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Functional Identification of a *Leishmania* Gene Related to the Peroxin 2 Gene Reveals Common Ancestry of Glycosomes and Peroxisomes

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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 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 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.

Glycosomes are organelles found only in the kinetoplastid protozoa (33, 34), which includes the trypanosomatid pathogens causing sleeping sickness (African trypanosomes), Chagas' disease (American trypanosomes), and leishmaniasis (*Leishmania* spp.). The glycosome compartmentalizes several important metabolic pathways, including the first six steps of glycolysis and enzymes involved in purine salvage and pyrimidine biosynthesis (33). Glycosomal compartmentalization has been hypothesized to be required for the high glycolytic rate of African trypanosomes, arguably the highest among all eukaryotes, while devoting less than 10% of total cellular protein to this pathway (34). In these parasites, virtually all energy is derived from glycolysis (33). The glycosome is also thought to be the site of the synthesis of the ether-linked lipids (32), which in *Leishmania* comprise the plasma membrane anchor of a major surface virulence determinant, the lipophosphoglycan (LPG) (56). The compartmentalization of these critical metabolic pathways has led to the consideration of the glycosome as a potential target for drug development.

Based on its single membrane, dense matrix, and lack of DNA, the glycosome is categorized as a member of the microbody family of organelles, which also includes the peroxisomes found in nearly all higher eukaryotes as well as the glyoxysomes of plants (8, 40). However, glycosomes perform many functions that peroxisomes do not. For example, the enzymes involved in glucose metabolism and nucleic acid synthesis that are glycosomal in kinetoplastids are cytoplasmic, not peroxisomal, in other organisms. At least some enzymes involved in ether-lipid synthesis (19) and β-oxidation of fatty acids (21, 28) occur in both glycosomes and peroxisomes.

Intriguing evidence for an evolutionary relationship between glycosomes and peroxisomes has come from the determination of the signals which target proteins to their matrices. At least two types of targeting signals directing proteins to peroxisomes and glycosomes are related at the sequence level. The type 1 peroxisomal targeting signal, a C-terminal Ser-Lys-Leu (SKL) (17), can also direct heterologous proteins to the trypanosome glycosome (47). However, mutational analysis has established that considerably more variation in the signal is tolerated for efficient glycosomal targeting in *Trypanosoma brucei* than for peroxisomal targeting (47). Several glycosomal proteins contain an SKL sequence, or one closely related to it, at their carboxy termini (46), and some of these have been demonstrated to function in glycosomal targeting (7, 48, 49). The functional relationship of type 2 targeting signals is less clear, as there are conflicting reports as to whether the type 2 peroxisomal targeting signal is capable of directing proteins to the glycosome (6, 49). However, the type 2 peroxisomal targeting motif is similar to one that directs an endogenous protein to glycosomes (6).

Until now, nothing was known about the genes involved in glycosomal biogenesis and their relationships to peroxisome biogenesis genes, due in part to the inability to generate stable

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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 the 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 (PAF1) (44, 54), which was recently renamed peroxin 2 (PEX2) (11). It also possesses the most characteristic motif of these proteins, a C_3HC_4 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 Biochemicals). Log-phase parasites (4×10^7) were transfected by using a Gene Pulser (Bio-Rad) and standard procedures (26), using 10 to 50 μg of plasmid or cosmid DNA. Transfectants were grown overnight without selection. The next day, half of the culture was plated on 0.9% agarose–M199–10% FCS plates containing the appropriate selective agent(s), and the remaining half was grown under selection in liquid culture. Geneticin (Sigma) was used at 10 μg/ml and hygromycin (Sigma) was used at 35 μg/ml in both liquid and agarose plate cultures.

