Glycosomes are membrane-bounded microbody organelles that compartmentalize glycolysis as well as other important metabolic processes in trypanosomatids. The compartmentalization of these enzymatic reactions is hypothesized to play a crucial role in parasite physiology. Although the metabolic role of glycosomes differs substantially from that of the peroxisomes that are found in other eukaryotes, similarities in signals targeting proteins to these organelles suggest that glycosomes and peroxisomes may have evolved from a common ancestor. To examine this hypothesis, as well as gain insights into the function of the glycosome, we used a positive genetic selection procedure to isolate the first Leishmania mutant (gim1-1 [glycosome import] mutant) with a defect in the import of glycosomal proteins. The mutant retains glycosomes but mislocalizes a subset of glycosomal proteins to the cytoplasm. Unexpectedly, the gim1-1 mutant lacks lipid bodies, suggesting a heretofore unknown role of the glycosome. We used genetic approaches to identify a gene, GIM1, that is able to restore import and lipid bodies. A nonsense mutation was found in one allele of this gene in the mutant line. The predicted Gim1 protein is related to the peroxin 2 family of integral membrane proteins, which are required for peroxisome biogenesis. The similarities in sequence and function provide strong support for the common origin model of glycosomes and peroxisomes. The novel phenotype of gim1-1 and distinctive role of Leishmania glycosomes suggest that future studies of this system will provide a new perspective on microbody biogenesis and function.
glycosome biogenesis mutants. We report here the isolation, via a positive genetic selection technique, of a Leishmania donovani cell line defective in efficient glycosomal import (gim1-1 mutant). This mutant line exhibits an unusual phenotype, not typically seen in peroxisome biogenesis mutants, in which the defect in glycosomal import is both partial and restricted to specific proteins. Even this limited defect has a pronounced effect on cellular metabolism, since the normally abundant lipid storage bodies were almost absent from the mutant cells. This unexpected alteration demonstrates that the glycosome plays an important role in Leishmania physiology.

We were able to identify a Leishmania gene that rescues the mutant to restore efficient glycosomal import. Interestingly, the encoded protein exhibits overall sequence similarity to mammalian peroxisome assembly factor 1 (PAPF1) (44, 54), which was recently renamed peroxin 2 (PEX2) (11). It also possesses the most characteristic motif of these proteins, a C12H6 cysteine ring motif near the carboxy terminus (39, 54). In the mutant strain, one copy of this gene was shown to contain a premature stop codon. The conservation of a gene involved in both glycosome and peroxisome biogenesis provides evidence at a new level for the theory that the two organelles evolved from a common ancestor. Our findings extend the evolutionary antiquity of the PEX2 gene family and the peroxisome lineage to a time preceding the divergence of trypanosomatids, one of the most ancient orders of eukaryotes.

**Materials and Methods**

**Tissue culture and transfection.** Promastigotes of L. donovani 51.1 were used in all experiments and were maintained in medium 199 (M199; Gibco) supplemented with 5% fetal calf serum (FCS; Atlanta Biologicals). Log phase parasites (4×107) were transfected by using a Gene Pulser (Bio-Rad) and standard procedures (26), using 10 to 50 μg of plasmid or cosm id DNA. Transfectants were grown overnight without selection. The next day, half of the culture was plated on 0.9% agarose in 10% FCS plates containing 150 μg/mL kanamycin and 10% fetal calf serum (FCS; Atlanta Biologicals). Log-phase parasites were transfected into the gim1-1/B-L cell line. Two of the 1,150 original transfectants passed this second screen and were characterized.

