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Phenotypic characterization of a glucose transporter null mutant in *Leishmania mexicana*

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Abstract

Glucose is a major source of energy and carbon in promastigotes of *Leishmania mexicana*, and its uptake is mediated by three glucose transporters whose genes are encoded within a single cluster. A null mutant in which the glucose transporter gene cluster was deleted by homologous gene replacement was generated previously and shown to grow more slowly than wild type promastigotes but not to be viable as amastigotes in primary tissue culture macrophages or in axenic culture. Further phenotypic characterization demonstrates that the null mutant is unable to import glucose, mannose, fructose, or galactose and that each of the three glucose transporter isoforms, LmGT1, LmGT2, and LmGT3, is capable of transporting each of these hexoses. Complementation of the null mutant with each isoform is able to restore growth in each of the four hexoses to wild type parasites. Null mutant promastigotes are reduced in size to about 2/3 the volume of wild type parasites. In addition, the null mutants are significantly more sensitive to oxidative stress than their wild type counterparts. These results underscore the importance of glucose transporters in the parasite life cycle and suggest reasons for their non-viability in the disease-causing amastigote stage.

Keywords

Leishmania mexicana; Glucose transporters; Null mutant; Hexose transport; Oxidative stress

1. Introduction

Membrane transporters play a central role in the biology of *Leishmania* and other kinetoplastid protozoa by allowing entry into the parasite of a plethora of nutrients important for survival. These nutrients must be transported across the parasite plasma membrane by specific permeases that often recognize a small family of structurally related substrates [1,2]. Previously, we described a null mutant of *Leishmania mexicana*, Δlmg t [3], in which the gene cluster encoding three glucose transporter genes, *LmGT1*, *LmGT2* and *LmGT3* [4], had been deleted by targeted gene replacement. The initial phenotypic characterization of this Δlmg t null mutant revealed that it was completely deficient in uptake of [2-³H]deoxy-D-glucose, that it grew more slowly and to a lower density in the promastigote stage compared to wild type

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promastigotes, and that it exhibited reduced viability as amastigotes within macrophages and was unable to grow as axenic culture form amastigotes. In the present work we have extended the characterization of the glucose transporter null mutant and established additional biologically important phenotypes. In particular, we demonstrate that the Δlmg t null mutants are unable to import the hexoses glucose, mannose, fructose and galactose but that each of the three isoforms, *LmGT1*, *LmGT2*, and *LmGT3*, is able to restore import of all four hexoses and promote the ability of the null mutant to grow on these hexoses. The null mutants are significantly reduced in size compared to wild type promastigotes, and they are significantly more sensitive to oxidative stress, which they would encounter upon entry into macrophages, a phenotype that could in part explain their non-viability as amastigotes.

2. Materials and Methods

2.1 Cell lines and culture

Promastigotes of *L. mexicana* wild type and the Δlmg t null mutant cell lines were cultured at 26 °C in RPMI 1640 medium (Gibco), pH 7.2, supplemented with 10% heat-inactivated fetal bovine serum (iFBS), 0.1 mM xanthine and 5 μ g ml⁻¹ hemin. Cell lines complemented with episomal glucose transporter genes, Δlmg t[*pGT1*], Δlmg t[*pGT2*], and Δlmg t[*pGT3*] [3], were grown in the presence of the drug G418 (100 μ g ml⁻¹). Continuous cultures were maintained by periodic dilution of logarithmic phase parasites, and new parasite cultures were initiated frequently from frozen stocks. Axenic amastigotes of wild type parasites [5] were obtained by incubating stationary phase promastigotes at 32.5 °C in DME-*Leishmania* medium (DME-L), pH 5.5 containing 30 mM MES buffer instead of HEPES, and supplemented with 20% iFBS and 10 mM glucose [6]. Continuous cultures were then maintained by periodic dilution in this medium.

2.2 Parasite growth curves

Growth curves for promastigotes were obtained by inoculating 1 $\times$ 10⁶ cells ml⁻¹ of wild type or null mutant cell lines into RPMI 1640 medium, pH 7.2, supplemented with 5% dialyzed iFBS (Gibco), containing either no sugar or 10 mM D-glucose, D-mannose, D-fructose, or D-galactose. Parasites were incubated at 26 °C and counted on a hemacytometer every 24 h. Growth curves of wild type axenic amastigotes in several sugars were obtained by incubating 1 $\times$ 10⁶ cells ml⁻¹ in DME-L medium containing 10 mM of each sugar or no sugar, and counting at 48 h intervals. Growth curves at several pH were obtained by inoculating 1 $\times$ 10⁶ cells ml⁻¹ of logarithmic-phase wild type, Δlmg t, or Δlmg t[*pGT2*] promastigotes into RPMI 1640 medium, plus 10% iFBS and 10 mM D-glucose. Media adjusted to pH 5.0 and pH 6.0 were buffered using MES, and media adjusted to pH 7.0 and pH 8.0 using HEPES. After filtration, the pH of each medium was measured again and that value was used as initial pH. Cells were counted by triplicate and pH of each medium was measured every 24 h.

