Depletion of GIM5 Causes Cellular Fragility, a Decreased Glycosome Number, and Reduced Levels of Ether-linked Phospholipids in Trypanosomes*

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Microbody division in mammalian cells, trypanosomes, and yeast depends on the PEX11 microbody membrane proteins. The function of PEX11 is not understood, and the suggestion that it affects microbody (peroxisome) numbers in mammals and yeast, because it plays a role in beta-oxidation of fatty acids, is controversial. PEX11 and two PEX11-related proteins, GIM5A and GIM5B, are the predominant membrane proteins of the microbodies (glycosomes) of Trypanosoma brucei. The compartmentation of glycosomal enzymes is essential in trypanosomes. Deletion of the GIM5A gene from the form of the parasite that lives in the mammalian blood has no effect on trypanosome growth, but depletion of GIM5B on a gim5a null background causes death. We show here that procyclic trypanosomes, adapted for life in the Tsetse fly vector, survive without GIM5A and with very low levels of GIM5B. The depleted cells have fewer glycosomes than usual and are osmotically fragile, which is a novel observation for a microbody defect. Thus trypanosomes require both GIM5B and PEX11 for the maintenance of normal glycosome numbers. Procyclic cells lacking GIM5A, like mouse cells partially defective in PEX11, have fewer ether-linked phospholipids, even when GIM5B levels are not reduced. Metabolite measurements on GIM5A/B-depleted bloodstream form trypanosomes suggested a change in the flux through the glycolytic pathway. We conclude that PEX11 family proteins play important roles in determining microbody membrane structure, with secondary effects on a subset of microbody metabolic pathways.

Microbodies are single-membrane-bound organelles found in diverse eukaryotes; examples include the peroxisomes of mammals and yeast, the glyoxysomes and peroxisomes of plants, and the glycosomes of kinetoplastid protists. Microbodies manifest considerable diversity with regard to the enzymes and metabolic pathways they contain: examples include catalase, and enzymes of fatty acid beta-oxidation, alcohol oxidation, ether-lipid biosynthesis, and, in the kinetoplastids, glycolysis and glycerol metabolism. The mechanisms of microbody biogenesis are in contrast conserved: indeed, the “PEX” proteins (peroxins) involved in microbody membrane formation and matrix protein import are the only markers that enable unambiguous identification of microbodies throughout evolution (1–3).

One of the PEX proteins, the membrane protein PEX11, is required for microbody division. Yeast, mammalian cells and trypanosomes with reduced PEX11 function have fewer, but larger peroxisomes than wild-type cells, whereas overexpression of PEX11 leads to an increase in microbody numbers or elaboration of excess microbody membrane (see for example Refs. 4–6). Despite these clear phenotypes, however, the way in which PEX11 influences peroxisome size and division is still very controversial. Initial results suggesting that mammalian PEX11 might act via interactions with COP I components were not supported by subsequent analyses in other species (summarized in Ref. 7), although there is some evidence for a role of GTPases in peroxisome division (8). Van Roermund et al. (9) suggested that yeast PEX11 plays a primary role in medium-chain fatty acid oxidation, a process that affects peroxisome number and size in Saccharomyces cerevisiae. They showed that a loss of yeast PEX11 blocked peroxisomal beta-oxidation of medium-chain fatty acids and that defects in medium-chain fatty acid oxidation conversely reduced peroxisome abundance. Elevated PEX11 expression in yeast, however, caused increased peroxisomal abundance even in lipid-free medium (7), suggesting that the link between PEX11 function and beta-oxidation might be indirect. Mammalian cells contain up to three different PEX11 isoforms, α, β, and γ, which complicates functional analysis (10, 11). Mouse cells lacking PEX11/8 showed a 40% reduction in fatty acid beta-oxidation (7); but the knockout cells had a 2-fold reduced peroxisome number, relative to wild-type cells, whether or not lipids were present in the medium.

Trypanosoma brucei, the sleeping sickness trypanosome, parasitizes the blood and tissue fluids of mammals and is transmitted in sub-Saharan Africa by Tsetse flies. The “bloodstream form” trypanosomes, which multiply in the mammal, depend upon substrate level phosphorylation during glycolysis for ATP generation, and the first nine enzymes of glycolysis and glycerol metabolism are correspondingly dominant in the microbodies (12). In contrast, the “procyclic forms” found in the insect midgut have an active mitochondrion and are capable of surviving with amino acids, instead of glucose, as the principal

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carbon source, so that they are no longer so dependent on the glycosome for ATP. In these forms, the first seven enzymes of glycolysis are still in the glycosome, but several are reduced in activity and additional glycosomal enzymes, from a variety of pathways, including ether-lipid biosynthesis, are present (12–14).

Originally, it was thought that compartmentation of glycolytic enzymes in trypanosomes was required to achieve a high glycolytic flux. However, it now seems more likely that the compartmentation of glycolysis serves a regulatory role, by compartmentalizing ATP/ADP and NAD(P)H and thus protecting the glycosomal enzymes, which are not subject to normal feedback inhibition mechanisms, from the ATP/ADP and NAD/ NADH ratios found in the cytosol (12). That compartmentation is important has been abundantly demonstrated: expression of two different glycolytic enzymes in the cytosol of bloodstream form was deleterious to cell growth (15, 16). Moreover, disruption of the enzyme import mechanism was lethal in both bloodstream and procyclic forms (17, 18).