**Selection for glycosome biogenesis defects.** The selection scheme was based on one used to select for peroxisome-deficient yeast strains (12). A related scheme was proposed for isolating glycosome-deficient T. brucei (9), although stable mutants have yet to be described. L. donovani cells (108) expressing Ble-Luc (designated wt/B-L) were mutagenized by exposure to 12 mg of ethyl methanesulfonate per mL for 4 h in 10 mL of medium with shaking. Stringent mutagenesis (~90% killing) was used to attempt to mutate both alleles. Surviving cells (108) were allowed to recover for 24 h before the selection procedure in 5 mL of medium containing 600 μg of plasmocin (Sigma) per mL for 2 h. This selection killed ~85% of the cells after 3 days. Once surviving cells had recovered to 108 cells, secondary selection in 800 μg of plasmocin per mL for 3 h was carried out, and half of the 107 cells was immediately plated on agarose containing M199-10% FCS plates containing 150 μg of plasmocin per mL. Plasmocin-resistant colonies appeared at a rate of approximately 4×10−4 and were picked into liquid culture and grown for Luc compartmentalization analysis. Differential digoxigenin solubilization was performed as described previously (47), and Luc activity in pellet and supernatant fractions was measured on a Monolight 2010 luminometer (Analytical Luminescence Laboratory). Some of the mislocalization mutants obtained are sister clones, it is not possible to reliably measure the frequency of the desired mutants. However, we estimate that 1 in 107 to 108 mutagenized cells had the mislocalization phenotype. 

**Immunoblot analysis.** Differential digoxigenin solubilization was performed on 4×105 cells from the unselected wt/B-L cells and the mutant gim1-1/B-L cells at increasing digoxigenin concentrations. For hypoxanthine-guanine phosphoribosyltransferase (HPRT) and glyceraldehyde phosphate dehydrogenase (GAPDH), immunoblot analysis, pellet and supernatant fractions were loaded per lane on sodium dodecyl sulfate–10% polyacrylamide gels. For Luc, 56PGK (the 56-kDa isozyme of phosphoglycerate kinase), and ribosomal protein P0 immunoblots, 4×105 cells equivalents were loaded per lane. Immunoblot analysis was done as described previously (36). The anti-HPRT antiserum was a gift from Buddy Ullman, the anti-GAPDH antiserum was a gift from Paul Michels, and the anti-P0 antiserum (45) was a gift of Steven Reed and Yassir Skeily. The anti-56PGK and anti-T. brucei glycosome antisera have been described elsewhere (36).

**Electron microscopy.** Promastigotes were collected by centrifugation at 1,000×g for 10 min. Pelleted cells were gently resuspended and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h on ice. Cells were washed twice in 0.1 M sodium cacodylate buffer by postfixation for 2 h at 5°C in 1% osmium tetroxide–5 mM calcium chloride–0.8% potassium ferricyanide in 0.1 M sodium cacodylate. Cells were washed twice in water and stained en bloc in 2% uranyl acetate for 12 h. Following centrifugation, the cell pellets were gently resuspended in 100 μL of low-gelling-temperature agarose (0.8%) in 0.1 M sodium cacodylate. The solidified agarose was cut into small pieces for subsequent processing. Cells were dehydrated through a graded ethanol series to propylene oxide and infiltrated and embedded in Epon-812–Tissueon. Sections were cut at a thickness of 70 nm on a Sorvall MT-5000 ultramicrotome with a Diatome diamond knife and stained in saturated uranyl acetate in 50% ethanol and Sato’s lead stain (42). Sections were observed and photographed on a Zeiss 109 transmission electron microscope. For counts of glycosomes and lipid bodies, section areas were selected based only on a high cell density, using magnifications too low to resolve the organelles of interest. A map of the cells in the area was sketched, and each cell to be subsequently evaluated was numbered on the map. Each cell was then video recorded at a primary magnification of ×7,000 and a further magnification of ×20,000 to determine the number of glycosomes and lipid bodies for each cell. The lipid bodies were easily identified since they lack a bounding membrane and have a very regular shape. Glycosomes were identified by virtue of their thin membrane, size, and granular contents. A total of 75 cells was analyzed for each of the wt/B-L, gim1-1/B-L, and rescued gim1-1+/gim1-1 cell lines. A cross-grating replica (Polaron Equipment, Bio-Rad) of known gridding dimension was video printed at a magnification of ×7,000 and used to measure the exact total area of all cells analyzed for each cell line. 

