to the organelle has not been characterized, although the bulk of glycosomal proteins contain the COOH-terminal tripeptide glycosomal peroxisomal targeting signal-1 (PTS-1) similar to the mammalian and fungal peroxisomal targeting signal. To characterize the mechanisms of glycosomal targeting, the gene encoding LdPEX5, designated LdPEX5, has been isolated from Leishmania donovani. LdPEX5 encodes a 625-amino acid protein with a molecular mass of 69.7 kDa. Like its homologs in yeast and humans, LdPEX5 predicts a protein with seven copies of a tetratricopeptide repeat in its COOH-terminal half. Several studies and generation of antibodies. Recombinant LdPEX5 bound xanthine phosphoribosyltransferase (XPRT), a PTS-1 containing glycosomal protein with a $K_d$ of 4.2 nM, but did not bind an XPRT in which the PTS-1 had been deleted. Moreover, binding studies with the COOH-terminal half of the LdPEX5 confirmed that this portion of the PEX5 protein was capable of binding the XPRT PTS-1 with an affinity of 17.3 nM. Confocal microscopy revealed that LdPEX5 was predominantly in the cytosolic milieu, and genetic analysis implied that LdPEX5 was an essential gene.

Parasites of the Trypanosomatidae family, including the human pathogens of the Leishmania and Trypanosoma genera, are evolutionarily primitive eukaryotes that exhibit a variety of molecular, biochemical, and cell biological characteristics peculiar to these organisms. Prominent among these is the glycosome, a unique peroxisome-like microbody that accommo-

dates glycolytic enzymes, as well as enzymes involved in fatty acid $\beta$-oxidation, glycerol metabolism, ether-lipid and pyrimidine biosynthesis, and purine salvage (1). The hallmark enzyme of peroxisomes, catalase, however, is absent in Trypanosoma brucei, the etiologic agent of African sleeping sickness, and Leishmania spp., which cause a spectrum of diseases collectively known as leishmaniases (2, 3). Although the enzymatic contents of glycosomes and peroxisomes differ, both types of microbodies share common structural features including that they are surrounded by a single phospholipid membrane bilayer, lack DNA, exhibit similar morphologies, are electron dense, sediment to equivalent densities in isopycnic gradients, and are presumed to share common mechanisms of protein targeting and biogenesis (4). Glycosomes appear to be indispensable to bloodstream forms of T. brucei, the causative agent of African sleeping sickness, because this stage of the parasite is entirely dependent upon glycolysis for ATP production (5). The role of the glycosome in Leishmania species is less clear, because this genus is capable of utilizing alternative fuel sources to produce cellular energy (6, 7). However, the multiplicity of key metabolic pathways that require glycosomal machinery for function suggests that proteins encompassed within this unique parasite organelle offer a number of potentially attractive targets for the therapy of trypanosomiases or leishmaniases.

Glycosomal, as well as peroxisomal, proteins are nuclear encoded, synthesized on cytosolic polyribosomes, and subsequently imported post-translationally without modification into the glycosomal matrix. This translocation across the glycosomal and peroxisomal membrane is mediated by a variety of targeting signals. The first peroxisomal targeting signal (PTS)\(^1\) to be recognized and characterized was the COOH-terminal tripeptide, serine-lysine-leucine archetype or PTS-1. PTS-1 appears to be both necessary and sufficient for protein import (8) and is a topogenic signal for proteins targeted to the peroxisome of mammals and yeast, the glyoxysome of plant cells, and the glycosome of trypanosomes and Leishmania (9–11). Mutational analysis has revealed that PTS-1 accommodates considerable acceptable degeneracy, because conservative substitutions at any of the three PTS-1 positions are tolerated (12). Although the majority of glycosomal proteins contains a PTS-1, other targeting signals have been described. These include PTS-2, an NH$_2$-terminal peptide found on T. brucei aldolase and hexokinase (13, 14), and an internal signal found on phosphoglycerate kinase and triosephosphate isomerase (15).