Plasmids and cosmids. The pX vector (29), which specifies geneticin resistance, was used to express bleomycin resistance protein (Ble)-luciferase (Luc) fusion proteins with and without a glycosomal SKL targeting signal. The *BLE* coding region was amplified from pUT333 (CAYLA Laboratories), using the primers 5′ BLE (*GACGCTCGAGAGGCCT*ATGGGCGAAATGACCGACCA; the start codon is underlined, and the sequence of the linker containing *Xho*I and *Stu*I recognition sites is italicized) and 3′ BLE (*GACGAAGCTT*GCGCATGA GATGCCTGCAAGCAA; linker containing a *Hin*dIII site is italicized). The product was cleaved with *Xho*I and *Hin*dIII and subcloned upstream and in frame with the *LUC* gene in pBSLUC1 (a gift from C. C. Wang and Jürg Sommer). This yielded plasmid pXB-L, which encodes a fusion protein consisting of Ble-Cys-Ala-Ser-Leu-Ile-Luc. pXB-LΔSKL was constructed in the same way, by subcloning the *BLE* gene PCR product upstream and in frame with the *LUC* gene in pLUH207 (48), which encodes a Luc lacking the SKL targeting signal. The targeted and untargeted *BLE-LUC* genes were excised from their respective plasmids on *Stu*I-*Bam*HI fragments and subcloned into pX digested with *Sma*I and *Bam*HI to yield pXB-L. Sequencing of the *BLE-LUC* junction confirmed that the two coding regions were joined in frame. Transfections for expression of Luc (as a nonfusion protein) used the plasmid PX63HYG, which specifies hygromycin resistance (10). Similarly, the cosmid backbone used for the *L. donovani* genomic DNA library contains the hygromycin resistance gene. This library has been described previously and is from strain Ld4 (41). Subclones of the relevant cosmids were made by deletion of restriction fragments followed by religation or by subcloning specific restriction fragments into pX63HYG.

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 (10^9) 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 (10^8) were allowed to recover for 24 h before the primary selection in 5 ml of medium containing 600 μg of phleomycin (Sigma) per ml for 2 h. This selection killed ~85% of the cells after 3 days. Once surviving cells had recovered to 10^8 cells, secondary selection in 800 μg of phleomycin per ml for 3 h was carried out, and half of the culture (5×10^7 cells) was immediately plated on 0.9% agarose–M199–10% FCS plates containing 150 μg of phleomycin per ml. Phleomycin-resistant colonies appeared at a rate of approximately 4×10^{-5} and were picked into liquid culture and grown for Luc compartmentalization analysis. Differential digitonin 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). Since it is likely that many 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 10^7 to 10^8 mutagenized cells had the mislocalization phenotype.

Immunoblot analysis. Differential digitonin solubilization was performed on 4×10^7 cells from the unselected wt/B-L cells and the mutant *gim1-1*/B-L cells at increasing digitonin concentrations. For hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and glyceraldehyde phosphate dehydrogenase (GAPDH) immunoblot analysis, pellet and supernatant fractions from 10^7 cell equivalents 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, 4×10^6 cell equivalents were loaded per lane. Immunoblot analysis was done as described previously (36). The anti-HGPRT 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 Yasir Skeiky. The anti-56PGK and anti-*T. brucei* glycosome antisera have been described elsewhere (36).

Electron microscopy. Promastigotes were collected by centrifugation at $1{,}000 \times g$ 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 then washed twice in 2% sucrose in 0.1 M sodium cacodylate, followed 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 pelleted cells were gently resuspended in 100 μl of 2% agarose (SeaPlaque low-gelling-temperature agarose) 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 tEpon-812 (Tousimis). Sections were cut at a thickness of 40 to 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 printed at a primary magnification of ×7,000 and then studied at a high magnification (×12,000 to ×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, *gim 1-1*/B-L, and rescued *gim1-1*+pGIM1 cell lines. A cross grating replica (Polaron Equipment, Bio-Rad) of known grating dimension was video printed at a magnification of ×7,000 and used to determine the exact total area of all cells analyzed for each cell line.

Rescue of the *gim1-1*/B-L cell line. The *L. donovani* cosmid library in cLHYG was transfected into the *gim1-1*/B-L cell line, and 1,150 individual hygromycin-resistant colonies were grown in 96-well microtiter plates in M199–10% FCS. After 14 days, a visible button of cells was present in all wells, and the plates were replica plated into 96-well plates containing 0 or 130 μg of phleomycin per ml. An eight-well multichannel pipettor was used to inoculate approximately 5×10^3 to 1×10^4 cells into each well. Plates were scored after 7 days for sensitivity to phleomycin. Eight colonies were identified as possibly phleomycin sensitive and were then rescued from the master plate and retested for phleomycin sensitivity. Two of the 1,150 original transfectants passed this second screen and were hypothesized to contain a cosmid restoring normal glycosomal import. Cosmids were isolated and transformed into *Escherichia coli*. Restriction mapping of genomic cosmid inserts was performed by using partial restriction digestion followed by Southern blotting using end-labeled T3 or T7 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, blotted to Nytran (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 *Nde*I-*Xho*I 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 pGIM1 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.). The entire coding region of GIM1, plus approximately 300 bp flanking sequence on either side, was amplified from both wild-type and *gim1-1* cells, using the high-fidelity polymerase *Pfu*I (Stratagene). The products were cloned into pBluescript and sequenced. Independently generated PCR products were directly sequenced to verify the presence or absence of the identified mutation in both cell types.