**DNA sequencing.** Large inserts of genomic cosmids were sequenced by using partial restriction digestion followed by Southern blotting using end-labeled T7 or T3 primers as probe. 

**RNA and DNA analyses.** RNA blot analysis was performed as previously described (37), using 5 μg of total RNA isolated from wild-type and gim1-1/B-L cells and separated on a 1.5% agarose formaldehyde gel. Samples of genomic DNA isolated from wild-type and gim1-1/B-L cells were digested with various restriction enzymes. The fragments were separated on a 0.85% agarose gel, plotted to Nitran (Schleicher & Schuell), and hybridized. Riboprobes for both RNA and DNA blots were synthesized from pBSGIM1, which is a pBluescript (Stratagene) subclone of the 1.3-kb NdeI-XhoI fragment containing GIM1. Final stringencies were 0.1× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate) at 65°C.

**DNA sequencing.** Both strands of the GIM1 insert were sequenced by using a combination of nested deletion (22), random sequencing, and primer walking. DNA sequencing was on an ABI automated sequencer using ABI dye terminator cycle sequencing (Applied Biosystems, Foster City, Calif.). Genomic DNA isolated from both wild-type and gim1-1/B-L cells, using the high-fidelity polymerase Pfu (Stratagene). The products were cloned into pBluescript and sequenced. Independently generated PCR products were sequenced to verify the presence or absence of the identified mutation in both cell types.
Isolation of a glycosome-defective cell line. It has proven possible to isolate loss-of-function mutants of Leishmania by mutagenesis and appropriate selections (24, 25, 27). The identification of Leishmania cell lines defective in glycosome biogenesis presents several challenges not faced in the isolation of yeast peroxisome-deficient cell lines. First, in contrast to yeast, Leishmania is diploid and there is no experimentally manipulable sexual cycle. Second, unlike peroxisome-deficient yeast, glycosome-deficient Leishmania may not be viable. Third, due to differences between yeast and trypanosomatid metabolism, the metabolic selection strategies used to identify peroxisome-deficient yeast (13, 18, 58, 60) would be unlikely to yield mutants defective in glycosome biogenesis or import. To address the last two concerns, we adopted a selection scheme based on a positive selection for mutants defective in protein import (12). This protocol made no assumptions about metabolic roles and additionally could yield mutants only partially defective in glycosomal compartmentalization.

The starting cells were L. donovani promastigotes stably transfected with a Leishmania expression vector encoding a fusion protein consisting of Ble (15), which specifies phleomycin resistance, fused to the amino terminus of luciferase (Luc) which contains a C-terminal SKL glycosomal targeting signal. The specificity of this signal is well established (47). The plasmid and clonal line are designated pXB-L and wt/B-L, respectively. If the Ble-Luc fusion protein is sequestered in the glycosome, it should be unable to bind the selective agent phleomycin. Thus, phleomycin selection will kill cells with intact glycosomal import. In cells with defects in glycosomal import, Ble-Luc would remain partially or completely cytoplasmic and could bind phleomycin, thus preventing its cytotoxic action. Such cells should survive phleomycin selection. Figure 1 verifies the effectiveness of phleomycin selection on wt/B-L cells. Wild-type cells expressing a cytosolic Ble-LucSKL fusion protein were resistant and grew well even at high concentrations of phleomycin.

We mutagenized wt/B-L cells and selected with phleomycin. Numerous colonies arose which were then examined to determine if any had mislocalized Ble-Luc to the cytoplasm. Subcellular fractionation was carried out by using differential digitonin solubilization. At low digitonin concentrations, the glycosomal membrane remains intact due to its low cholesterol content, while the plasma membrane is solubilized, releasing cytosolic proteins (43). Following subcellular fractionation, Luc assays were performed to differentiate clones with Luc mislocalized to the cytosol from the majority of clones that displayed normal Luc targeting. Of 120 colonies tested, 5 showed evidence of mislocalization. As these were not necessarily independent, we concentrated on the clone gim1-1/B-L in which 50% of the Luc activity was cytosolic (Fig. 2). In comparison, in wild-type cells, Ble-Luc cofractionates with the glycosome-containing organelar pellet, while Ble-LucSKL is localized almost exclusively to the cytosolic fraction.