2.3 Macrophage infection

BALB/c bone marrow-derived macrophages were infected with wild type and Δlmg t stationary phase promastigotes (cell/parasite ratio 1:10) and centrifuged at 20°C for 5 min [7]. Cells were resuspended in Dulbecco's Modified Eagle medium supplemented with 4 mM L-glutamine, 1 mM sodium pyruvate, 25 mM glucose and 10% iFBS. Cultures were seeded in 4-well Lab-TekII Chamber Slides (Nalgen Nunc International Corp.), or in cell culture dishes (13 $\times$ 100 mm) and incubated at 34°C in a humidified 5% CO₂ incubator. After 3 h, adherent macrophages were washed 3 $\times$ with PBS and fresh medium was added. Five days post-infection, cells grown in the chamber slides were fixed with cold methanol and stained using Giemsa Stain (Sigma). Intracellular amastigotes were purified from macrophages three days after infection as described [8]. Parasites were settled onto poly-L-lysine coated coverslips, and stained using the Hema³ Stain Kit as described by the manufacturer (Fisher).

2.4 Light microscopy

Logarithmic phase wild type, $\Delta lmg1$, $\Delta lmg1[pGT2]$ promastigotes were washed twice with PBS, allowed to settle onto poly-L-lysine coated coverslips for 30 min at room temperature, and fixed with 100% cold methanol at -20°C for 10 min. The coverslips were mounted on glass slides in 30 μl PermMount (Fisher). Promastigotes, amastigotes and infected macrophages were observed under a 60 $\times$ oil immersion lens with a Nikon Microphot-FX phase contrast microscope. Images were captured using MagnaFire software (Optronics).

2.5 Uptake assays

Assays for uptake of [$6\text{-}^3\text{H(N)}$] D-glucose (PerkinElmer Life Sciences), [$2\text{-}^3\text{H(N)}$] D-mannose (Moravek), [$5\text{-}^3\text{H}$] D-fructose (American Radiolabeled Chemicals) and [$6\text{-}^3\text{H}$] D-galactose (American Radiolabeled Chemicals) in wild type and $\Delta lmg1$ promastigotes from *L. mexicana* were performed essentially as reported [9]. Logarithmic- and stationary-phase wild type and $\Delta lmg1$ promastigotes were assayed for sugar uptake at several substrate concentrations between 100 μM and 1 mM. Uptake of 1 μM [$2,8\text{-}^3\text{H}$] adenosine (Moravek) was used as control. Uptake assays were performed between 0 and 50 seconds, and the data were fitted to a straight line by linear regression. Sugar uptake using the complemented cell lines ($\Delta lmg1[pGT1]$, $\Delta lmg1[pGT2]$ and $\Delta lmg1[pGT3]$) was performed using the cells in middle-late logarithmic phase of growth.

2.6 Cell volume determination

Cell volumes of wild type, $\Delta lmg1$, and $\Delta lmg1[pGT2]$ promastigotes were measured according to the method developed in the laboratory of Dr. Steven Beverley (Washington University, St. Louis, MO). Briefly, logarithmic phase wild type, $\Delta lmg1$, and $\Delta lmg1[pGT2]$ promastigotes were washed and resuspended at 2×10^9 cells ml^{-1} in PBS. Then 400 μl of cell suspension was added into 1.2 ml of working solution containing 20 $\mu\text{Ci ml}^{-1}$ $^3\text{H}_2\text{O}$ (PerkinElmer Life Sciences) and 266 μM [^{14}C] carboxyl-inulin (PerkinElmer Life Sciences) in PBS. After parasites were incubated at 30°C for 15 min, three aliquots of 500 μl were withdrawn and cells were pelleted at 12,000 rpm for 3 min. The supernatant was removed carefully without disturbing the cell pellet and 50 μl of supernatant was added to 500 μl of 0.6 N perchloric acid and incubated at 4°C with gentle shaking overnight. Similarly, cell pellets were resuspended in 500 μl of 0.6 N perchloric acid and incubated at 4°C with gentle shaking overnight. The following day, 200 μl of the supernatant and cell pellets were analyzed by liquid scintillation counting to determine the activities of [^3H] and [^{14}C] in each fraction, and the internal cell volume was calculated from the difference of these activities.