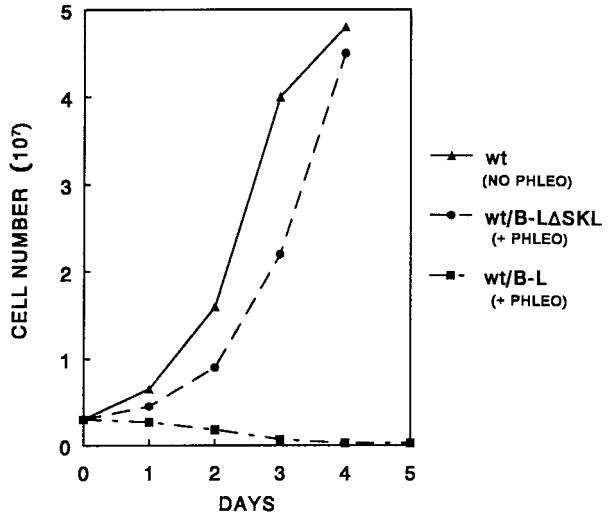

FIG. 1. Efficiency of phleomycin selection on *L. donovani* cell lines expressing Ble-Luc compared to a cell line expressing untargeted Ble-LucΔSKL. Stably transfected cells (3×10^6) were exposed to phleomycin (600 μg/ml) for 2 h, washed, and grown in fresh M199. For comparison, growth of wild-type cells in nonselective medium is shown. (No obvious differences were noted between the lines in nonselective medium.) Cells were counted at 24-h intervals.

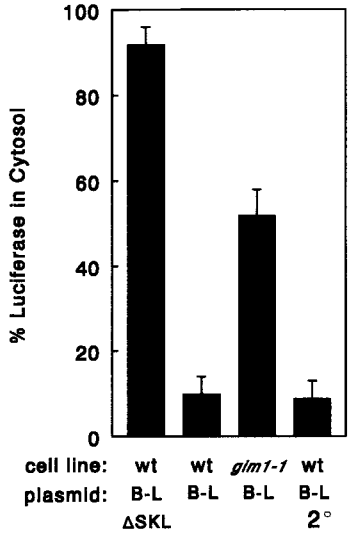

FIG. 2. The *gim1-1*/B-L cell line mislocalizes Ble-Luc fusion protein to the cytosol. Cells from the indicated cell lines were fractionated into organellar pellet and cytosolic supernatant fractions following differential digitonin solubilization of the plasma membrane. The percentage of Luc activity present in the cytosolic fraction is shown. The bar marked wt/B-L 2° indicates wild-type cells transfected with pXB-L isolated from the *gim1-1*/B-L cell line.

Nucleotide sequence accession number. The nucleotide sequence of the pBSGIM1 insert has been deposited in GenBank with accession number U80074.

RESULTS

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-LucΔSKL 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 organellar pellet, while Ble-LucΔSKL 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 organellar 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.