After gel electrophoresis and staining of purified glycosomal membranes from trypanosomes, only two protein bands, migrating at 24 and 26 kDa, could be detected by Coomassie Blue or silver staining (19). The 24-kDa band corresponds to trypanosome PEX11 (TbPEX11) (6). Overexpression of TbPEX11 in trypanosomes caused elongation and clustering of the glycosomes, whereas depletion of PEX11 resulted in a decrease in the number of glycosomes, with a corresponding increase in their size (6). Attempts to completely delete the PEX11 genes were unsuccessful, suggesting that they were essential. The 26-kDa band represents two very closely related proteins, GIM5A and GIM5B, each of which contains two transmembrane domains (20). Previous results suggested that, as for PEX11, both the N and the C termini are exposed to the cytosol, with only a short loop, located between the two transmembrane domains, facing the glycosomal matrix. GIM5A and GIM5B differ almost exclusively within the intra-glycosomal loop and assemble into heterodimers (20); whether or not homodimers are normally also present is uncertain. A recent sequence comparison (10) and our own results (see below) indicate that the GIM5 proteins are in fact isoforms of PEX11.

In previous experiments, we constructed bloodstream trypanosomes in which the GIM5A gene was completely absent, and the endogenous GIM5B gene had been replaced by a new copy under control of a tetracycline-inducible promoter (20). These parasites grew normally in the presence of tetracycline, but stopped growing and died when GIM5B expression was reduced to less than 10% by withdrawal of tetracycline. Thus our results indicated that bloodstream trypanosomes required both PEX11 and the PEX11-related protein, GIM5B, for survival. Neither GIM5 nor PEX11 is likely to be acting via fatty acid beta-oxidation, because bloodstream form trypanosomes are thought to lack this pathway. No defects in the import of glycosomal enzymes were detected in the GIM5-depleted cells (20). However, the results also suggested that bloodstream trypanosomes with defective glycosomes died too rapidly for such defects to be detected (20).

We have recently shown that PEX2-depleted procyclic trypanosomes show clear defects in glycosome biogenesis, whereas the equivalent bloodstream forms, like the GIM5-depleted bloodstream forms, die much more rapidly, with no detectable glycosomal defect (17). We suspect that it is generally possible to detect glycosomal defects in bloodstream forms, because glycosomal integrity is essential for the ATP supply of the parasites. To obtain further insight into the functional relationship between GIM5 and PEX11, we have here therefore investigated the effects of GIM5 depletion in procyclic trypanosomes.

**EXPERIMENTAL PROCEDURES**

Trypanosomes and Transfection—Trypanosomes were cultured in either HMI-9 medium (bloodstream cell lines) (21) or MEM-PROS medium (procyclic cell lines), supplemented with 10% fetal calf serum. Cells were transfected as described previously (23). In all experiments, the “wild-type” (WT) cells were phleomycin-resistant bloodstream or procyclic trypanosomes of strain 272 stably expressing the tetracycline repressor from the plasmid pH449 (23).

**Construction of the GIM5 Conditional Knockout Cell Line**—A schematic representation of the construction of the conditional GIM5 knockout line is shown in Fig. 2 (A–D). The GIM5-encoding gene was inserted into the tetracycline-inducible expression vector pH678 (containing hygromycin resistance (23)) to yield pH813. After transfection of the “wild-type” cell line (see Fig. 2A), trypanosome clones resistant to phleomycin (BLE) and hygromycin (HYG), expressing the Tet repressor (TETR BLE) and bearing an inducible copy of GIM5B (GIM5B HYG), were isolated and used as the starting point for the knockout of GIM5; their genotype is TETR BLE GIM5B HYG GIM5AB/GIM5AB (see Fig. 2B) (20).

For deletion of the GIM5AB loci, containing both the GIM5A and GIM5B genes, primers C2992 and C2993 (Table I) were used to amplify a 1.1-kb (5′-region) genomic fragment located upstream of GIM5A, and primers C2996 and C2997 were used to amplify a 0.4-kb fragment (3′-region) downstream of GIM5B (20). Briefly, the targeting fragments were inserted either side of the NEO or BSD genes bearing actin 5′-splice sites and 3′-untranslated regions. For BSD, the relevant fragment was amplified using C2994 and C2995. Cells bearing the single knockout were selected in the presence of 10 μg/ml 5′-blasticidin (TETR BLE GIM5B HYG Δgim5ab:BSD/GIM5AB). The conditional GIM5 double knockout TETR BLE GIM5B HYG Δgim5ab:BSD/Δgim5ab:NEO (see Fig. 2C), in this report abbreviated to Δgim5a/Δgim5b, was obtained using 12 μg/ml G418, 10 μg/ml 5′-blasticidin, and 250 ng/ml tetracycline. Tetracycline (250 ng/ml) was added daily to maintain GIM5 expression, because the drug is unstable. The correct integration of the constructs into the genome was monitored by Southern blotting (not shown).

For deletion of the GIM5B loci, without affecting GIM5A, primers C2192 and C2192 (Table I) were used to amplify a 0.6-kb genomic fragment (5′-region) located upstream of GIM5A, and primers C2996 and C2997 were used to amplify a 0.4-kb fragment (3′-region) downstream of GIM5B. The targeting fragments were inserted on either side of the NEO or BSD genes bearing actin 5′-splice sites and 3′-untranslated regions. In this experiment, wild-type trypanosomes (strain 449) were used for transfection. A schematic representation of this strategy is shown in Fig. 2 (E and F). However, single GIM5B knockout clones could not be obtained by selecting either with G418 (12 μg/ml) or blasticidin (10 μg/ml). The reason for this failure is unknown, because it is clearly possible to reduce the level of GIM5B to below 50% without affecting growth.