We next ruled out several trivial explanations for the mislocalization phenotype. To investigate the possibility that a plasmid-borne defect could explain the cytosolic fusion protein, pXB-L was reisolated from the gim1-1/B-L clone and transfected into wild-type cells. As seen in Fig. 2, the retransfected plasmid specified a fusion protein that cofractionated with the organelar pellet, with little remaining in the cytosol. To address the chance that an overall increase in fusion protein expression might overwhelm the glycosomal import pathways, immunoblot analysis was performed to compare fusion protein expression in unselected cells with expression in the gim1-1/B-L cell line. No significant difference in fusion protein expression was observed (Fig. 3, LUC). No differences in plasmid copy number or plasmid structure were detected between the gim1-1/B-L and wt/B-L clones (data not shown). The mislocalization defect in the gim1-1/B-L clone is therefore a cell-specific defect affecting the import of at least one protein into glycosomes.
The *gim1-1* mutant has few lipid bodies and mislocalizes a subset of glycosomal proteins. The properties of *gim1-1* were further explored by analyses of protein compartmentalization and ultrastructure. Western blot analysis was used to evaluate the localization of other glycosomal proteins. wt/B-L and *gim1-1*/B-L cells were solubilized with increasing concentrations of digitonin prior to subcellular fractionation. Figure 3 demonstrates that a subset of proteins was localized to the cytosol to a much greater extent in the *gim1-1*/B-L cell line than in the original parental cell line. These include the Ble-Luc fusion protein (solid arrow) and a Luc nonfusion protein (which contains an SKL signal) which was expressed from a second plasmid transfected into the two cell lines. Similarly, the glycosomal enzyme HGPRT (2) was inefficiently compartmentalized. This result further confirms that the defect in the *gim1-1*/B-L cell line is cell specific and affects localization of some proteins normally found within the glycosome. In contrast, other proteins appear to be compartmentalized normally. At digitonin concentrations that release cytosolic proteins such as the ribosomal protein P0, both GAPDH and the 56PGK remained in the organellar pellet in *gim1-1*/B-L cells and wt/B-L cells. (Also marked in the PGK immunoblot is the location of other phosphoglycerate kinase isoforms, hypothesized by analogy to other trypanosomatids [35, 36] to be a mixture of cytosolic and glycosomal forms.) GAPDH and 56PGK therefore appeared to be efficiently localized in *gim1-1*/B-L cells. Further studies using an antibody raised against *T. brucei* glycosomes, which detects at least eight proteins of unknown identity in *Leishmanina*, suggested that almost all glycosomal proteins were similarly compartmentalized in wild-type and mutant cells, since only one of the proteins detected was apparently mislocalized (data not shown). Taken together, this evidence demonstrates that *gim1-1*/B-L is a leaky mutant and is probably still capable of assembling glycosomes which contain at least a substantial complement of glycosomal proteins.

Electron microscopic analysis of wt/B-L, *gim1-1*/B-L, and the rescued *gim1-1*/B-L cell lines (see below) demonstrated that most cellular structures appeared identical in all three cell lines (Fig. 4). As predicted from the glycosomal protein targeting data, glycosomes were seen distributed throughout the cytoplasm in all cell lines (Fig. 4A to C). No major differences in glycosome morphology or number were apparent, with 17 glycosomes per 100 μm² for wild-type cells and 19 per 100 μm² for *gim1-1*/B-L cells. Unexpectedly, these analyses demonstrated an approximately 10-fold decrease in the number of lipid bodies in *gim1-1*/B-L cells (0.9 per 100 μm²) relative to wt/B-L cells (7.8 per 100 μm²). These lipid bodies have been postulated to serve as an energy reserve (30).