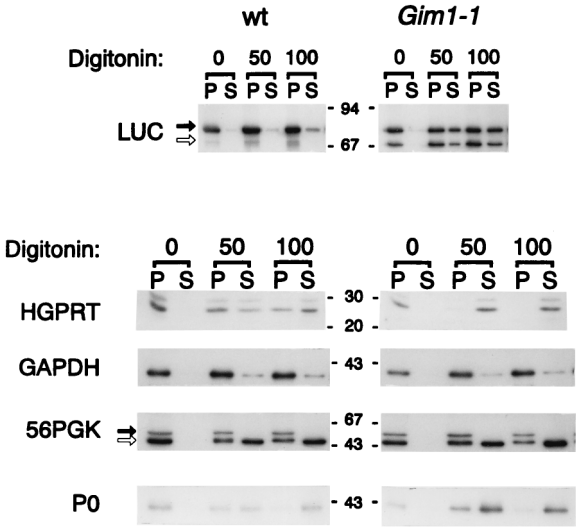

FIG. 3. Distribution of glycosomal proteins in the *gim1-1*/B-L cell line. Increasing amounts of digitonin (indicated in micrograms per milliliter) were used to solubilize wt/B-L (wt) and *gim1-1*/B-L (*Gim1-1*) cells. Aliquots of the organellar pellet (P) and cytosolic supernatant (S) fractions were separated on sodium dodecyl sulfate–10% polyacrylamide gels and immunoblotted with the indicated antibodies. For the LUC panel, wild-type and *gim1-1*/B-L cells expressing the Ble-Luc fusion protein plus a Luc nonfusion protein were used (both of these contain a C-terminal SKL targeting signal). The solid arrow indicates the 81-kDa Ble-Luc fusion protein; the open arrow indicates the 67-kDa Luc. In the 56PGK panel, the solid arrow indicates the 56-kDa minor glycosomal isozyme, and the open arrow points to the region where both the cytosolic and the second glycosomal phosphoglycerate kinase isozyme are expected to migrate. On staining, the lane containing the 50-μg/ml supernatant fraction for *gim1-1*/B-L in the P0 panel was found to be overloaded in comparison with the same lane in the wt/B-L panel. Positions of protein standards are shown in kilodaltons.

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 isozymes, hypothesized by analogy to other trypanosomatids [35, 36] to be a mixture of cytosolic and glycosomal forms.) GAPDH and 56PGK therefore appeared t 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 *Leishmania*, 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 μm^2 for wild-type cells and 19 per 100 μm^2 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 μm^2) relative to wt/B-L cells (7.8 per 100 μm^2). 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 organellar 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 cGim1BΔ3), 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 *Xho*I-*Nde*I 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 μm^2 in *gim1-1*/B-L+pGIM1 cells versus 0.9 per 100 μm^2 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 μm^2).