2.7 Protein content determination

To determine the protein content, parasites were lysed in buffer containing 50 mM Tris, pH 7.6, 300 mM NaCl and 0.1% Triton X-100, as described [10]. The amount of protein in each lysate was determined using the detergent compatible DC Protein Assay (BioRad).

2.8 Sensitivity to hydrogen peroxide (H_2O_2) and Blasticidin S

Wild type and null mutant cell lines were exposed to increasing concentrations of H_2O_2 and the parasite viability was monitored as described [11], with modifications. Briefly, logarithmic phase wild type, $\Delta lmg1$, and $\Delta lmg1[pGT2]$ promastigotes were washed and resuspended at 5×10^6 cells ml^{-1} in incubation buffer containing 5 mM KCl, 80 mM NaCl, 2 mM MgCl_2 , 16.2 mM Na_2HPO_4 , 3.8 mM NaH_2PO_4 , 50 mM glucose, 0.15% bovine serum albumin, adjusted to pH 7.4. Parasites were incubated with various concentrations of H_2O_2 from 0-1 mM for 1 h. Parasites were then washed and resuspended in RPMI 1640 medium plus 10% iFBS and 10 mM glucose, at 26°C . After 24 h of growth, parasites were counted in triplicate on a hemacytometer, and the cell density was plotted against the concentration of H_2O_2 employed.

For blasticidin S (InvivoGen) experiments, parasites were incubated in the same culture medium at 26°C with various concentrations of drug (0-87 μ M) for 48 h. Parasites were counted in triplicate on a hemacytometer, and results were analyzed as for H₂O₂. The EC₅₀ was calculated using GraphPad Prism version 4.0a for Windows (GraphPad Software).

2.9 alamarBlue™ Reduction

Wild type, Δ lmg1, and Δ lmg1[pGT2] middle-late logarithmic phase promastigotes, grown in RPMI 1640 medium, pH 7.4, supplemented with 10% iFBS and 10 mM glucose, were plated in 96 well plates at various densities in the same cell culture medium. alamarBlue™ (Invitrogen) was added to 10% of the culture volume. Dye reduction was monitored spectrophotometrically at two wavelengths (570 and 600 nm), using the Multiskan Ascent plate reader (Thermo Electron Corp.). Each sample was evaluated in triplicate at several times after adding the dye. Percentage reduction was calculated as described by the manufacturer (Invitrogen). Statistical comparisons were done using Student's *t*-test. *p* value <0.005 were considered significant. Cells were also counted in triplicate on a hemacytometer at different time points.

2.10 Starvation in PBS

Wild type, Δ lmg1, and Δ lmg1[pGT2] middle-late logarithmic phase promastigotes ($\sim 5 \times 10^7$ cells ml⁻¹) were washed twice in PBS, resuspended in the same buffer and counted to determine the initial cell density. Then, cells were incubated at 26 °C, and samples were removed at various times for counting. Viability was determined by using trypan blue staining (0.04%, v/v). From four independent experiments, the initial cell density (0 h) was between $2-3.5 \times 10^7$ cells ml⁻¹. Statistical comparisons were done using Student's *t*-test. *p* value <0.05 were considered significant.

3. Results and Discussion

3.1 Ability of Δ lmg1 promastigotes and of individual glucose transporter isoforms to take up hexoses

Our previous results showed that Δ lmg1 promastigotes were incapable of taking up the glucose analog [³H]2-deoxy-D-glucose [3]. To determine whether the *L. mexicana* glucose transporters also mediate the uptake of other hexoses, uptake assays were performed with wild type and Δ lmg1 promastigotes using the monosaccharides glucose, mannose, fructose and galactose. Fig. 1 shows that wild type promastigotes are able to take up all four hexoses. In contrast, we could not detect any sugar uptake mediated by Δ lmg1 promastigotes indicating that the glucose transporters are indeed responsible for uptake of all four sugars. Identical results were obtained when Δ lmg1 cells were assayed in the logarithmic or stationary phase of growth, or when Δ lmg1 parasites were grown in sugar-depleted medium or medium containing 10 mM mannose or galactose as the sole sugar (data not shown). It was suggested previously that *L. mexicana* might possess an efficient and specific glucose-independent mannose uptake system [12]. Our results do not support that hypothesis since Δ lmg1 parasites are unable to take up mannose, an activity that would remain in this null mutant if an independent mannose permease existed. Previous studies have shown that galactose is a poor inhibitor of glucose uptake mediated by *Leishmania* and *Trypanosoma* hexose transporters [1,13,14] and that bloodstream *T. brucei* cannot take up galactose [15]. Nonetheless, the results in Fig. 1 confirm that galactose can serve as a substrate for the *L. mexicana* glucose transporters. These results are also consistent with the reported ability of [³H]galactose to label glycoconjugates in *L. amazonensis* [16] and *L. donovani* [17], but not in *T. brucei* [18]. The ability of *Leishmania* species to take up and metabolize galactose is also consistent with the existence of a gene (systematic name LmjF35.2740) in the *L. major* genome (hppt://www.genedb.org/) that encodes a putative galactokinase, the enzyme that converts galactose to galactose 1-phosphate. In contrast, the