**Rescue of gim1-1 identifies a gene related to human PEX2.** A functional rescue approach was used to screen a wild-type *L. donovani* genomic DNA cosmid library for sequences that restore efficient glycosomal targeting to the *gim1-1*/B-L mutant cell line. Rescue of the mislocalization defect in the *gim1-1*/B-L cell line renders the cells phleomycin sensitive by virtue of the restoration of efficient glycosomal import of the Ble-Luc fusion protein. Briefly, the *gim1-1*/B-L mutant was transfected with a genomic library cloned into the cLHYG Leishmania cosmid shuttle vector (41). Individual hygromycin-resistant clones were grown in liquid culture and replica plated into media with and without phleomycin. Of 1,150 independent transfectants tested, two clones which were reproducibly phleomycin sensitive were identified. Analysis of Ble-Luc compartmentalization showed the wild-type pattern, with little fusion protein remaining in the cytoplasm (Fig. 5A). This result indicated that glycosomal targeting was restored in these clones. To confirm that the rescue was cosmid mediated and not the result of a reversion, the cosmids isolated from the two phleomycin-resistant clones were retransfected into *gim1-1*/B-L cells. The resultant lines were tested for phleomycin resistance and Luc compartmentalization. In contrast to an irrelevant cosmid, both of these cosmids (designated cGIM1A and cGIM1B) restored the wild-type phenotype of phleomycin sensitivity and Ble-Luc targeting to the organelar pellet (Fig. 5A). Thus, the cosmids each carry a gene that allowed this functional rescue.

Restriction mapping of cGIM1A and cGIM1B revealed that they shared a common region (Fig. 5B). To further map the gene (or genes) that rescued the *gim1-1* phenotype, we tested various deletion clones of the cosmids for the ability to restore import. A 6-kb segment rescued Ble-Luc import (Fig. 5, clone cGim1B3), and random fragments within this region were sequenced. BlastX searches (3, 16) of the sequences identified a region specifying an amino acid sequence with homology to human PEX2, previously known as PAF1, and homologous yeast proteins that have been identified as being required for peroxisome biogenesis in higher eukaryotes. A 1.3-kb XhoI-NdeI DNA fragment containing the corresponding open reading frame was subcloned into the expression vector pX63HYG to yield pGIM1. This plasmid fully restored normal targeting of the Ble-Luc fusion protein when transfected into *gim1-1*/B-L cells. It also restored the high numbers of lipid bodies to the parasites (13.4 per 100 μm² in *gim1-1*/B-L+pGIM1 cells versus 0.9 per 100 μm² in *gim1-1*/B-L cells). The number of glycosomes per unit area was slightly less than in wild-type cells (12 versus 17 per 100 μm²).

The DNA sequence of the pGIM1 insert reveals an open reading frame of 990 bp encoding a polypeptide of 330 amino acids with a calculated molecular mass of 38 kDa. Like other
glycosomal proteins, including the two integral membrane proteins characterized previously (38). Gim1 is very basic, with an isoelectric point of 9.67. As shown in Fig. 5, Gim1 has significant homology with the human peroxisomal integral membrane protein PEX2 (PAF1) (44), with 27% identity and 55% similarity at corresponding residues. Gim1 is also related to the proposed PEX2 homologs from the yeast Pichia pastoris (59) (Per6p; 28% identity and 57% similarity) and the filamentous fungus Podospora anserina (5) (car1; 23% identity and 54% similarity), all of which are known to be required for peroxisome biogenesis in their respective organisms. This level of relatedness is comparable to that of human and fungal proteins (~27% identity). In addition to the overall amino acid similarity, a C4HC4 cysteine ring motif in the carboxy-terminal half of Gim1 aligns with the C4HC4 motif of the PEX2 proteins (though car1 contains several insertions in this region). This motif is hypothesized to be involved in protein-protein interactions (14, 55). Gim1 is slightly shorter than human PEX2 and possesses a 24-residue amino-terminal extension relative to all known PEX2 family proteins except car1, which has an unrelated amino-terminal extension (Fig. 6).