**Subcellular Fractionation with Digitonin**—For subcellular fractionation of procyclic cell lines with digitonin, 2 × 10⁶ cells were washed twice with 10 ml of STEN (250 mM sucrose, 25 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, pH 7.4) and resuspended in 300 ml of STEN supplemented with digitonin (150 μg/ml final concentration). After

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**Table I**

Oligonucleotides used in this study

| Oligonucleotides | Forward Primer | Reverse Primer |
|------------------|----------------|----------------|
| C2992            | 5′-ATCGGATCCTAGCTTGCATGCCTGCAAGG-3′ |                   |
| C2993            | 5′-ATCGACTACGCTTCCTTTGACACTACACGG-3′ |                   |
| C2994            | 5′-ATCGACTACGCTTCCTTTGACACTACACGG-3′ |                   |
| C2995            | 5′-ATCGACTACGCTTCCTTTGACACTACACGG-3′ |                   |
| C2996            | 5′-ATCGACTACGCTTCCTTTGACACTACACGG-3′ |                   |
| C2997            | 5′-ATCGGATCCTAGCTTGCATGCCTGCAAGG-3′ |                   |
| C792             | 5′-ATCGGATCCTAGCTTGCATGCCTGCAAGG-3′ |                   |
| C793             | 5′-ATCGGATCCTAGCTTGCATGCCTGCAAGG-3′ |                   |
| C1921            | 5′-GCCCGTGCAATCTTCATTGGCTGTTTCTACG-3′ |                   |
| C1922            | 5′-ATTTCATTAGTTCTACGTACCTGGTG-3′ |                   |

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1 The abbreviations used are: MEM-PROS, modified Eagle’s medium for procyclics; PC, phosphatidylcholine; PE, phosphatidylethanolamine; BLE, blasticidin; HYG, hygromycin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; CD, conserved domain; ALD, aldolase; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde-3-phosphate.
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incubation for 1 h at ice, the cells were fractionated by centrifugation (2 min 20,000 × g, at 4 °C) into a cytosome-enriched fraction (s, supernatant) and a glycosome-enriched fraction (p, pellet). The pellet was resuspended in 300 μl of STEN, and equal volumes of lysis buffer (2% v/v IGEFAL CA-630, Sigma, 500 mM NaCl, 50 mM Tris-HCl, pH 7.4) were added to both fractions.

Immunoprecipitation and Western Blot Analysis—Immunoprecipitation with protein-A-agarose beads and Western blot analysis with ECL detection (Amersham Biosciences) were all performed as described previously (6). Antibodies used for the immunoprecipitation and detection of PEX11 and aldolase were previously described (6). Antibodies used for the immunoprecipitation and detection of GIM5A and GIM5B were described previously (20).

Metabolic Labeling—Parasites were labeled in vivo with [35S]methionine (24). 2 × 10^7 trypanosomes were incubated in 400 μl of modified MEM medium (lacking methionine), supplemented with fetal calf serum to GIM5, recognizing both GIM5A and GIM5B, was described previously (20). Antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was a gift from Paul Michels, Christian de Duve Institute of Cellular Pathology, Brussels, Belgium. The antisera to the cytosolic marker protein CSM was kindly provided by Antonio Estevez (ZMBH, Heidelberg). The anti-serum to GIM5, recognizing both GIM5A and GIM5B, was described previously (20).

Results

GIM5A and GIM5B Are Structurally Related to PEX11—We originally reported that there were no homologues to GIM5A and GIM5B in the databases, apart from the closely related Leishmania major GIM5 sequences (28). However, a repeated database search revealed that both GIM5A and GIM5B contain a conserved domain (CD) structure that has been identified recently for PEX11 proteins (CD: LOAD pex11.6, pex11; PSSM-Id: 3679). Alignment of the GIM5A and GIM5B sequences with a subset of PEX11a, b, and γ sequences from different mammals and yeast illustrates the extremely low level of similarity between these proteins, but also reveals the presence of several conserved amino acid residues (Fig. 1). GIM5A, GIM5B, and TbPEX11 are all 14–15% identical to S. cerevisiae PEX11, whereas GIM5A and GIM5B are 13–14% identical to TbPEX11. This level of identity is similar to that between mammalian and yeast PEX11s (for example, human PEX11β is 19% identical to S. cerevisiae PEX11). The calculated molecular masses of GIM5A/B and different PEX11s are within a narrow size range (24–26 kDa), and the proteins all share similar membrane topology (28) and basic isoelectric point. That GIM5A/B and the various PEX11 proteins indeed are related can also be concluded from the phylogenetic analysis recently shown by Li et al. (10). Unlike trypanosome PEX11, GIM5A and GIM5B have neither a potential NTP binding site nor a potential consensus peroxisomal membrane targeting signal (6, 28). These two motifs are not conserved in all PEX11 sequences and have also not been shown to be functional.

Depletion of GIM5A/B in Procyclic Forms—We already knew that the depletion of PEX11 in trypanosomes caused a reduction in glycosome numbers (6) and that the trypanosome PEX11 was fully functional in yeast (6, 28). Why, then, should trypanosomes need an additional set of PEX11-related sequences? And why, if GIM5 is simply another PEX11, should depletion of these proteins be lethal in bloodstream forms when PEX11 levels were unaffected?

Our previous experiments had shown that depletion of GIM5B on a gim5a null (gim5a) background was so rapidly lethal in the bloodstream form of T. brucei that it was impossible to draw any conclusions about the phenotype of the depleted cells (20). After 24 h of depletion, showing a decrease of the GIM5B level to about 50% of normal (20), the majority of the parasites had still a normal morphology. However, after 48 h, with a decrease of the GIM5B level to roughly 20% of normal (20), more than 80% of the parasites were severely affected: their cells were rounded up, which is characteristic for dying cells, and had a reduced 4’,6-diamidino-2-phenylindole DNA staining (not shown). We therefore decided to deplete GIM5 in the procyclic form of T. brucei using exactly the same approach (Fig. 2, A–D) as was applied for bloodstream forms. Attempts to completely delete all GIM5A and GIM5B genes were unsuccessful, indicating that the encoded proteins were probably essential. We were, however, able to create cell lines that contained a single, tetracycline-inducible copy of GIM5B (GIM5Bγ), and no copies of either GIM5A (gim5a) or GIM5B in the original GIM5 locus (Fig. 2, C and D). In these cells, which completely lacked the GIM5A protein, the expression of GIM5B was dependent on the addition of tetracycline to the medium. In the rest of this paper, this cell line will be called GIM5A/GIM5Bγ.