\(^{1}\) The abbreviations used are: PTS, peroxisomal targeting signal; PEX, peroxin; XPRT, xanthine phosphoribosyltransferase; PCR, polymerase chain reaction; bp, base pair(s); ORF, open reading frame; His$_6$-LdPEX5, His$_6$-tagged LdPEX5; ELISA, enzyme-linked immunosorbent assays; PBS, fetal bovine serum; PBS, phosphate-buffered saline; CT-LdPEX5, COOH terminus LdPEX5; TPR, tetratricopeptide repeats; HGPSRT, hypoxanthine-guanine phosphoribosyltransferase; PAGE, polyacrylamide gel electrophoresis; NTA, nitrotriacetic acid.
The mechanism by which Pts-1, the predominant topogenic signal, directs protein import into the glycosome is not known. Experiments in yeast and human cells (16, 17) have revealed that Pts-1 initially docks with a cytosolic protein designated peroxin 5 (PEX5). The soluble complex then interacts specifically with the membrane-associated peroxins, PEX13 and PEX14, for subsequent translocation into the organelle (18–20). To initiate a dissection of the molecular mechanisms that contribute to glycosome import and biogenesis, we have isolated the gene encoding the PEX5 homolog of L. donovani (LdPEX5), the causative agent of visceral leishmaniasis, over-expressed LdPEX5 in Escherichia coli, and purified recombinant LdPEX5 protein to homogeneity for biochemical characterization and immunolocalization. LdPEX5 exhibited a high affinity, specific interaction with the Pts-1 of the L. donovani xanthine phosphoribosyltransferase (XRPT) enzyme and has been localized predominantly to the leishmanial cytosol by confocal microscopy. Genetic experiments have implied that a functional LdPEX5 gene is essential for parasite survival.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents—**[32P]dCTP (300 Ci/mmol) and GeneScreen Plus membrane were purchased from NEN Life Science Products. All restriction enzymes and DNA modifying enzymes were acquired from either Life Technologies, Inc. or Roche Molecular Biochemicals. Ready-To Go Beads (dCTP) were obtained from Amersham Pharmacia Biotech. All other reagents were of the highest quality commercially available.

**Cell Culture**—The wild type, L. donovani line, DJ700, was grown as the extracellular promastigote form in completely defined Dulbecco's modified Eagle-Leishmania medium that was routinely supplemented with hemin and xanthine.

**Molecular Cloning of LdPEX5—**Degenerate sense (5'-CAGGCS-GAGAACGARMARGA-3') and antisense (5'-GTSGCCCNARTRT- TCCA-3') oligonucleotides were used as primers to amplify a 456-base pair (bp) fragment from L. donovani genomic DNA using polymerase chain reaction (PCR) technology. PCR was performed on an MJ Research instrument with standard ingredients and Taq polymerase using a 5-min hot start at 94 °C followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s. The PCR fragment was cloned into the Topo TA vector (Invitrogen, Carlsbad, CA) and sequenced on a Perkin Elmer Applied Biosystems 377 DNA automated sequencer using dye terminator cycle methodology to verify that the translation product was homologous to PEX5 family members. The 456-bp PEX5 fragment was then used as a probe to isolate the full-length PEX5 from an L. donovani cosmid library using previously reported high stringency conditions of hybridization and washing (21). The complete LdPEX5 open reading frame (ORF) was sequenced from clones only containing the LdPEX5 coding sequence. Cell pellets from 1-liter cultures were resuspended in 20 ml of 40 m M Tris, pH 8.0, 200 m M NaCl (binding buffer) containing mini EDTA-free protease inhibitor mixture (Roche Molecular Biochemicals), and the cells were lysed by two passes through a French Press. Clarified lysates were applied to a 1.5 × 8.0-cm Ni2+-NTA column (Qiagen) equilibrated in binding buffer at a flow rate of 0.3 ml/min. The column was washed with 100 ml of binding buffer, and His6-LdPEX5 was eluted with a 20-ml 10–50 m M imidazole (pH 8.0) step gradient in binding buffer. Fractions were analyzed by SDS-PAGE and Coomassie blue staining (26), and those containing a homogeneous protein migrating with an apparent mass of 75 kDa were pooled, dialyzed against 4 liters of 100 mM Tris, pH 8.0, 50 m M β-mercaptoethanol, and concentrated to 5.0 mg/ml in a Centricon 10 concentrator (Amicon, Beverly, MA). The NH2 terminus of the recombinant protein was validated by amino acid sequencing on an Applied Biosystems 473 gas phase sequencer.