To determine if GIM1 complements the primary lesion or acts as a suppressor, we performed additional analyses of GIM1 transcripts and genes. Northern blot analysis using the GIM1 gene detected a 1.4-kb GIM1 transcript at similar levels in both wt/B-L and gim1-1/B-L cells (Fig. 7A). Southern analysis using multiple restriction enzymes, including those shown in Fig. 7B, indicates that GIM1 is a single-copy gene. No obvious alterations such as large insertions, deletions, or rearrangements were detected. We then amplified, cloned, and sequenced both alleles from the gim1-1/B-L strain. One allele contained a point mutation that converted a glutamine codon (CAA) to a stop codon (TAA) at amino acid 240 (Fig. 6). This mutation would truncate the protein prior to the cysteine ring motif. The second allele was identical to that of the parent wild-type strain and the cosmid throughout the coding region. An additional 230 bp of flanking sequence on either side of the gene was analyzed, and no changes were observed. The presence of this mutation in one allele of the mutant and its absence from the wild-type parent were verified by direct sequencing of independent PCR products.

FIG. 4. Electron micrographs of wt/B-L (A and D), gim1-1/B-L (B and E), and rescued gim1-1/B-L (C and F) cells. Representative organelles are labeled: g, glycosomes; l, lipid bodies; m, mitochondrion. Panels on the left show a section through the cells. Panels on the right show enlargements of glycosomes marked with arrowheads in cells on the left. The mitochondria and glycosomes in panels B and E appear darker because the section was thicker and stained well. Left panels, bar = 1 μm; right panels, bar = 200 nm.
DISCUSSION

This report describes the isolation of an L. donovani cell line with a defect in efficient import of proteins targeted to the glycosome and the identification of the GIM1 gene that rescues the defect. The product of the GIM1 gene, Gim1, shares substantial amino acid sequence similarity with the PEX2 family of proteins from humans and yeast, all of which are peroxisomal integral membrane proteins known to be required for peroxisome biogenesis. This represents the first identification of a gene involved in glycosomal biogenesis.

The results of this study support the view that peroxisomes and glycosomes have a common evolutionary origin. The similarity in protein targeting signals, the partial overlap in metabolic pathways, and now conservation of a gene involved in biogenesis all point to a common ancestor for all microbody organelles. Since kinetoplastids are the first organisms on the evolutionary tree to possess these unique organelles, we can speculate that the endosymbiotic event giving rise to extant microbodies may have occurred after the separation of the most divergent eukaryotes, such as Giardia, Microsporidia, and Trichomonas. Kinetoplastids are also the first mitochondriate organisms, suggesting that the acquisition of the progenitors of both mitochondria and microbodies could have occurred at similar points in the evolution of eukaryotes.

Although the absence of peroxisomes has a devastating effect on human development (affecting the function of brain, liver, and kidney and leading to perinatal death), peroxisomes are not required for cellular viability. Thus, it has been possible to identify numerous mutants with peroxisome biogenesis defects by using selection and screening strategies focused on the loss of a peroxisomal metabolic pathway. However, the different metabolic role of glycosomes suggested that they might be essential in Leishmania, and we therefore chose a positive selection strategy that did not require the loss of metabolic functions. We do not know whether only mutants with partial defects in glycosomal biogenesis are viable, and future studies seeking new gim mutants will provide the answer to this question. Although leaky mutants are often not considered useful for isolating complementing genes, we were able to use the gim1-1 mutant to isolate the GIM1 gene.