When we removed tetracycline from the gim5a/GIM5Bγ cells, the growth of the parasites was unaffected (Fig. 3), although there was a strong reduction in the level of GIM5B protein (see later in Fig. 5A, lane 2). This indicates that procyclic trypanosomes, in contrast to bloodstream forms, need only a relatively low level of GIM5B to survive. We did however note that the movement of the cells was severely reduced. Attempts to create cells expressing GIM5A but not GIM5B failed, appar-
ently for technical reasons (see “Experimental Procedures”), so we do not know if such cells can survive or not.

**Glycosome Morphology and Cellular Fragility in GIM5-depleted Procyclic Trypanosomes**—To find out whether GIM5 is involved in glycosome biogenesis and division, we examined glycosomal morphology in the \(\Delta\text{gim5a}\)/GIM5B-depleted procy-
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Depletion of GIM5B in the procyclic cell line Δgim5a/GIM5BΔ does not affect growth. The cell line was grown with (solid lines) or without (dotted lines) tetracycline (0.250 μg ml−1) in the culture media. Cultures were initiated at 5 × 105 cells ml−1. Cultures that had been diluted each day to 5 × 105 cells ml−1 (indicated by vertical arrows).

GIM5 Depletion Does Not Affect Import of Glycosomal Matrix Proteins—To answer the question of whether GIM5A/B depletion affected the import of glycosomal matrix proteins or not, we examined the distribution of glycosomal proteins after digitonin fractionation of the different cell lines. The fractionation procedure was monitored using the cytosolic protein CSM (Fig. 5A, panel 1) and the glycosomal membrane protein PEX11 (Fig. 5A, panel 3) as controls. Western blotting of the different fractions derived from the procyclic Δgim5a/GIM5BΔ cell line, grown for 4 days in the presence of tetracycline, showed clearly that the glycosomal matrix proteins aldolase (ALD) and glyceraldehyde phosphate dehydrogenase (GAPDH) were only detectable in the glycosomal enriched pellet fraction and not in the cytosol (Fig. 5A, panel 4, ALD; and panel 5, GAPDH).

In the same Δgim5a/GIM5BΔ cell line, but now 2–4 days after tetracycline removal, only a very small amount of GIM5B was detectable in the glycosome-enriched pellet fraction (Fig. 5A, panel 2), suggesting that the depletion of this protein was almost complete. The reduction in GIM5B was now accompanied by an appearance of a portion of the glycosomal matrix proteins ALD (Fig. 5A, panel 4) and GAPDH (Fig. 5A, panel 5) in the cytosol-enriched fraction. One possible explanation for the presence of glycosomal matrix proteins in the cytosol-enriched fraction was that some matrix protein had leaked out of the glycosomes during the fractionation procedure, which would correlate with the enhanced fragility of the cells. An alternative explanation was that the glycosomes retained matrix proteins that had been synthesized before GIM5B depletion, but that newly synthesized proteins were no longer imported into the glycosomes. The latter explanation seemed unlikely, because the amounts of ALD and GAPDH in the glycosome-enriched fractions remained constant over the period of 2–4 days after tetracycline removal (Fig. 5A, panels 4 and 5), whereas the cells were rapidly and continually dividing (see Fig. 3). Severely impaired glycosomal protein import in the GIM5B-depleted cells should in contrast have resulted in gradual dilution of ALD and GAPDH in the glycosomes.

To scrutinize the two explanations further, we examined the distribution of newly synthesized, metabolically labeled ALD and GAPDH after 48 h of GIM5B depletion (Fig. 5B). Both labeled proteins were found predominantly in the glycosomal fraction, implying that most newly synthesized ALD and GAPDH were imported properly into the glycosomes even when GIM5A was absent and GIM5B was depleted. The small amount of ALD and GAPDH in the cytosolic fraction could be due either to a rather minor defect in import or to glycosomal leakage upon fractionation. The reduction in GIM5 had no effect on the overall levels of ALD and GAPDH, suggesting that an increase in glycosomal volume, visible in the immunofluorescence pictures, was able to compensate for the decrease in glycosome number. PEX11 levels were also unaffected.

It was recently shown that PEX14-depleted procyclic cells, in which glycosomal matrix enzymes are redistributed to the cytosol, have a decreased glycosome number.
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Phospholipid Analysis—Membranes of wild-type procyclic T. brucei cells are known to contain a relatively high amount of ether lipids, comprising both glycerol ethers (with alkylacyl moieties) and plasmalogens (with alk-1-enacyl moieties) (29). The first step in the biosynthesis of both types of ether lipids is the addition of an acyl-CoA to dihydroxyacetone phosphate (DHAP) by DHAP-acyltransferase, creating 1-acyl dihydroxyacetone phosphate. Next, an acyl group is exchanged for the acyl group by alkyl-DHAP synthase. The third enzyme is alkyl/acyl DHAP reductase, giving alkyl or acyl glycerol 3-phosphate. All these three enzymes are found in the glycosome (13, 14). We therefore tested whether GIM5 could have a function in glycosomal ether-lipid metabolism by examining the effects of GIM5 depletion on trypanosome phospholipid composition. Phosphatidylycholine (PC) and phosphatidylethanolamine (PE) together account for over 70% of all phospholipids found in T. brucei (29) and are also major components of the glycosomal membrane, which lacks detectable sphingomyelin, phosphatidylinositol, and phosphatidyl serine (30). The PC and PE fractions were isolated and analyzed from wild-type and GIM5B-depleted cell lines, grown with or without tetracycline. We found that the PE/PC ratio in the GIM5A/B-depleted cells (grown without tetracycline) was significantly lower (0.30 ± 0.02, n = 3) than in wild-type cells (0.53 ± 0.12, n = 3). Subsequent analysis of the PC species composition revealed that in the GIM5A/B-depleted cells all the main ether-lipid species were drastically reduced: these were plasmalogen PC (16:0, 18:2), plasmalogen PC (18:0, 18:2), and alkylacyl PC (18:0, 18:2) (Table II). Semi-quantitative analysis of the PE species showed similar results: a strong decrease of most PE-ether-lipid species in GIM5A/B-depleted cells compared with wild-type cells (Fig. 7). As in normal procyclic trypanosomes, ether-lipid species accounted for the majority of the PE species but for less than 25% of the PC species (Fig. 7 and Table II) (29), so the decrease in ether-lipids in GIM5A/B-depleted cells correlates with the observed decrease in their PE/PC ratio.