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

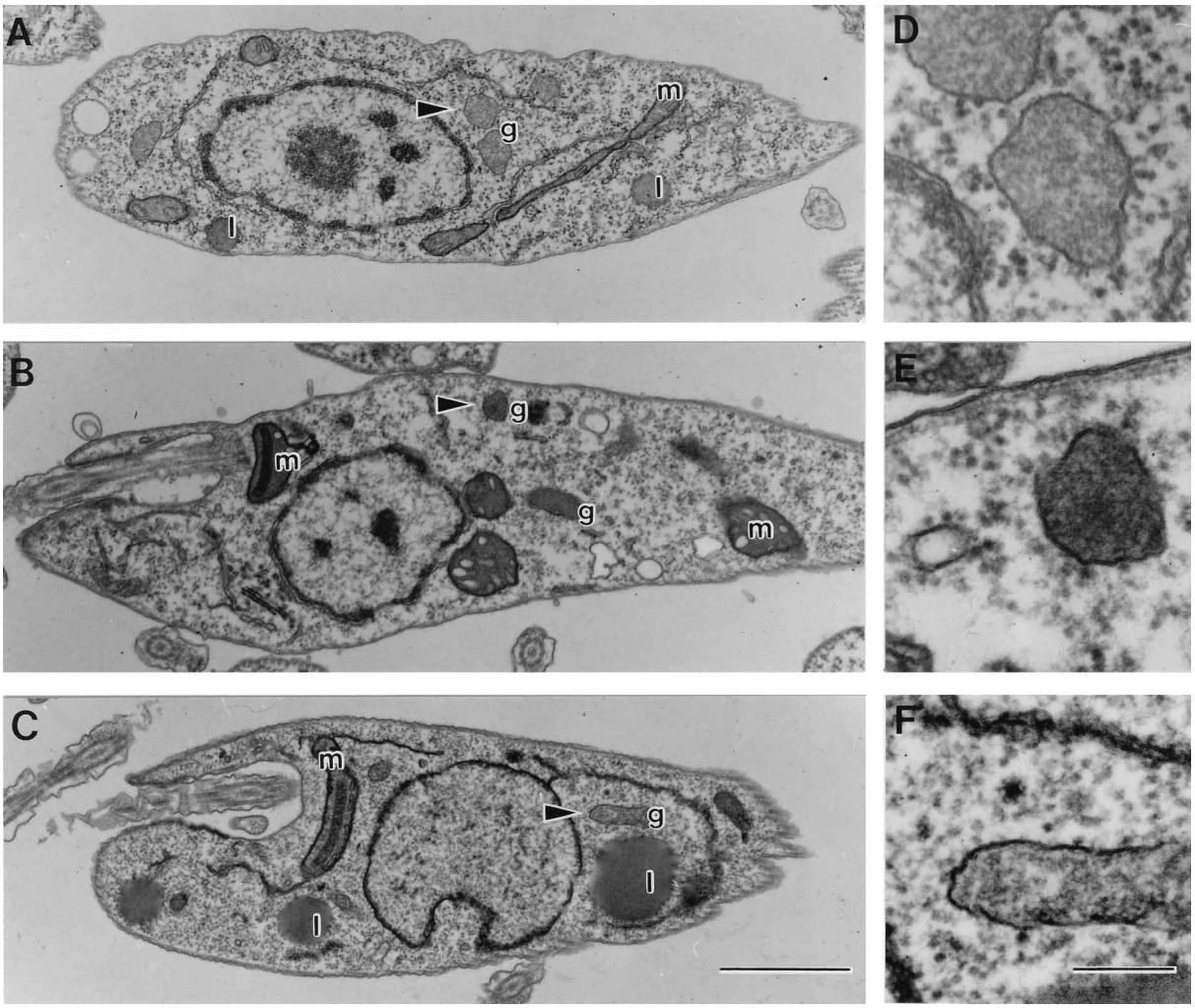

FIG. 4. Electron micrographs of wt/B-L (A and D), *gim1-1*/B-L (B and E), and rescued *gim1-1*/B-L (C and F) cells. Representative organelles are labeled: g, glycosomes; l, lipid bodies; m, mitochondrion. Panels on the left show a section through the cells. Panels on the right show enlargements of glycosomes 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.

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 C_3HC_4 cysteine ring motif in the carboxy-terminal half of Gim1 aligns with the C_3HC_4 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*/BL 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 line and its absence from the wild-type parent were verified by direct sequencing of independent PCR products.