The function of PEX2 is unknown. Its localization within the peroxisomal membrane and its conserved cysteine ring motif have led to speculation that PEX2 homologs are involved in protein translocation into the microbody matrix (59). PEX2 gene defects are one cause of the fatal human peroxisomal disorder Zellweger's syndrome (44). Mutants in PEX2 family genes described thus far are defective in the import of all peroxisomal proteins examined (44, 54, 59) (although a Pichia mutant is slightly leaky). In contrast, the gim1-1 mutant mislocalizes to the cytosol only a subset of proteins with the type 1 signal. These include the marker enzyme Luc, which has a C-terminal SKL, and HGPRT, with a C-terminal SKV (2). Curiously, another type 1 protein, GAPDH (= SKM, as found in the Leishmania mexicana gene [20], as well as S6PGK, a protein predicted by analogy with the trypanosome (1) and Critidia fasciculata (51) homologs to lack a type 1 or 2 signal, are efficiently imported into the organelar pellet. Perhaps most similar to the gim1-1 phenotype in this respect is the pex5 (pay32) mutant of the yeast Yarrowia lipolytica. In this mutant, some type 1 peroxisomal proteins remain in the cytoplasm while others cofractionate with peroxisomes, apparently trapped as translocation intermediates (52). Since PEX5 is not homologous to GIM1, it is clear that defects in a variety of genes could lead to a partial defect in organelle biogenesis. It will be interesting to determine how the gim1-1 defect allows some type 1 proteins entry into the glycosome while others remain in the cytosol. It is possible to envision that competition at the glycosome membrane for scarce intact import machinery causes a significant amount of proteins with weak glycosomal targeting signals to remain unimported in the cytosol.

The presence of a mutant allele, gim1-1, in the mutant strain strongly supports the hypothesis that GIM1 rescues by virtue of complementation of the initial lesion. The presence of both wild-type and mutant alleles in the gim1-1 strain suggests that
themutantallelemaybehaveasadominantnegativemutation withrespecttothephenotypethatweobserved.If,ashypoth-
esized,PEX2homologsareinvolvedinprotein-proteininter-
actions,adefectiveGim1proteincouldresultinnonproductive
interactionsyieldingadominantnegativephenotype.Another
alternativeisthatthe
\textit{gim1-1}
phenotypeissimplytheresultof
agenedosageeffect.Ineithercase,thepresenceofawild-type
allele may account for the restricted nature of the import
defect.Althoughnotcommon,dominantnegativeallelesfor
twogenesinvolvedinperoxisomebiogenesishavebeen de-
scribed(53).Interestinglythesedominantnegativeallelesyield
aperoxisomeimportdefectwhenpresentwithawild-type
allelein diploid cellsoftheyeast
\textit{Hansenula polymorpha}
but
peroxisomedeficiencywhenaloneinhaploids.Inthefuture,
we will attempttodisrupt
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paucityofstrategiesformanipulatinggeneexpression),suc
suchstudieswillbeimportantbecausetheywillallowustofurther
exploretheroleoftheglycosomeinparasitevirulence and
viability.

Our observations on the phenotype of the mutant, which
exhibitsonlyveryrestricteddefectsinproteinimport,provide
preliminary support for the theory that glycosomes are critical
organelles. We observed a dramatic decrease in the number of
lipid bodies in \textit{gim1-1} cells compared to wild-type cells. Rescue
withthe
GIM1 generestoredhighnumbersoflipidbodies.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6}
\caption{Comparison of the amino acid sequences deduced from the \textit{L. donovani} \textit{GIM1} gene and other genes in the \textit{PAF} gene family. Sequences shown: Gim1, \textit{L. donovani}; PAF1, human PEX2; car1, \textit{Podospora anserina} PEX2; Per6p, \textit{Pichia pastoris} PEX2. Black shading indicates identity with Gim1, and gray shading indicates similarity. Open circles mark the cysteines in the C_3HC_4 ring motif; asterisks designate stop codons. The location of the premature stop codon found in the \textit{gim1-1} mutant allele is marked by an arrow. Only the domains homologous to Gim1 are shown; car1 and Per6p extend another 98 and 82 amino acids, respectively.}
\end{figure}