Interestingly, no significant differences in either the PC and PE species composition or the lipid class composition could be observed between the GIM5A/B-depleted cell lines grown with or without tetracycline (Table II and Fig. 7). Thus, the GIM5A deletion alone (Δgim5a) induced the observed reduced ether-lipid content; a subsequent, additional depletion of GIM5B had no further effect on phospholipid metabolism. Therefore, GIM5A plays a role in ether-lipid biosynthesis, whereas the observed changes in glycosome size (Fig. 4) upon depletion of GIM5B were unlikely to be caused by alterations in phospholipid metabolism.

Metabolite Analysis—The preceding results showed that the GIM5A/B-depleted cell line, lacking GIM5A but still expressing GIM5B (grown with tetracycline), was defective in ether-lipid biosynthesis, although glycosome numbers, glycosome size, and the import of matrix proteins into the glycosome were unaffected. The impairment of ether-lipid biosynthesis in this cell line might have been caused by an enzyme defect or disturbed import or export of substrates or products. Repeated attempts to express native GIM5A and GIM5B in Escherichia coli were unsuccessful, with results suggesting that expression could be lethal. This unfortunately precluded further studies of the pure proteins but would be consistent with a possible role...
as a transporter or porin. We therefore turned again to in vivo mutant studies in trypanosomes.

The enzymes of trypanosome ether-lipid biosynthesis are not yet characterized in detail, and no glycosomal metabolite transporters have yet been identified. One obvious candidate for a transporter whose activity is required for ether-lipid biosynthesis would be the transporter that mediates import of DHAP, the first substrate of the pathway.

DHAP transporter activity would be very difficult to assess in procyclins, given our patchy and superficial understanding of their lipid metabolism, but bloodstream form glycosomal metabolism is quite well understood. In bloodstream forms under aerobic conditions, glucose is metabolized to pyruvate and glyceraldehyde. The experimental ratio of pyruvate to glyceraldehyde ranges between 9:1 and 4:1, pending on the culture conditions used (31). To achieve this ratio, glyceraldehyde 3-phosphate (G3P) is exported from the glycosome and converted to DHAP by a mitochondrial glyceraldehyde-3-phosphate dehydrogenase. DHAP can then be reimported into the glycosome and converted to glyceraldehyde-3-phosphate by triosephosphate isomerase (Fig. 8). The reduction of DHAP to G3P enables re-oxidation of NADH within the glycosome, thus preserving the glycosomal redox balance. If the recomversion of G3P to DHAP is prevented via inhibition of the mitochondrial oxidase, bloodstream trypanosomes react by switching to an anaerobic form of glycolysis. All the G3P is converted to glycerol, and the ratio of glycerol to pyruvate becomes 1:1. Under these conditions, the cells can survive for at least 4 h, although they probably cannot persist indefinitely. Such cells are very rapidly killed by addition of 1–2 mM glycerol, which presumably exerts a mass-action effect, mimicking the oxidase defect, resulting in an increase in glycerol production by bloodstream trypanosomes.

To find out whether GIM5 was involved in DHAP/G3P transport, wild-type and Δgim5a/GIM5BΔ bloodstream cell lines (20) were cultured in media with glucose as the defined carbon source for 4 h at 37°C in the presence of 1 mM GTP, 10 μM ΔGTP, and 2 mM 32P-labeled orthophosphate (33). After harvest, cells were rapidly washed and homogenized, and the phospholipids were extracted and separated by reverse-phase HPLC. The phospholipid species were analyzed by mass spectrometry and quantified by evaporative light scattering detection as described under “Experimental Procedures.” The most abundant PC peak number represents their elution order of the HPLC PC-species separation, and the PC species description comprises the sn-1 and sn-2 position.

### Table II

| Peak | PC species | Wt | +Tet | −Tet |
|------|------------|----|------|------|
| 1    | PtdCho 22:6, 22:6 | 3.3 ± 0.1 | 2.4 ± 0.4 | 2.4 ± 0.0 |
| 2    | PtdCho 22:6, 22:5 | 4.0 ± 0.4 | 4.9 ± 0.8 | 4.5 ± 0.2 |
| 3    | PtdCho 18:3, 22:3 | 6.0 ± 0.5 | 7.3 ± 1.2 | 6.8 ± 0.4 |
| 4    | PtdCho 20:4, 22:6 | 1.8 ± 0.1 | 2.4 ± 0.1 | 2.6 ± 0.1 |
| 5    | PtdCho 20:4, 22:5 | 3.3 ± 0.3 | 4.8 ± 0.1 | 5.1 ± 0.1 |
| 6    | PtdCho 18:2, 22:5 | 7.6 ± 0.4 | 13.3 ± 2.1 | 11.5 ± 0.3 |
| 7    | PtdCho 18:1, 22:6 | 7.7 ± 0.7 | 7.7 ± 2.1 | 7.2 ± 0.5 |
| 8    | PtdCho 18:1, 18:2 | 3.7 ± 0.2 | 4.8 ± 0.7 | 4.5 ± 0.0 |
| 9    | PtdCho 18:3, 22:3 | 5.5 ± 0.2 | 7.2 ± 1.1 | 6.8 ± 0.0 |
| 10   | PtdCho 18:2, 22:3 | 2.8 ± 0.3 | 4.0 ± 0.6 | 4.2 ± 0.2 |
| 11   | PtdCho 18:0, 22:6 | 6.6 ± 0.5 | 4.8 ± 0.5 | 5.9 ± 0.4 |
| 12   | PlasCho 16:0, 18:2 | 4.1 ± 0.3 | 2.3 ± 1.2 | 2.7 ± 0.2 |
| 13   | PtdCho 18:1, 18:1 | 4.0 ± 1.2 | 4.5 ± 1.2 | 3.6 ± 0.9 |
| 14   | PtdCho 18:0, 22:5 | 4.2 ± 1.0 | 2.1 ± 0.3 | 3.1 ± 0.9 |
| 15   | PtdCho 18:0, 18:2 | 4.3 ± 0.6 | 6.4 ± 1.0 | 5.9 ± 0.7 |
| 16   | PlasCho 18:0, 18:2 | 3.9 ± 1.0 | 1.0 ± 0.7 | 1.3 ± 0.2 |
| 17   | AlkCho 18:0, 18:2 | 7.9 ± 3.2 | 2.4 ± 0.9 | 2.3 ± 1.5 |
| 18   | PtdCho 18:0, 18:1 | 4.9 ± 0.5 | 3.2 ± 1.2 | 4.5 ± 0.7 |