homologous sequence in the *T. brucei* genome (systematic name Tb927.5.290) encodes a pseudogene, implying that this parasite cannot salvage galactose from its hosts. This latter observation is consistent with the demonstration [19] that UDP glucose 4'-epimerase is an essential enzyme in *T. brucei* required to generate UDP-galactose for incorporation of galactose into glycoconjugates.

To evaluate the role of each LmGT isoform in transporting specific hexoses, uptake assays were performed using the Δlmg t null mutant complemented individually with the *LmGT1*, *LmGT2*, or *LmGT3* genes expressed from the episomal vector pX63NEO [3]. The results (Fig. 2) demonstrated that all three glucose transporter isoforms are able to mediate the uptake of the four hexoses. This broad substrate specificity is characteristic of the kinetoplastid glucose transporters and distinguishes them from their mammalian counterparts [1,13]. We note that glucose transporters encoded by the episomal expression vector are somewhat under-expressed compared to wild type parasites (Fig. 2, data not shown). We have also tested the ability of the four hexoses to inhibit glucose uptake mediated by the three glucose transporter isoforms, performing uptake assays in which 5 mM and 50 mM concentrations of each sugar were used as inhibitors. Fig. 3 shows that D-glucose, D-mannose or D-fructose effectively inhibit transport of glucose in Δlmg t promastigotes expressing *LmGT1*, *LmGT2* and *LmGT3* genes (Fig. 3A); however, 50 mM D-galactose does not significantly inhibit glucose uptake mediated by LmGT2 and LmGT3 proteins, but inhibits glucose uptake mediated by LmGT1 protein by about 60% (Fig. 3B). Thus, the inhibition patterns of the three isoforms are very similar with the exception of galactose, which is a low affinity ligand for all three permeases. The lower EC₅₀ of LmGT1, compared to LmGT2 or LmGT3, may be explained, at least in part, by the fact that LmGT1 has a higher K_m for glucose (1.22 ± 0.22 mM) compared to LmGT2 or LmGT3 (109 ± 22 μ M and 208 ± 40 μ M, respectively) [3]. This difference is predicted [20] to result in a lower EC₅₀ for galactose.

3.2 Hexoses support growth in wild type but not Δlmg t promastigotes

Our previous results have demonstrated that glucose supports growth in wild type but not in Δlmg t promastigotes [3]. We extended this characterization by also determining the ability of mannose, fructose and galactose to support growth of wild type and Δlmg t promastigotes. No significant differences in growth were observed for wild type promastigotes incubated in the presence of 10 mM glucose, mannose or fructose (Fig. 4, left panel); however, when the unique sugar source was galactose wild type promastigotes grew poorly, similar to cells growing in the absence of sugar. These results are consistent with the ability of glucose, fructose and mannose, but not galactose, to be phosphorylated by the same hexokinase [21] and with the ability of glucose 6-phosphate, fructose 6-phosphate, and mannose 6-phosphate to be interconverted by the enzymes phosphoglucose isomerase and phosphomannose isomerase [12] and thus to serve equivalent nutritional roles.

In contrast, Δlmg t promastigotes grew at similar rates in RPMI 1640 medium in either the presence or absence of glucose, mannose, fructose, or galactose (Fig 4, central panel). These results demonstrate that external sugar sources do not support growth in the Δlmg t null mutant. The residual growth is presumably supported by oxidation of amino acids, which can also serve as a fuel source for *Leishmania* promastigotes [22].

Complementation of the Δlmg t null mutant with *LmGT1*, *LmGT2*, or *LmGT3* expressed on an episome restored growth to wild type levels in RPMI 1640 medium containing each of the four hexoses (Fig. 5). This result is consistent with the observation (Fig. 2) that each permease is able to mediate the uptake of glucose, mannose, fructose, and galactose. We note that in a previous report from our laboratory [3] revealed that complementation with *LmGT2* and *LmGT3*, but not *LmGT1*, can restore growth of Δlmg t amastigotes in primary murine macrophages.