For oligomerization studies a non-His-tagged version of LdPEX5 was also overexpressed in E. coli using a plasmid-LdPEX5 expression construct containing only the LdPEX5 coding sequence. Cell pellets from 1-liter cultures were resuspended in 20 ml of 40 mM Tris, pH 8.0, and the cells were lysed with a French Press. Solid (NH4)2SO4 was added to the clarified lysates to a concentration of 20% and incubated at 4 °C for 1 h. The precipitate was harvested by centrifugation, redissolved in 3 ml of 100 mM Tris, pH 9.0, and dialyzed 16 h at 4 °C against 2 liters of 100 mM Tris, pH 9.0. The dialyzed LdPEX5 solution was clarified by centrifugation, and 5.0 mg of protein were loaded onto a Mono Q column (Amersham Pharmacia Biotech) equilibrated with 100 mM Tris, pH 9.0, 100 mM NaCl at flow rate of 1.0 ml/min. The column was developed with a 40-ml 200–500 mM NaCl linear gradient. Fractions containing a homogeneous 75-kDa band on SDS-PAGE were pooled and submitted for NH2-terminal analysis to verify that the protein was LdPEX5 (Protein Microchemistry Center, University of Victoria, Victoria, Canada). LdPEX5 Antibodies and Immunoblotting—Polyclonal antiserum against LdPEX5 were generated in rabbits by Cocalico Biologicals Inc. (Reamstown, PA) using His6-LdPEX5 as an immunogen and standard injection protocols. Antiserum titers were determined by enzyme-linked immunosorbent assay (ELISA) on microtiter plates (Beckton Dickinson Labware, Lincoln Park, NJ) coated with 1 μg of His6-LdPEX5 well using goat anti-Rabbit IgG-HRP (Bio-Rad). Antiserum was reacted with rabbit horseradish peroxidase-conjugated secondary antibody (27). ELISA assays were developed with 2,2'-azinoisobutyryl-(3-ethylbenzothiazoline) sulfonic acid and H2O2.

Immunoblotting was performed with 1 × 105 L. donovani promastigotes fractionated by SDS-PAGE after transfer of proteins to a polyvinylidene difluoride membrane (NEN Life Science Products) (28).
Blots were blocked with 3% fetal bovine serum (FBS) diluted in phosphate-buffered saline (PBS) containing 0.1% Tween-20 and probed first with the LdPEX5 antisera diluted 1:10000 in blocking buffer followed by the goat anti-rabbit horseshadish peroxidase conjugate (1:5000) and developed with the Renaissance chemiluminescence reagent (NEG Life Sciences, Beverly, MA).

**Creation and Expression of xprtΔALK**—The molecular and biochemical characterization of the Leishmania PEX5 gene has been recently reported (29). The COOH-terminal tripeptide of XPR is alanine-lysine-leucine (ALK), a tripeptide that conforms to the glycosomal PTS-1 (12, 30). A 408-bp 3′ fragment from XPR was amplified from the pBACE- L. donovani expression vector (29) using a Taq DNA polymerase (Roche Molecular Biochemicals), the sense primer 5′-TACCTCGGC-GACGGGTGGCCT-3′, and the antisense primer 5′-TCTACAAAGGTGAACGGGGTGCCCT-3′, after 30 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min. The PCR product was then ligated into the Topo-TA vector (Invitrogen) for sequencing. A 426-bp SalI/KpnI fragment encompassing the 3′ portion of the *L. donovani* XPR was excised from the Topo TA vector and cloned into the corresponding sites of pBACE-XPR (29) to create xprtΔALK, an XPR that lacked the AKL COOH-terminal tripeptide. XPR and xprtΔALK were transformed into *S. cerevisiae* (31) *E. coli* and expressed in low phosphate induction medium, and the wild type and truncated XPR proteins were purified to homogeneity as described (29).

**Binding Assays**—A Pro-Bind flat bottom ELISA plate (Beckton Dickinson Labware, Lincoln Park, NJ) was coated with 3 μg/well of wild type XPR or xprtΔALK in 100 μl of PBS at 4 °C for 16 h. Unbound protein was removed by washing twice with PBS, and the plates were blocked with 200 μl of 3% FBS in PBS for 1 h at 25 °C. The blocking solution was discarded, and 100 μl of His6-LdPEX5 (0.8–800 nM) diluted in 3% FBS, 0.1% Tween 20 in PBS was added to each well and incubated at room temperature for 2 h. Unbound His6-LdPEX5 was discarded, and the wells were washed four times with 200 μl of 0.1% Tween 20 in PBS. LdPEX5 was quantitated by standard ELISA protocols using LdPEX5-specific rabbit antisera (1:1000) and goat anti-rabbit horseshadish peroxidase-conjugated secondary antibody (1:3000) (Roche Molecular Biochemicals) diluted in PBS containing 3% FBS and 0.1% Tween 20. ELISA assays were developed using 2,3,5-aminonitrobenzaldehyde (sulfonic acid) as the chromogenic substrate. Plates were quantitated on a Dynatech Laboratories MR700 plate reader at 410 nm.