Effects of GIM5 Depletion in Trypanosomes

Electron microscopy of the procyclic GIM5B-depleted cell line Δgim5a/GIM5BΔ revealed mitochondrial defects at the ultrastructural level. A and B, different UltraThin sections of the same cell line. For ultrastructural analysis, Δgim5a/GIM5BΔ procyclic trypanosomes were cultured for 48 h without tetracycline, followed by a 60-min fixation at room temperature in 4% paraformaldehyde, 0.05% glutaraldehyde, and 2% sucrose in PBS. The cells were post-fixed with reduced osmium and embedded in Epon 812. Mito, mitochondrion; Gly, glycosome; Nuc, nucleus. Arrowsheads point to protrusions of the glycosomal membrane.
source, in the presence or absence of tetracycline. The pyruvate, glucose, and glycerol concentrations were determined after 24 h. We already knew that depletion of GIM5B, on a gim5A null background, rapidly kills the bloodstream cell line, but we nevertheless hoped to be able to detect an increase in glycerol formation before depletion had become critical. The results are shown in Table III.

The wild-type cells cultured on glucose media produced pyruvate and glycerol in a 4:1 ratio, accounting together for about 60% of the consumed glucose carbon; the rest was presumably used in macromolecular biosynthesis (biomass) or other pathways. The concentrations of the different metabolic end products produced by the Δgim5a/GIM5B+ cell line (with tetracycline, expressing GIM5B and growing almost as well as wild-type cells) were indistinguishable from those of the wild-type cells. After tetracycline removal and consequent GIM5B depletion, the bloodstream cells initially divided then gradually stopped growing, as previously observed (20). (Decreases in cell number start later (20).) Correspondingly, the glucose was converted more completely into pyruvate. Had GIM5A/B been a simple DHAP/G3P exchanger, we would have expected an increase in glycerol production. The results of GIM5A/B depletion were diametrically opposite: there was now no detectable net production of glycerol at all (see Table III). Assuming that of the total glucose carbon consumed, only a relatively small amount was used for macromolecular biosynthesis (biomass) or other pathways, then even for normal aerobic metabolism, a glycerol concentration of at least 0.4 mM might have been expected, which is far above the detection limit (about 0.02 mM) of the assay used. If a DHAP/G3P exchanger were defective, an easily detectable 1.89 mM glycerol with a corresponding decrease in pyruvate concentration would ensue. These results
mitochondrial alternative oxidase. The ‘mass action effect’ triggered by.

Gly3P, glyceraldehyde 3-phosphate; G3P, glycerol 3-phosphate; 1,3-BiPG, 1,3-bisphosphoglycerate; PEP, phosphoenolpyruvate; AOX, mitochondrial alternative oxidase. The ‘mass action effect’ triggered by.

FIG. 8. Schematic representation of glycosomal metabolism in bloodstream form trypanosomes. DHAP, dihydroxyacetone phosphate; Gly3P, glyceraldehyde 3-phosphate; G3P, glycerol 3-phosphate; 1,3-BiPG, 1,3-bisphosphoglycerate; PEP, phosphoenolpyruvate; AOX, mitochondrial alternative oxidase. The ‘mass action effect’ triggered by the addition of glycerol to the culture medium is indicated by a bold arrow.

did not exit the glycosome sufficiently fast, it might exert a mass-action effect, driving glucose metabolism almost completely toward pyruvate production, as was observed for our GIM5A/B-depleted cells (Table II). We simulated such a condition in our glucose media supplemented with glycerol. Growth of both the wild-type and Δgim5a cells was inhibited relative to the cells cultured on glucose media containing glycerol alone, and, as expected, a strong shift toward pyruvate production when glycosome function is compromised by culturing the different cell lines on glucose media supplemented with glycerol. Growth of both the wild-type and Δgim5a cells was inhibited relative to the cells cultured on glucose media containing glycerol alone, and, as expected, a strong shift toward pyruvate production when glycosome function is compromised.

Therefore provide no evidence whatsoever for the hypothesis that GIM5A or GIM5B functions as a DHAP/G3P exchanger, and indeed argue against this idea.