It may be noteworthy that the Δlmg null mutant grows better than wild type parasites in hexose-deficient medium (compare left and middle panels of Fig. 4, note difference in scales of y-axes), suggesting that the null mutant may have undergone some level of adaptation to hexose depletion. Furthermore, this adaptation is apparently reversible, as the Δlmg null mutant grows poorly in hexose-deficient medium if it is complemented with any of the three *LmGT* permease genes (compare Fig. 5 to Fig. 4).

3.3 Growth of wild type axenic amastigotes in several sugars

We have previously demonstrated that *L. mexicana* requires glucose to survive as axenic amastigotes in DME-L medium [3], because deletion of this sugar from culture medium coupled with its removal from iFBS by dialysis resulted in failure of amastigotes to grow. Similar to promastigotes, the substitution of glucose in the medium by either mannose or fructose allowed the amastigotes to grow (Fig. 4, right panel). However, axenic amastigotes grew faster in the medium containing fructose than glucose or mannose. No growth was observed when the amastigotes were incubated in sugar-depleted or galactose-containing media.

3.4 Decreased size of Δlmg null mutants

Microscopic examination of Δlmg null mutant promastigotes suggests that they are smaller than either wild type parasites or null mutants that have been complemented with the *LmGT2* glucose transporter gene on an episomal expression vector ($\Delta lmg[pGT2]$) (Fig. 6). To quantify this reduction in size, we measured cell volume by monitoring partitioning of $^3\text{H}_2\text{O}$ and ^{14}C carboxyl-inulin into the three cell lines, and we determined the protein content per cell. The ratio for wild type/ Δlmg cells was 1.57 ± 0.10 (n=7) for the cell volume and 1.53 ± 0.10 (n=13) for the protein content measurement, confirming that the Δlmg null mutants are significantly smaller than wild type promastigotes. Episomal complementation with *LmGT2* was able to restore the size of Δlmg parasites, as the ratio for wild type/ $\Delta lmg[pGT2]$ cells was 1.01 ± 0.07 (n=3) for the cell volume and 1.00 ± 0.02 (n=3) for protein content. These results complement the previous observation that Δlmg promastigotes grow slowly and to a lower cell density compared to wild type parasites [3].

It is notable that restriction of nutrients such as glucose and amino acids leads to a pronounced reduction in cell size in many eukaryotes. This size reduction is thought to be controlled by a signal transduction pathway in which the TOR protein kinase plays a central role [23]. This TOR pathway is highly conserved among eukaryotes, and potential homologs of the TOR, raptor, and GβL proteins, that constitute the functional protein kinase, are encoded within the *L. major* genome (systematic names LmjF36.6320 and LmjF34.4530 for TOR, LmjF25.0610 for raptor, and LmjF10.0780 for GβL proteins). These observations suggest that the reduced size of Δlmg null mutants might be mediated by a TOR-raptor-GβL complex.

3.5 Increased susceptibility of Δlmg promastigotes to oxidative stress

As intracellular parasites, *Leishmania* are exposed to reactive oxygen species (ROS) such as H_2O_2 , superoxide anion (O_2^-) and hydroxyl radical ($\text{OH}^\bullet$), which arise from a number of different mechanisms including those generated from its own aerobic metabolism [24] and by the host immune response [25]. To evaluate whether Δlmg parasites are more sensitive to oxidative stress, we exposed both wild type and Δlmg promastigotes to increasing concentrations of a natural ROS H_2O_2 , and monitored parasite viability. Fig. 7A demonstrates that Δlmg promastigotes exhibited an EC_{50} for H_2O_2 that was ~5-fold lower than that for wild type parasites ($25.9 \pm 4.4 \mu\text{M}$ vs. $122.9 \pm 12 \mu\text{M}$ (n=4), respectively) and were thus more susceptible to oxidative stress than their wild type counterparts. To confirm that this phenotype is dependent upon deletion of the glucose transporter genes, we complemented the Δlmg cell line with the *LmGT2* gene expressed on the pX63NEO episome. The expression of *LmGT2* in

the null mutant was able to restore the EC₅₀ value ($140.5 \pm 4.1 \mu\text{M}$, (n=3)) to the levels observed in wild type cells (Fig. 7A). Furthermore, we compared the metabolic reducing potential of wild type and Δlmgt promastigotes by monitoring the ability to reduce alamarBlue™, a dye that is reduced by a variety of compounds including NADPH, NADH, FMNH₂, FADH₂, and cytochromes (manufacturer's data sheet, Invitrogen). After 2 and 4 h incubation with the dye, no significant increase in the number of cells was observed; however, the capacity of 6×10^7 cells ml⁻¹ to reduce the dye was decreased to a modest but statistically significant degree in Δlmgt promastigotes compared to wild type cells ($32.7 \pm 4.7\%$ and $37.3 \pm 4.8\%$ decrease in reducing potential compared to wild type parasites at 2 and 4 h, respectively (n= 4), $p < 0.005$). The complementation of the null mutant with an episomal copy of *LmGT2* gene restored the capacity to reduce the dye to levels observed in wild type cells (Fig. 7B).