**Proteolysis of LdPEX5**—LdPEX5 (1 mg/ml) was dissolved in 50 mM Tris, pH 7.4, and digested with trypsin (50 μg/ml) at 25 °C for 2 h. 200 μl aliquots were removed every 20 min for analysis by SDS-PAGE. A 38-kDa protease-resistant core protein distal to the trypsin cleavage sites was detected by polynucleotide dye fluorescence membrane (28) for NH2-terminal sequencing. This core domain corresponded to the COOH-terminal portion of LdPEX5 and was designated CT-LdPEX5.

**Expression and Purification of NT-LdPEX5-His6 and His6-CT-LdPEX5**—A 1.173-bp NdeI/XhoI fragment from pBACE-His6-LdPEX5 was subcloned into the corresponding sites of the pET 30b vector (Novagen, Madison, WI) to create NT-LdPEX5-His6. This construct encoded the NH2-terminal domain (amino acids 1–465) of LdPEX5 with a COOH-terminal His tag. A COOH-terminal fragment of LdPEX5 was amplified from pBACE-His6-LdPEX5 using a sense primer, 5′-AAGGGAACT-CCATATGCTGCAGAAGGTGACGAAC-3′, and a BamHI site (restriction sites underlined) by PCR with 25 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 45 s, and extension at 72 °C for 2 min. The PCR product was subcloned into the Topo-TA vector and sequenced. A 966-bp NdeI/ BamHI fragment was then cloned into the NdeI and BamHI sites of the pET 15b vector (Novagen) yielding His6-CT-LdPEX5, a DNA fragment encoding the majority of the COOH terminus of LdPEX5 (amino acids 303–625) preceded by an NH2-terminal His tag. The pET 30b NT- LdPEX5-His6 and pET 15b His6-CT-LdPEX5 constructs were transferred into the ER2566 strain of *E. coli* (New England Biolabs, Beverly, MA), and expression was induced in 1 l cultures for 4 h at 37 °C with 0.8 mM isopropyl-β-D-thiogalactopyranoside (Roche Molecular Biochemicals). Bacteria were harvested by centrifugation, and the cell pellets were suspended in 20 ml of 50 mM Tris, pH 8.0. 0.2 mM NaCl containing EDTA-β-naphtylmethylphosphonate (Roche Molecular Biochemicals) and lysozyme (14 kDa). Proteins were monitored using Coomassie blue staining. NT-LdPEX5 was harvested and resuspended in PBS, allowed to adhere to glass coverslips coated with polylysine (Sigma) for 30 min, and fixed for 10 min at −20 °C with methanol. The coverslips were then washed three times in PBS containing 0.2% Triton X-100 and blocked with 3% FBS diluted in PBS. Preimmune and LdPEX5 antisera were diluted 1:2000 in blocking solution and incubated at 25 °C for 1 h. Goat anti-rabbit IgG conjugated to ALEXA 594 dye (Molecular Probes, Eugene, OR) was used as the secondary antibody, and the stained parasites were visualized on a Leica confocal laser scanning microscope as described (32).

**Subcellular Fractionation**—Log phase DH70 *L. donovani* promastigotes (1 × 106) were mixed with an equal volume of silicic acid powder (Sigma), resuspended in 10 ml of 20 mM HEPES, 1 mM EDTA, 1 mM dithiothreitol, 250 mM sucrose, and mini-tab protease inhibitor mixture (Roche Molecular Biochemicals), and lysed in a tight-fitting Dounce homogenizer as reported (33). The lysed cell suspension was centrifuged at 1,500 × g for 10 min to remove the silicic carbide, unlysed cells, and nuclei. The supernatant was then centrifuged at 50,000 × g for 2.5 h at 4 °C. The resulting fractions were then collected from the bottom of the gradient, and each fraction was assayed for pyruvate kinase and hexokinase (34) enzymes, cytotoxic markers, and glycosomal markers, respectively (35). To assay for native LdPEX5, 20-μl aliquots from each fraction were diluted to 100 μl with 0.2% dH2O, applied to wells of an ELISA plate, and incubated at 27 °C for 20 h to adsorb proteins to the well. Native LdPEX5 was detected by ELISA as described above.