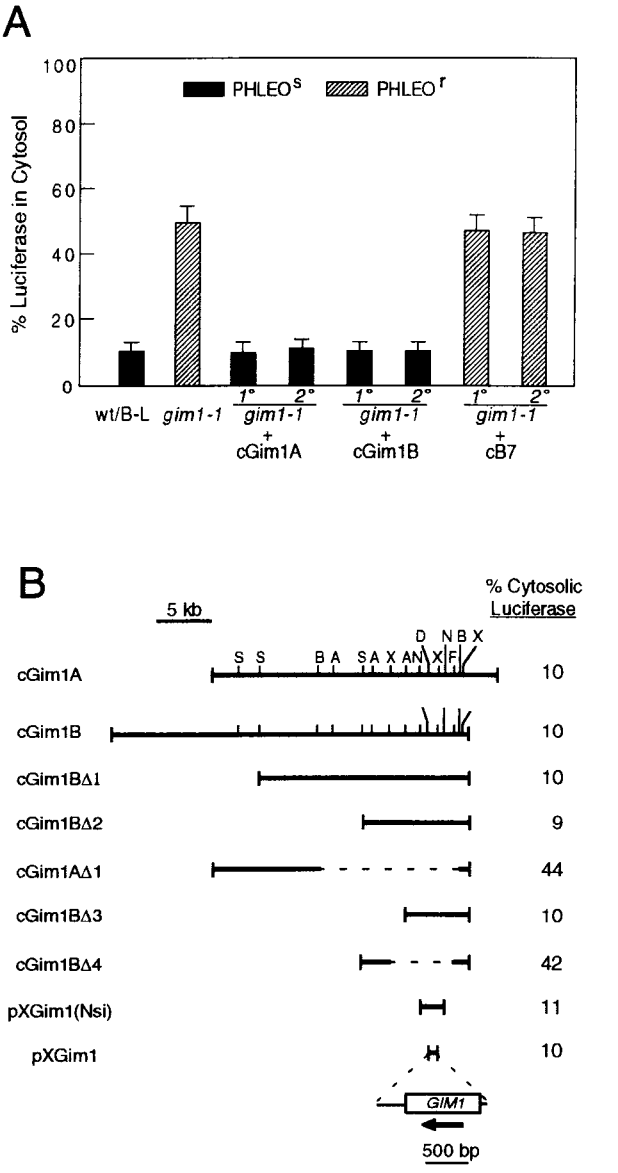

FIG. 5. The *GIM1* gene rescues the *gim1-1*/B-L cell line. (A) Compartmentalization of Ble-Luc, as determined by Luc activity. Bars are shaded according to the phleomycin sensitivity of the cells line (r, resistant; s, sensitive). All lines contain pXB-L. Any additional plasmids or cosmids that they contain are indicated, with cB7 being an irrelevant, random cosmid. 1° transfectants are those arising after transfection with the cosmid library; 2° transfectants are those transfected with cosmid DNA isolated from the primary clone. (B) Restriction maps and relative positions of cosmid inserts cGim1A and cGim1B are shown at top; the deletion constructs used in rescue studies are shown below. Cytosolic Luc activity determinations for *gim1-1*/B-L cell clones arising following transfection with each deletion construct are shown at the right. The box represents the *GIM1* coding region, and the arrow designates the direction of transcription. A, *Apa*I; B, *Bgl*II; N, *Nsi*I; D, *Nde*I; S, *Sma*I; F, *Sfi*I; X, *Xho*I.

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 glycosome 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 56PGK, a protein predicted by analogy with the trypanosome (1) and *Crithidia fasciculata* (51) homologs to lack a type 1 or 2 signal, are efficiently imported into the organellar 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