As a control, we also determined the EC₅₀ for blasticidin S, a drug that specifically inhibits protein synthesis by interfering with peptide bound formation on the ribosome. After a 48 h incubation with various concentrations of this drug, wild type, Δlmgt , and $\Delta\text{lmgt}[pGT2]$ promastigotes showed similar EC₅₀ values ($2.29 \pm 0.92 \mu\text{M}$, $2.25 \pm 0.16 \mu\text{M}$, and $2.60 \pm 0.76 \mu\text{M}$ (n=3), respectively; Fig. 7C), confirming that the higher sensitivity to H₂O₂ observed in Δlmgt promastigotes was not due to a generalized hypersensitivity of this cell line to toxic insults.

The increased sensitivity to oxidative stress and the decreased reducing capacity of the Δlmgt null mutant may reflect the inability of the null mutants to take up and metabolize glucose via the pentose phosphate pathway [26]. This pathway is responsible for synthesis of NADPH and thus for maintaining the reducing environment of the cytosol [24]. Moreover, the increased sensitivity of Δlmgt null mutants to oxidative stress may play a significant role in the inability of these parasites to survive in the oxidative environment inside mammalian host macrophages [3].

3.6 Sensitivity of Δlmgt to nutrient limitation and altered pH

In addition to oxidative stress, *Leishmania* parasites are exposed to nutrient limitation [27] and reduced pH [28] when they enter macrophages. To determine whether the reduced viability of Δlmgt null mutant parasites in macrophages might be due to impaired ability to survive these stresses, we monitored the relative ability of wild type and Δlmgt promastigotes to survive starvation and reduced pH.

Wild type and Δlmgt promastigotes exhibited a similar ability to survive starvation in PBS for periods of up to 24 h. At 48 h, only six out of eight experiments showed a statistically significant (n= 3, $p < 0.05$) difference in the survival between both cell lines. However, extended starvation for 72 or 96 h resulted in a modest but significant ($p < 0.05$) reduction in viability of Δlmgt null mutants compared to wild type parasites in all eight experiments. Thus in eight independent experiments, the number of mutant parasites was reduced by $48.7 \pm 11.5\%$ (72 h) and $48.4 \pm 9.8\%$ (96 h) compared to wild type parasites. Hence increased sensitivity to nutrient deprivation, possibly resulting from the reduced amounts of the storage carbohydrate β -mannan that are available in Δlmgt parasites [29], may contribute to the impaired viability of the null mutants inside macrophages. However, complementation of the null mutant with an episomal copy of the *LmGT2* gene did not restore the survival of starved Δlmgt cells to wild type levels (data not shown), despite the fact that glucose uptake was restored (Fig. 2).

To monitor possible sensitivity to reduced environmental pH, wild type and Δlmgt promastigotes were incubated in culture media adjusted to various initial pH values from 5.0 to 8.1. Wild type promastigotes showed similar growth properties at pH 6.0 (5.3), 7.0 (6.8), or 8.1 (7.3) but grew to a significantly lower density at pH 5.0 (5.2), where the values in parentheses represent the final pH of the medium at day 7 (Fig. 8). Previously, we reported

that Δlmg promastigotes grew more slowly and to a lower cell density than wild type parasites at pH 7.0 [3]. This growth pattern was also observed at pH 6.0, sometimes reaching higher cell density than at pH 7.0 (Fig. 8). However, the growth of Δlmg promastigotes was not impaired at pH 5.0 compared to wild type cells. These results suggest that the non-viability of Δlmg amastigotes is not due to increased sensitivity to acid pH that they encounter in the phagolysosome. However, unlike wild type promastigotes, the null mutants showed poor growth at pH 8.1 (8.2). This may be because they did not acidify the medium during the growth as did wild type parasites, presumably due to failure to secrete organic acids that are the end products of glucose metabolism [30,31]. Higher environmental pH may have a negative effect on the transport function of several nutrients, as many of the leishmanial transporters have been proposed to act as proton-symporters [27]. The *LmGT2* complemented null mutant showed growth properties that were similar to wild type parasites (Fig. 8). Moreover, wild type parasites cultivated in glucose-depleted medium grew in the same way as Δlmg parasites in glucose-replete medium; specifically, growth was optimal at pH 6.2 (6.8), followed by pH 7.2 (7.5), but was poor at pH 8.1 (8.1) (data not shown).