**Molecular Constructs for Targeted Gene Replacement and Transfections**—A sense oligogeneotide, 5′-CAAGGGCTTCGTCTGCTACTCGGACAG-CCAGAG-3′ and an antisense primer, 5′-CGGCGACGGCTGAGACATAGGATG-3′ (underlined) were used to amplify a 610-bp 5′ flank of LdPEX5 by PCR. Similarly, a 680-bp sequence 3′ to the XPR coding region was amplified using the sense primer 5′-CCCCGGGCTACAGCAGGAGGACAGGACAG-AGCACAG-3′ and the antisense primer 5′-CATGACCTTGGACAGAGCCAGGACTGATA-3′ (underlined), respectively, were used to amplify a 610-bp 5′ flank of LdPEX5 by PCR. Specifically, a 680-bp sequence 3′ to the XPR coding region was amplified using the sense primer 5′-CCCCGGGGTCTACAGCAGGAGGACAGGACAG-AGCACAG-3′ and the antisense primer 5′-CATGACCTTGGACAGAGCCAGGACTGATA-3′ (underlined), respectively. To generate the knockout constructs, the 610-bp 5′ and 680-bp 3′ flanks were cloned into the HindIII/SalI and Smal/BglII sites of the pX63HYG and pX63NEO vectors (36), which contain the hygromycin phosphotransferase and neomycin phosphotransferase resistance markers, respectively. The presence of the LdPEX5 flanking regions and their orientation were confirmed by restriction mapping. The drug resistance cassettes were designated pX63-NEO-Ldpx5 and pX63-HYG-Ldpx5, and the linearized plasmids used for the transfections were designated X63-NEO-Ldpx5 and X63-HYG-Ldpx5. Transfection protocols followed standard procedures (36).

**RESULTS**

Molecular Cloning of the LdPEX5 Gene—A PCR-based strategy was employed to clone the *L. donovani* PEX5 (LdPEX5) homolog. A multiple sequence alignment of the human, *Saccharomyces cerevisiae*, and *Caenorhabditis elegans* PEX5 proteins revealed two regions of marginally conserved amino acid sequence, corresponding to residues 342–346 (Qaene) and 488–494 (WNKLGAT) of the human PEX5 (boxed in Fig. 1), that were used to design degenerate PCR primers that accommodated for the codon bias of *Leishmania* (37). A 456-bp PCR fragment was then amplified from *L. donovani* genomic DNA and sequenced. Partial alignment of the 152-amino acid PCR translation product with human PEX5 revealed 32% amino acid identity and 55% similarity when conservative amino acid substitutions were considered (Fig. 1). The full-length LdPEX5 was then isolated on a cosmid using the 456-bp PCR fragment as a probe and sequenced. Translation of the LdPEX5 nucleotide sequence revealed an ORF of 1875 bp encoding a 625-amino acid protein with a calculated molecular mass of 69.7 kDa (Fig. 1). This ORF was preceded by an in frame termination...
tion codon 123–125 bp upstream from the presumed initiation Met codon (GenBank™ accession number AF198051). Reverse transcription-PCR of \textit{LdPEX5} RNA using a nondegenerate sense primer designed to the \textit{L. donovani} mini-exon (23) revealed a spliced leader junction site 235 bp upstream from the first Met. However, it should be noted that it is impossible to predict the \textit{LdPEX5} initiation codon with any certainty, because the predicted NH\textsubscript{2}-terminal 131 amino acids of \textit{LdPEX5} included 25 Met residues (19%) and do not exhibit homology with \textit{PEX5} proteins from higher eukaryotes.

A multiple sequence alignment of \textit{LdPEX5} with peroxisomal \textit{PEX5} proteins from human, \textit{S. cerevisiae}, and \textit{C. elegans} revealed a limited homology among the four proteins that was localized strictly to the COOH-terminal domain (Fig. 1). The COOH terminus of \textit{LdPEX5} (residues 300–625), like the peroxisomal \textit{PEX5} proteins (16), is comprised of seven tandem tetratricopeptide repeats (TPR) (bracketed underlines in Fig. 1). Pairwise alignments between \textit{LdPEX5} and the three other \textit{PEX5} proteins disclosed a 22–27% overall identity, percentages that increased to 30–37% when only the COOH-terminal domain is considered.

The NH\textsubscript{2}-terminal portion of \textit{LdPEX5} (amino acids 1–300) is not homologous to the other three aligned \textit{PEX5} proteins. This NH\textsubscript{2}-terminal polypeptide exhibits an unusual amino acid preference for Met, Gln, and Ala residues and appears to contain three tandem 22-amino acid repeats corresponding to residues 52–73, 74–95, and 101–122, each of which includes the motif QQQQA (double underline in Fig. 1). Secondary structure analysis of the \textit{LdPEX5} NH\textsubscript{2}-terminal domain using the GOR4 secondary structure prediction algorithm (38) implied that these Gln-rich repeats would adopt an \textit{\alpha}-helical conformation, although the biological role of these structural elements is clearly unknown. In addition, the NH\textsubscript{2}-terminal domain encompasses three copies of a pentapeptide, WXXX(F/Y), at positions 51–55, 174–178, and 291–295 (shaded boxes in Fig. 1), that are also conserved among \textit{PEX5} family members.