The lack of increased glycerol production by the GIM5A/B-depleted cells, and the clear shift toward pyruvate production, raised the question whether GIM5B might be involved in glycerol transport, import and/or export, across the glycosomal membrane. Although glycerol can diffuse across membranes, there are some indications that a plasma membrane carrier can also play a role in its transport in trypanosomes (32). If glycerol could not exit the glycosome sufficiently fast, it might exert a mass-action effect, driving glucose metabolism almost completely toward pyruvate production, as was observed for our GIM5A/B-depleted cells (Table II). We simulated such a condition in our glucose media supplemented with glycerol. Growth of both the wild-type and Δgim5a cells was inhibited relative to the cells cultured on glucose media containing glycerol alone, and, as expected, a strong shift toward the production of pyruvate was observed. In contrast to the cells cultured on glucose media, a net glycerol consumption was measured, suggesting that in both cell lines glycerol can be imported into the cells and glycosomes, and consequently can be metabolized. (The redox balance can presumably be maintained by multiple cycles of DHAP reduction and mitochondrial re-oxidation.) In the GIM5A/B-depleted cells, similar mass-action effects were observed. (Notably, we found no indication of glycerol toxicity, further substantiating our conclusion that the cells were still capable of DHAP/G3P exchange.) These results implied that GIM5A and GIM5B are probably not required for glycerol import into trypanosomes and glycosomes under our conditions. A role of GIM5B in glycosomal glycerol export could not be excluded; alternatively, it may be that a regulatory mechanism, which senses the growth rate or metabolic status, causes a shift toward pyruvate production when glycosome function is sub-optimal. Notably, rapid export of glycerol is an important event in adaptation to low osmolarity in yeast (33).

DISCUSSION

GIM5A and GIM5B clearly resemble PEX11 proteins at the levels of domain organization and sequence. We have here shown that, like other PEX11s, the GIM5 proteins play a role in microbody division. Thus trypanosomes, like mammalian cells, express three PEX11 isoforms: the original T. brucei PEX11 (TbPEX11), GIM5A, and GIM5B. Procyclic trypanosomes that have normal TbPEX11 and GIM5B, but lacked GIM5A, had reduced levels of ether lipids and were moderately fragile (Table IV). Upon GIM5B depletion, the cells still grew normally in culture but exhibited severe fragility and had fewer glycosomes (see Table IV). TbPEX11, GIM5A, and GIM5B therefore have non-redundant, but overlapping functions. It therefore seems most appropriate to discuss our results concerning GIM5A/B in the context of other observations concerning PEX11.

The PEX11 protein of S. cerevisiae was initially characterized as being essential for the division of peroxisomes (4). Overexpression caused peroxisome proliferation, and depletion of the gene resulted in the retention of very few, very large peroxisomes that could only poorly segregate into new buds. Mammalian cells have, like trypanosomes, at least three PEX11 homologues, whose respective roles are not yet entirely clear (5, 10). PEX11y mRNA has so far been found only in liver and the protein function is uncertain (10). PEX11e mRNA is present in a few organs and levels are markedly increased after treatment with agents that induce peroxisome proliferation; it is required for peroxisome proliferation in response to 4-phenylbutyrate but is dispensable for peroxisome proliferator-activated receptor α-mediated peroxisome proliferation (10). In PEX11α knockout mice, cipropibrate caused normal peroxisome proliferation, but mitochondria developed unusual parallel cristae, clustered around lipid droplets (10). This is an intriguing parallel with the abnormal mitochondrial cristae seen in the GIM5A/B-depleted procyclic trypanosomes. PEX11β mRNA is constitutively expressed, and its overexpression is sufficient to induce peroxisome proliferation (5). Most strikingly, mouse cells lacking PEX11β, which showed 2-fold reduced peroxisome abundance, also resembled the GIM5A/B mutant cells in that they exhibited a 20% reduction in ether lipid biosynthesis (11).

Our results indicate that GIM5 also plays a role in ether-lipid biosynthesis, because Δgim5a cells showed a 40–70% reduction in ether-lipid species. Ether lipids reduce membrane fluidity and ion permeability in model membranes (34), and therefore, depletion of ether lipids could result in increased membrane fluidity. Cells lacking GIM5A contained a reduced amount of polyunsaturated phospholipid species, such as 22:6, 22:6 diacylglycerol PC (Table II), which could perhaps partially compensate for the effects caused by the reduced ether-lipid content. It is important to remember, however, that depletion of GIM5B on a Δgim5a background resulted in defective glycosome division and a marked increase in cell fragility without further alterations in ether lipids. These results suggest that neither the glycosomal division defect, nor the severe fragility, are caused by ether-lipid changes, that the effects on ether lipids are not caused by a change in glycosome size, and that additional metabolic or membrane defects must be present in the GIM5B-depleted cells.

GIM5A and GIM5B form heterodimers (20). Although GIM5A genes were dispensable for growth when GIM5B was still produced, glycosomal function was clearly affected. One possible explanation is that the GIM5B homodimer is only partially functional. More simply, it may be that the absolute amount of GIM5 (either A or B or both) in the glycosomal membrane determines its properties. Results from the past few years have taught us that in cultured trypanosomes, essential enzymes are often present in roughly 10-fold higher levels than
are required for normal growth, and attempts at further depletion are unsuccessful (see for example Refs. 16 and 35). The same pertains for PEX11, which can support normal procyclic trypanosome growth (with increased glycosomal size) at roughly 15% of normal levels (6). Because we were unable to delete both GIM5A and GIM5B, it is possible the low level of GIM5B that persists in GIM5B-depleted \( /H9004\) gim5a cells provides sufficient GIM5 activity for survival.

Although the reduction in ether lipids is a common feature of both PEX11-depleted mouse brain cells and \( /H9004\) gim5a cells, the ether-lipid defect observed was insufficient to impair peroxisome division. Also, bloodstream trypanosomes are capable of lipid synthesis, the major product is myristate (36); neither beta-oxidation nor ether-lipid biosynthesis have been detected, and our recent results\(^2\) indicate that the level of the mRNA encoding 3-keto-acyl CoA thiolase is much lower in bloodstream forms than in procyclics. Thus the changes in both ether lipids and beta-oxidation that have been seen in PEX11- or GIM5-depleted cells are most likely to be secondary symptoms of a more general defect. What functions of PEX11/GIM5 are consistent with all these results?