3.7 Δlmg parasites transform into amastigotes within macrophages

It has been suggested [8] that Δlmg parasites fail to differentiate into amastigotes thus explaining the non-viability of this mutant inside macrophages. However in our initial studies on the Δlmg mutant [3], we observed transformation of the mutant into amastigotes followed by the inability of these parasites to replicate intracellularly. To further establish this point, murine bone marrow-derived macrophages were infected at a multiplicity of 10 with stationary phase wild type and Δlmg promastigotes. As established previously [3], equal numbers of each parasite line invade macrophages at early time points, but wild type parasites replicate intracellularly over a period of days whereas Δlmg parasites decrease dramatically in number. At day 5 after infection, macrophages were stained with Giemsa and examined by bright field microscopy for intracellular parasites. Both wild type (Fig. 9A) and Δlmg (Fig. 9B) infections generated non-flagellated amastigotes of identical morphology within vacuoles, although the number of intracellular parasites surviving was much lower for the null mutant compared to the wild type line. Similarly, amastigotes could be isolated as described [8] at three days post-infection from both wild type (Fig. 9C) and Δlmg (Fig. 9D) infected macrophages, confirming that the null mutants do transform into amastigotes. These results are consistent with the notion that glucose transporters are required for replication of amastigotes within their host cells. A critical role for these permeases is also supported by the observation that established culture form amastigotes of wild type *L. mexicana* do not replicate if they are inoculated into glucose-free medium (Fig. 4 and [3]).

3.8 Summary

Results reported here further extend the biological characterization of the glucose transporter null mutant in *L. mexicana*. The observation that these null mutants are unable to import glucose, mannose, fructose, or galactose establishes that these permeases are the only high affinity transporters that recognize these four hexoses. Furthermore, expression of each permease in the null mutant background revealed that each transporter mediates the uptake of all four hexoses and each permease alone can support growth of promastigotes. Nonetheless, significant differences have been shown to exist between the three isoforms. In particular, *LmGT2* mRNA is strongly upregulated in promastigotes compared to amastigotes, whereas *LmGT1* and *LmGT3* mRNAs are expressed at similar levels in both life cycle stages [4]. Furthermore, *LmGT2* and *LmGT3* are localized in the pellicular plasma membrane surrounding the cell body, whereas *LmGT1* is preferentially expressed in the flagellar membrane [3]. The reasons why the parasite elaborates three distinct glucose transporters with different patterns of expression and subcellular localization is not clear; however, phenotypic

analysis of null mutants in individual permease genes may help to resolve distinct biological functions.

The increased susceptibility of Δlmg null mutants to oxidative stress may explain in part the non-viability of these parasites as amastigotes within macrophages, where the parasites are exposed to various ROS. Reduced trypanothione is essential for viability of promastigotes and amastigotes [32] and is required for various endogenous metabolic processes such as the detoxification of methylglyoxal [24,33] that is generated from spontaneous decay of triose phosphates. NADPH is the principal determinant of the redox state of the cell and maintains trypanothione in its reduced form [24]. Since NADPH is produced by metabolism of glucose 6-phosphate via the pentose phosphate pathway [26], it is possible that reduced flux through this pathway in the glucose transporter null mutants accounts for the increased susceptibility of these parasites to oxidative stress.

The inability of Δlmg null mutants to import the four hexoses examined raises the question of whether they are able to synthesize the many glycoconjugates and complex carbohydrates that are constituted from these sugars and have been shown to play important roles in the biology of *Leishmania* parasites [12,34,35]. In a separate study [29] we have demonstrated that a variety of these carbohydrate-containing species are still synthesized in Δlmg null mutants, albeit at reduced levels. The carbohydrate moieties appear to be synthesized by gluconeogenesis, as radiolabel from glucogenic precursors such as amino acids or glycerol can be incorporated into these macromolecules. Despite the ability of Δlmg null mutants to synthesize carbohydrates by gluconeogenesis, they do not proliferate as amastigotes suggesting that gluconeogenesis by itself may be insufficient to support growth in this life cycle stage.

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Abbreviations

iFBS	heat inactivated fetal bovine serum
DME-L	Dulbecco's modified Eagle's medium- <i>Leishmania</i>
MES	2-morpholinoethanesulphonic acid
HEPES	<i>N</i> -[2-hydroxyethyl] piperazine- <i>N'</i> -[2-ethanesulphonic acid]
PBS	phosphate buffered saline, pH 7.4
ROS	reactive oxygen species

Fig 1.