**Molecular Characterization of the \textit{LdPEX5} Locus**—Northern blot analysis revealed a single 3.7-kilobase \textit{LdPEX5} transcript \textit{L. donovani} promastigotes (Fig. 2A). Southern blot analysis indicated that the \textit{LdPEX5} locus contained only a single gene copy, because digestion of genomic DNA with EcoRI, NcoI, or SacI, each of which cut \textit{LdPEX5} once, excised only two fragments of dissimilar sizes that hybridized with the full-length ORF (Fig. 2B). Digestion with XhoI, another single cutter, only resulted in a single hybridization signal, presumably because the enzyme cleaved \textit{LdPEX5} into two approximately equal sized fragments. The restriction map of the \textit{LdPEX5} locus comprised from the nucleotide sequence and Southern blot data.
Southern blot analysis of genomic DNA and nucleotide sequence.

**C**

resolved on an agarose gel, and probed with LdPEX5

otes was digested with the indicated panel of restriction endonuclease,

cated (residues 303–625) NH2-terminal His-tagged version of

test whether the COOH-terminal portion of LdPEX5 contained

TPRs postulated to form the PTS-1 binding pocket (16, 39). To

respond to the COOH-terminal domain that encompassed the

(Fig. 1). Thus, the 38-kDa fragment LdPEX5 fragment corre-

sequence of LQKVTNSTD matching residues 303–311 of LdPEX5

ngle 38-kDa fragment, CT-LdPEX5, with an NH 2-terminal se-

ent homogeneity on Ni 2

LdPEX5 gel, blotted onto nylon membrane, and probed with the

mRNA was electrophoresed on a 0.8% formaldehyde agarose

gene 38-kDa fragment, CT-LdPEX5, with an NH 2-terminal se-

is displayed in Fig. 2C.

Purification and Characterization of Recombinant LdPEX5—pBAce-His6-LdPEX5 was overexpressed in E. coli, and the recombinant His6-LdPEX5 protein was purified to apparent homogeneity on Ni2+-NTA agarose (Fig. 3). The ability of LdPEX5 to recognize PTS-1 was then assessed by ELISA using L. donovani XPRT with and without (xprtΔAKL) the COOH-terminal PTS1 tripeptide, Ala-Lys-Leu (29), as the cargo protein. Fig. 4A demonstrates that LdPEX5 binds specifically to wild type Xprt but not to xprtΔAKL. Scatchard plot analysis revealed the interaction of Xprt and LdPEX5 to be high affin-

ity with a KD of 4.2 ± 0.57 nM (Fig. 4B). These data establish that LdPEX5 is an authentic high affinity PTS1 receptor.

A limited trypsin digestion of His6-LdPEX5 produced a single 38-kDa fragment, CT-LdPEX5, with an NH2-terminal sequence of LQKVTNSTD matching residues 303–311 of LdPEX5 (Fig. 1). Thus, the 38-kDa fragment LdPEX5 fragment corre-

sponded to the COOH-terminal domain that encompassed the TPRs postulated to form the PTS-1 binding pocket (16, 39). To test whether the COOH-terminal portion of LdPEX5 contained all the information necessary for PTS-1 recognition, a truncated (residues 303–625) NH2-terminal His-tagged version of LdPEX5, His6-CT-LdPEX5, was expressed and the His6-CT-LdPEX5 protein purified from E. coli (Fig. 3). His6-CT-LdPEX5 bound Xprt with a KD of 17.3 ± 5.1 nM, whereas no specific interaction between His6-CT-LdPEX5 and xprtΔAKL could be detected (Fig. 4, C and D). Thus, the COOH-terminal portion of LdPEX5 encompasses all the elements necessary for recognition of the Xprt PTS-1.

Quaternary Structure of LdPEX5—His6-CT-LdPEX5 was freshly purified and subjected to size exclusion chromatography on an Amersham Pharmacia Biotech Superdex-200. The protein migrated with a molecular mass of ~280 kDa, consistent with the
the expected size of 36 kDa that is predicted from the nucleotide sequence, intimating that His$_{6}$-CT-LdPEX5 behaves as a monomer in solution. Conversely, purified NT-LdPEX5-His$_{6}$ assembled into a large aggregate with an apparent mass $>2 \times 10^{6}$ Da (Figs. 4 and 5).