the mutant allele may behave as a dominant negative mutation with respect to the phenotype that we observed. If, as hypoth-
esized, PEX2 homologs are involved in protein-protein inter-
actions, a defective Gim1 protein could result in nonproductive
interactions yielding a dominant negative phenotype. Another
alternative is that the \textit{gim1-1} phenotype is simply the result of
a gene dosage effect. In either case, the presence of a wild-type
allele may account for the restricted nature of the import
defect. Although not common, dominant negative alleles for
two genes involved in peroxisome biogenesis have been de-
scribed (53). Interestingly these dominant negative alleles yield
aperoxisomeimportdefectwhenpresentwithawild-type
allele in diploid cells of the yeast \textit{Hansenula polymorpha} but
peroxisome deficiency when alone in haploids. In the future,
we will attempt to disrupt \textit{GIM1} in order to definitively char-
acterize its role in glycosome biogenesis and to generate a true
glycosome-deficient \textit{Leishmania} cell line. Although disruption of
critical genes is still more difficult in trypanosomatid than in
organisms such as yeast (due to the lack of a sexual segregation
system, the slow growth and ploidy of the parasite, and the
paucity of strategies for manipulating gene expression), such
studies will be important because they will allow us to further
explore the role of the glycosome in parasite virulence and
viability.

Our observations on the phenotype of the mutant, which
exhibits only very restricted defects in protein import, provide
preliminary support for the theory that glycosomes are critical
organelles. We observed a dramatic decrease in the number of
lipid bodies in \textit{gim1-1} cells compared to wild-type cells. Rescue
with the \textit{GIM1} gene restored high numbers of lipid bodies. We
can pose several possible explanations for this finding. The first
is that Gim1 has pleiotropic functions and independently af-
facts both glycosomes and lipid bodies. Another explanation
could be that disruption of glycolysis caused by the mislocal-
ization of one or more glycolytic enzymes to the cytosol may
force\textit{gim1-1} cells to utilize their lipid reserves as an energy
source. Finally, glycosomally located ether-lipid biosynthesis
could be disrupted, negatively affecting the biosynthesis of lipid
bodies more directly. It is known from studies of patient’s with
Zellweger’s syndrome that while some peroxisomal proteins
are stable and functional when mislocalized to the cytosol,
some, including enzymes of ether lipid synthesis, are not (23).

An issue of great potential interest is the biosynthesis of an
important surface glycolipid, LPG, which is anchored to the
membrane by a lipo-alkyl-ether-lipid. LPG has been implicated
in several critical roles in the \textit{Leishmania} infectious cycle (57).
Disruption of ether-lipid synthesis in the glycosome in \textit{gim1-1},
as suggested by the lack of lipid bodies, might have significant
effects on the expression of LPG and parasite virulence. The
diversity of pathways contained within the glycosome, com-
bined with the possibility of isolating mutants with highly re-
stricted defects, as described here, suggests that the analysis of
glycosome biogenesis mutants will provide new insights into
the functional role of subcellular compartmentalization.

\textit{Leishmania} parasites cause mucocutaneous, cutaneous, and
visceral infections, killing approximately 400,000 of the 367
million people at risk each year (4). No effective vaccines
against this group of organisms are currently available, and
treatment generally involves use of toxic compounds. Drug
discovery is therefore of major importance, and the glycosome
stands out as a potential new drug target (31, 50). It seems
likely that the ability to inhibit the import of glycosomal pro-
teins would prove deleterious to the parasite. Even preventing
the import of a subset of glycosomal proteins would likely be
toxic since it would effectively segregate enzymes within a metabolic pathway into different subcellular compartments. Whether specific inhibitors of Gim1 function could be developed awaits further study, but given the rather moderate similarity of Gim1 and human PEX2, it seems likely that structural differences in biogenesis proteins could be exploited for drug development.

ACKNOWLEDGMENTS

This work was supported in part by NIH grants AI22645 to M.P. and AI30186 and AI31078 to S.M.B. The University of Puget Sound provided support for instrumentation used in transmission electron microscopy.

We thank Kayde Radford and Neil Cochran for excellent technical assistance and Tom Westlake of the SBRI DNA core facility for sequencing work. We also thank Joel Goodman for suggestions and assistance and Tom Westlake of the SBRI DNA core facility for providing support for instrumentation used in transmission electron microscopy.

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