Uptake of hexoses by wild type (WT) and $\Delta lmg1$ promastigotes from *L. mexicana*. (A) Logarithmic-phase wild type and $\Delta lmg1$ promastigotes were assayed for ability to take up 1 mM D-glucose, D-mannose, D-fructose or D-galactose. Filled symbols represent uptake by wild type parasites, and open symbols by $\Delta lmg1$ parasites. For each time point, uptake of three replicate samples was measured and averaged; errors bars represent standard deviations of these three values. This experiment is representative of two independent experiments.

Fig 2.

Hexose uptake mediated by $\Delta lmgf$ promastigotes complemented with each glucose transporter isoform. Logarithmic-phase cells were assayed for ability to transport 250 μ M D-glucose, D-mannose, D-fructose, or D-galactose for the indicated period of time. *LmGTs* represent $\Delta lmgf$ cells expressing the *LmGT1*, the *LmGT2*, and the *LmGT3* genes respectively from the pX63NEO expression vector. $\Delta lmgf$ represents the uncomplemented null mutant.

Fig 3.

Inhibition of glucose uptake mediated by $\Delta lmgT$ promastigotes complemented with each glucose transporter isoform. Logarithmic-phase cells were assayed for ability to inhibit the transport over a 60 s time interval of 100 μ M D-glucose (A) when they were incubated in the presence of no inhibitor (No Inh.) or 5 or 50 mM D-glucose (Glc), D-mannose (Man), D-fructose (Fru), or D-galactose (Gal), or (B) various concentrations of D-galactose (0.05-50 mM). Assays were performed using $\Delta lmgT$ cells expressing the *LmGT1*, *LmGT2* or *LmGT3* genes from the pX63NEO expression vector.

Fig 4.

Growth of *L. mexicana* wild type (WT) and $\Delta lmg1$ cells in several sugars. Wild type promastigotes (left), $\Delta lmg1$ promastigotes (middle), and wild type axenic amastigotes (right) were grown in media containing 10 mM glucose, mannose, fructose, galactose, or no sugar. For each time point, cells were counted in triplicate and the average and standard deviations (error bars) were plotted. These results are representative of at least two independent experiments.

Fig 5.

Growth in various hexoses of $\Delta lmgT$ promastigotes complemented with each glucose transporter isoform. Promastigotes complemented with *LmGT1* (left), *LmGT2* (middle), or *LmGT3* (right) were grown in 10 mM glucose, mannose, fructose, galactose or no sugar. For each time point, cells were counted in triplicate and the average and standard deviations (error bars) of these three values were plotted. These data are representative of at least two independent experiments.

Fig 6. Phase contrast micrographs of *L. mexicana* wild type (WT), Δlmg t, and Δlmg t complemented with *LmGT2* (Δlmg t[pGT2]) promastigotes. The same magnification (60x objective) was used in all three images.

Fig 7.

Sensitivity of wild type and $\Delta lmgT$ null mutants to oxidative stress. Promastigotes of wild type (squares), $\Delta lmgT$ (circles), or $\Delta lmgT[pGT2]$ (triangles) were incubated with various concentrations of (A) H_2O_2 or (C) blasticidin S as described in Material and Methods. The ordinate is plotted on a $\log_2$ (A) or $\log_{10}$ scale (C). 100% cell density represents parasites not treated with drug. In (B), wild type, $\Delta lmgT$ and $\Delta lmgT[pGT2]$ promastigotes were incubated with alamarBlueTM, at various cell densities, for 4 h. Percentage of alamarBlueTM reduction is plotted at each cell density assayed. Data points represent the average and standard deviations (error bars) of three measurements. In (B), error bars are too small to be seen. These data are representative of three independent experiments in each case.

Fig 8.

Sensitivity of wild type and $\Delta lmg1$ null mutants to environmental pH. Growth of wild type (squares) $\Delta lmg1$ (circles), and $\Delta lmg1[pGT2]$ (triangles) promastigotes in RPMI adjusted at various initial pH values, indicated at the left top of each figure. Data points represent the average and standard deviations (error bars) of three measurements. The pH values in parentheses represent the final pH of the medium at day 7. These data are representative of at least two independent experiments.

Fig 9. Infection of macrophages with wild type and $\Delta lmg1$ parasites. Murine bone marrow-derived macrophages infected with (A) wild type or (B) $\Delta lmg1$ stationary phase promastigotes, stained with Giemsa. Amastigotes isolated from (C) wild type or (D) $\Delta lmg1$ infected macrophages, stained with the Hema³ Stain kit. The same magnification (60x objective) was used in all images.