**Subcellular Localization of LdPEX5**—To establish the intracellular location of LdPEX5, polyclonal antibodies were generated against the purified recombinant protein. The antisera recognized only a single protein of $\sim$75 kDa on Western blots of crude *L. donovani* lysates (Fig. 6). This polypeptide was not recognized by preimmune sera. Confocal microscopy was then employed to determine the subcellular compartment for LdPEX5 by indirect immunofluorescence. As shown in Fig. 7A, the staining pattern for LdPEX5 was uniform throughout the cytoplasmic compartment. Preimmune antisera showed only background stain. In contrast, a discrete punctate pattern of staining was observed with antibodies generated against the *L. donovani* HGPRT, a glycosomal enzyme (32).

The environmental milieu of LdPEX5 was also confirmed biochemically after subcellular fractionation (33) of a *L. donovani* homogenate on a discontinuous sucrose gradient and assaying each fraction for LdPEX5 by ELISA. Under these sedimentation conditions, glycosomes migrate to the bottom of the gradient (Fig. 7B, fractions 1–5), whereas the cytosolic fraction remains at the top (fractions 9–13). After fractionation, $>90\%$ of LdPEX5 was found in the cytosolic fractions. This pattern paralleled the distribution of pyruvate kinase, a cytosolic marker (35). Approximately 5–10\% of LdPEX5 was present in fractions 1–4 in which the glycosomal enzymes HGPRT and hexokinase (32, 35) were found.

**Genetic Analysis of LdPEX5 Function**—Targeted gene replacement offers a genetic tool to determine gene/protein function in intact parasites. *LdPEX5/ldpex5* heterozygotes, authenticated by Southern blot analysis, were created from wild type parasites by transfection with either X63-NEO-$\Delta ldpex5$ and X63-HYG-$\Delta ldpex5$. Four separate experiments to create the homozygous $\Delta ldpex5$ line from the *LdPEX5/ldpex5:HYG* strain by transfection with X63-NEO-$\Delta ldpex5$. Four separate experiments to create the homozygous $\Delta ldpex5$ line from the *LdPEX5/ldpex5:HYG* strain failed to produce the desired knockout, however.

**DISCUSSION**

The glycosomal PTS-1 receptor gene, *LdPEX5*, has been cloned from *L. donovani*, and the gene product has been functionally characterized and localized. The LdPEX5 protein exhibits substantial structural resemblance to peroxisomal PEX5 proteins from higher eukaryotes, including significant amino
acid homology within the COOH-terminal half of the protein, seven TPRs, and three copies of a PEX5-specific pentapeptide sequence, WXXX(F/Y), that are theorized to interact with PEX14, another component of the peroxisomal protein translocation machinery (39). TPRs are present in a multiplicity of proteins with diverse biological functions (cell cycle regulation, neurogenesis, protein transport, protein folding, etc.) and presumably serve as structural elements in protein-protein interactions (40, 41), including those involved in PTS-1 recognition (16). Although there is considerable amino acid sequence divergence among TPRs, the crystal structure of protein phosphatase 5 reveals common TPR secondary structure motifs that are presumed to mediate these protein-protein interactions (40). Thus, the COOH-terminus of LdPEX5 is involved in PTS-1 recognition, whereas the NH2-terminal half of LdPEX5 provides a platform for the recruitment of other essential proteins required for glycosomal assembly. The resemblance between LdPEX5 and PEX5 proteins from peroxisome-containing higher eukaryotes, coupled with the relatively recent discovery and characterization of other peroxin gene sequences in both L. donovani (42) and T. brucei (43), lends further support for a common evolutionary ancestry for the glycosome and peroxisome. The ongoing genome sequencing projects for both genera of parasites should disclose other genes encoding components of the glycosomal import and biogenesis pathway.

Functional analysis of purified recombinant LdPEX5 proteins, His6-LdPEX5 and His6-CT-LdPEX5, confirmed that LdPEX5 was capable of binding with nanomolar affinity the L. donovani XPRT (29), a PTS-1-containing protein. The Kp value of His6-LdPEX5 for XPRT was 2 orders of magnitude lower than the interaction of the Pichia pastoris PEX5 for the PTS-1-containing tetradecapeptide (16). This PTS-1 recognition by LdPEX5 was mediated through the TPR-containing COOH-terminal portion, as His6-CT-LdPEX5 also recognized XPRT with high affinity. That the high affinity interactions of His6-LdPEX5 and His6-CT-LdPEX5 with XPRT were mediated through PTS-1 recognition was established, because a deletion construct of XPRT, xprtAAKL, lacking the PTS-1 tripeptide was not recognized by either the full-length or truncated His-tagged LdPEX5 proteins. These data demonstrate that the PTS-1 is the minimum sequence on XPRT required for LdPEX5 recognition.