PEX11 Proteins Are Structural Components of the Peroxisomal Membrane—

The PEX11 proteins determine membrane curvature and associate with specific lipids to determine correct peroxisomal membrane composition. The remarkably low

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**Table III**

| carbon source | cell line | tet | Cell increase, fold | glucose consumed | glycerol produced | glycerol consumed | pyruvate produced |
|--------------|-----------|-----|---------------------|------------------|-------------------|------------------|------------------|
| 2 mM glucose | wildtype  | +   | 17                  | 256 ± 41         | 63 ± 21           | -                | 237 ± 32         |
|              | \(\Delta gim5a/GIM5B\)\(^a\) | +   | 10                  | 234 ± 5          | 46 ± 4            | -                | 203 ± 16         |
|              | \(\Delta gim5a/GIM5B\)\(^a\) | -   | 6.1                 | 297 ± 87         | below detection limit | 508 ± 16         |
| 2 mM glucose + 2 mM glycerol | wild type | + | 12.3                | 205 ± 45         | -                 | 61 ± 11          | 456 ± 91         |
|              | \(\Delta gim5a/GIM5B\)\(^a\) | +   | 10.5                | 199 ± 10         | -                 | 38 ± 8           | 318 ± 13         |
|              | \(\Delta gim5a/GIM5B\)\(^a\) | -   | 5.5                 | 245 ± 6          | -                 | 104 ± 12         | 408 ± 56         |

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**Table IV**

|                        | \(\Delta gim5a/GIM5B\)\(^a\) cell line | \(\Delta gim5a/GIM5B\)\(^a\) cell line |
|-----------------------|-----------------------------------------|---------------------------------------|
| GIM5                  | GIM5A ‘GIM5B’ (tetracycline)            | GIM5A ‘GIM5B’ (–tetracycline)         |
| Growth                | Normal growth as for wild-type          | Normal growth as for wild-type        |
| Glycosome morphology  | Similar to wild-type                    | Reduced glycosome numbers, faint aldolase fluorescence in the cytosol |
| Glycosomal protein import | Not affected                             | Not affected, but “leakage” of matrix proteins into the cytosol |
| Glycolysis            | Normal, as for wild-type                | Changed metabolic flux, no glycerol produced |
| Cellular fragility    | Moderately osmotically fragile          | Main ether-lipid specie are drastically reduced |
| Phospholipids         | Not done                                 | Changes in mitochondrial morphology   |
| Ultrastructure        |                                         |                                       |

\(^a\) For the complete genotype of the \(\Delta gim5a/GIM5B\)\(^a\) cell line, see “Experimental Procedures.”
conservation, at the primary sequence level, of PEX11s between species (Fig. 1) is consistent with this idea, especially because trypanosome PEX11 is able to complement a S. cerevisiae Δpex11 mutant (6, 28). Measurements of the phospholipid:protein ratio of carbonate-stripped peroxisomal membranes yielded values of 1054 (37) or 200 nmol of phospholipid/mg of protein (38) for rat liver; for the peroxisomes of oleic acid-grown Candida tropicalis a value of 430 nmol of phospholipid/mg of protein was obtained (39). Whole glycosomes were reported to contain 188 nmol of phospholipid/mg of protein without detectable sphingomyelin or phosphatidylinositol or phosphatidylserine, whereas the carbonate-stripped membranes were reported to contain 580 nmol of phospholipid/mg of protein (30). In Candida peroxisomal membranes, abundant neutral lipids were also detected but not quantified (39). In bloodstream trypanosomes, the major glycosomal membrane proteins are the 50-kDa dimers of GIM5 (20) or PEX11 (6), with four transmembrane helices per dimer. (For the purpose of calculation, we will ignore the many other proteins that must be present in much lower abundance.) 1 mg of a 50-kDa protein is equivalent to 20 nmol. Taking the highest phospholipid estimate, we have 1000 nmol of phospholipid for every 20 nmol of protein, corresponding to 50 phospholipid molecules per GIM5 or PEX11 dimer. If the phospholipids are distributed over both leaflets, the amount of phospholipid is insufficient to surround the proteins as a monolayer: to surround four clustered transmembrane alpha helices of 15-nm diameter one would need about 100 molecules of phospholipid (assuming 1-nm diameter for a head-group). Thus, assuming the published phospholipid measurements are correct, even if considerable amounts of other lipids are present, PEX11 family proteins must make a critical contribution to the microbody membrane structure and characteristics.

According to this theory, all the effects seen upon depletion of, or mutation of, PEX11 family proteins would be consequent to altered microbody membrane composition and characteristics. Reduced PEX11/GIM5 levels would directly decrease the absolute amount of microbody membrane; given continued production and import of all other components, this would necessitate a decrease in the surface:volume ratio. Increased division and import of all other components, this would necessarily decrease the absolute amount of microbody membrane; given continued production and import of all other components, this would necessitate a decrease in the surface:volume ratio. Increased division and import of all other components, this would necessarily decrease the relative amounts of PEX11 and GIM5A/B are similar in the two stages. The results of mutant and metabolic studies in both trypanosomes and yeast indicate that microbodies are impermeable to NAD(P)(H), acetyl CoA, and ATP/ADP (12, 40), although a candidate ATP/ADP carrier (unrelated to PEX11) has been found (41). Conversely, the apparent permeability of peroxisomes to solutes such as succrose does suggest that the membranes may contain a porin; so far the only candidate identified is a 31-kDa protein of unknown sequence from Candida (42). The link between porin or transporter function and microbody division is not immediately evident, unless the porin were involved in the transport of substrates required for membrane synthesis. In yeast and mammals, complex feedback mechanisms regulate peroxisome number and size in response to the metabolic status of the cells, so that a transporter defect could indeed indirectly result in altered enzyme levels. Bloodstream trypanosomes are a particularly useful model here, because there is no evidence that their glycosomes are involved in the synthesis of lipids (see above), and there is no evidence of any regulatory link between glycosomal metabolic status and glycosome number or size.

None of the alternatives listed above is entirely satisfactory by itself, but neither are they mutually exclusive. We therefore suggest that the main function of PEX11 family proteins is to determine the overall structure and composition of the microbody membrane. This in turn will affect both the intrinsic membrane permeability and the activities of membrane-associated transporters and enzymes.

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