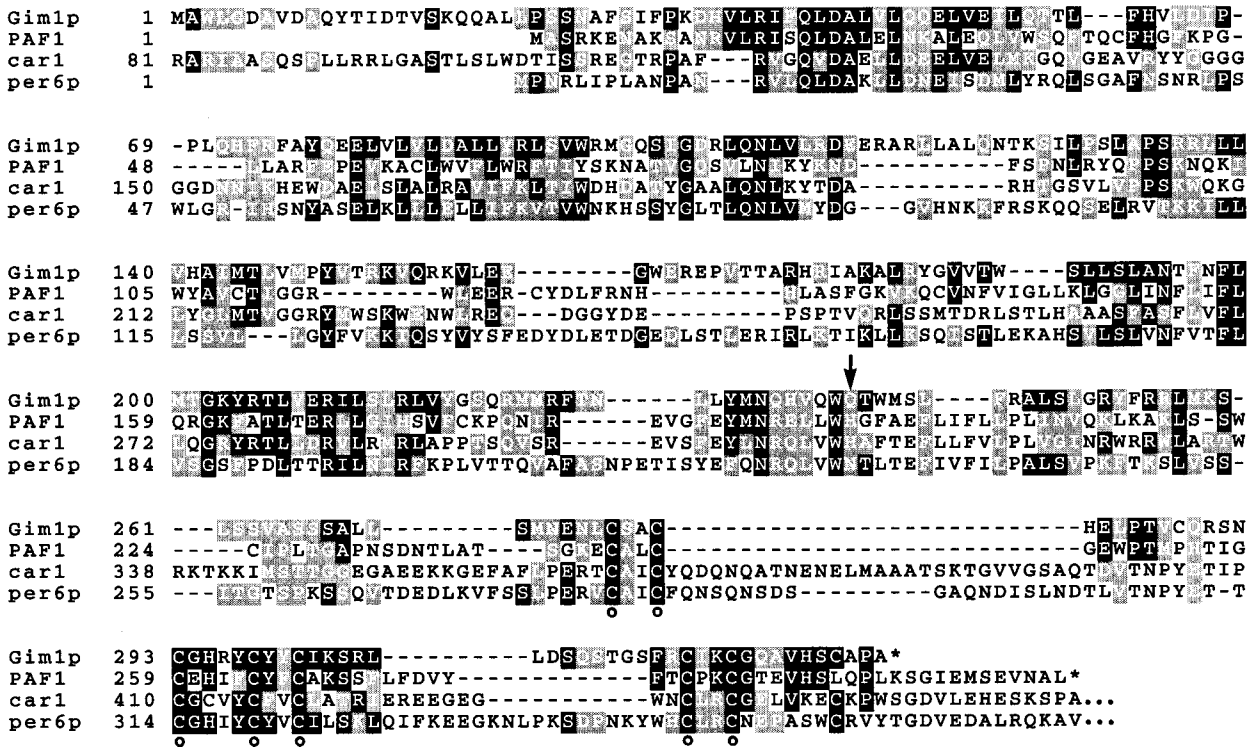

FIG. 6. Comparison of the amino acid sequences deduced from the *L. donovani GIM1* gene and other genes in the *PAF* gene family. Sequences shown: Gim1, *L. donovani*; PAF1, human PEX2; car1, *Podospora anserina* PEX2; Per6p, *Pichia pastoris* PEX2. Black shading indicates identity with Gim1, and gray 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 *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.

the mutant allele may behave as a dominant negative mutation with respect to the phenotype that we observed. If, as hypothesized, PEX2 homologs are involved in protein-protein interactions, a defective Gim1 protein could result in nonproductive interactions yielding a dominant negative phenotype. Another alternative is that the *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 described (53). Interestingly these dominant negative alleles yield a peroxisome import defect when present with a wild-type allele in diploid cells of the yeast *Hansenula polymorpha* but peroxisome deficiency when alone in haploids. In the future, we will attempt to disrupt *GIM1* in order to definitively characterize its role in glycosome biogenesis and to generate a true glycosome-deficient *Leishmania* cell line. Although disruption of critical genes is still more difficult in trypanosomatids 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 *gim1-1* cells compared to wild-type cells. Rescue with the *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 affects both glycosomes and lipid bodies. Another explanation could be that disruption of glycolysis caused by the mislocalization of one or more glycolytic enzymes to the cytosol may force *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 *lyso*-alkyl-ether-lipid. LPG has been implicated in several critical roles in the *Leishmania* infectious cycle (57). Disruption of ether-lipid synthesis in the glycosome in *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, combined with the possibility of isolating mutants with highly restricted defects, as described here, suggests that the analysis of glycosome biogenesis mutants will provide new insights into the functional role of subcellular compartmentalization.

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 proteins would prove deleterious to the parasite. Even preventing the import of a subset of glycosomal proteins would likely be

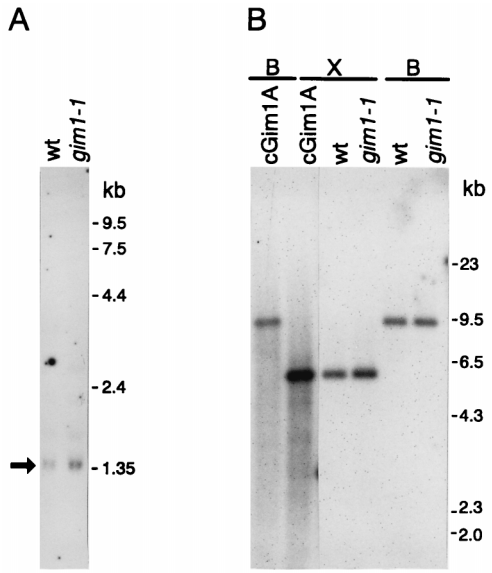

FIG. 7. The *GIM1* gene encodes a 1.7-kb transcript and is a single-copy gene. (A) Northern analysis. Total RNA (5 μg) from wild-type (wt) and *gim1-1*/B-L (*gim1-1*) cells was separated on a formaldehyde-agarose gel, transferred to a filter, and hybridized with an antisense riboprobe from pBSGIM1. RNA size markers are shown at the right. (B) Southern analysis. Genomic DNA (6 μg) from wild-type and *gim1-1*/B-L cells was digested with *Bgl*II or *Xho*I and separated on a 0.85% agarose gel, transferred to a filter, and hybridized with an antisense riboprobe from pBSGIM1. In each cell line, the probe hybridizes to a single band which comigrates with control cGim1A cosmid cut with the same enzyme. DNA size markers are shown at the right.

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.

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