Whether motifs other than PTS-1 influence the affinity of LdPEX5 for glycosomal proteins is unknown. Similar LdPEX5 binding experiments were also performed using the firefly luciferase, the L. donovani HGPRT, and the Trypanosoma cruzi HGPT. Luciferase is a peroxisomal enzyme (44), whereas the trypanosomatid HGPTs have a glycosomal milieu (32, 45). Furthermore, the COOH-terminal tripeptides of each of these three proteins, Ser-Lys-Leu (luciferase), Ser-Lys-Val (L. donovani HGPT), or Ser-Lys-Tyr (T. cruzi HGPT), conform to the relatively degenerate PTS-1 for glycosomal targeting (12) and can directly control the glycosomal localization of luciferase to the L. donovani glycosome (41). Of the three, only luciferase bound to His6-LdPEX5 with nanomolar affinity, whereas the HGPTs bound weakly, precluding an accurate determination of binding affinity (data not shown). It is interesting to note that HGPT is among a subset of glycosomal proteins that mislocalize in a pex2 L. donovani mutant, whereas other glycosomal are compartmentalized normally (46). That the weak interaction between HGPT and LdPEX5 influences mislocalization is, however, purely conjectural. Indeed, the variance in binding affinity among peroxisomal/glycosomal enzymes to His6-LdPEX5 could reflect sequence differences in the COOH-terminal tripeptide or discrepancies among other structural features of these proteins that contribute to LdPEX5 recognition. Recent studies using the yeast two-hybrid system have indicated that the binding affinity of PEX5 with a cargo protein is dependent not only upon the cognate PTS-1 tripeptide but also upon residues immediately adjacent to the tripeptide signal (47, 48). The role of the COOH-terminal tripeptide sequence in LdPEX5 binding affinity can now be tested by varying the XPRT PTS-1 sequence and determining binding parameters to His6-LdPEX5.

Gel permeation chromatography revealed that LdPEX5, like human PEX5 (39), forms a homotetramer, whereas CT-LdPEX5 behaves as a monomer in solution. These biophysical studies imply that the LdPEX5 oligomerization domain is located within the NH2-terminal portion of the protein. Deletion analysis of human the long form of PEX5 demonstrated that the tetramerization domain of this protein is confined to the NH2-terminal amino acid residues 1–251 (39). Interestingly, the NH2-terminal NT-LdPEX5-His6 (residues 1–391) also oligomerized. However, the extent of oligomerization of this truncated protein was much greater than that observed for the human PEX5 NH2 terminus.

Immunofluorescence and subcellular fractionation experiments indicated that LdPEX5 was primarily (90–95%) localized within the parasite cytosol with a small component (5–10%) associated with the glycosomal fraction. A similar bimodal distribution has been noted for human and yeast PEX5 (49, 50), lending support to a model of PEX5 shuttling between the cytosolic and peroxisomal subcellular compartments, a process that is dependent upon interactions with docking proteins on the peroxisomal surface (51). Two docking proteins, PEX13 and PEX14, are known to form strong interactions with PEX5 and to themselves (39, 52) and are, like PEX5, known to be essential for peroxisome biogenesis (20, 52). Although it is presently unknown whether analogous components of the peroxisomal translocation machinery exist for the glycosome in trypanosomatids, the presence of multiple WXXX(F/Y) repeats, found on peroxisomal PEX5 proteins and purported to be PEX14 association motifs (39), within the LdPEX5 NH2 terminus and the recently reported T. brucei PEX5 (53) implies that the PTS-1 import machinery into the glycosome will be analogous to that observed for the peroxisome of higher eukaryotes. Whether the glycosomal import machinery is essential for the viability of trypanosomatids is as yet unknown. However, the repeated inability to generate LdPEX5 null mutants by targeted gene replacement from the LdPEX5/ldpex5 heterozygotes using constructs proven to generate heterozygotes from wild type parasites implies that LdPEX5 is an essential gene in L. donovani. The cloning of the LdPEX5 has provided essential biochemical, molecular biology, and immunological reagents required to perform a thorough molecular dissection of the glycosomal protein import pathway to identify other components of glycosome biogenesis in this pathogenic protozoan.

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Peroxisomal Targeting Signal-1 Receptor Protein PEX5 from *Leishmania donovani*: MOLECULAR, BIOCHEMICAL, AND IMMUNOCYTOCHEMICAL CHARACTERIZATION

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