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Remodeling of protein and mRNA expression in *Leishmania mexicana* induced by deletion of glucose transporter genes

Xiuhong Feng^a, Torben Feistel^a, Cosmo Buffalo^a, Ashley McCormack^b, Elizabeth Kruvand^c, Dayana Rodriguez-Contreras^a, Natalia S. Akopyants^c, P.K. Umasankar^d, Larry David^e, Armando Jardim^f, Stephen M. Beverley^c, and Scott M. Landfear^{a,*}

^aDepartment of Molecular Microbiology and Immunology, Oregon Health & Science University, Portland, OR 97239, USA

^bVaccine and Gene Therapy Institute, Oregon Health & Science University, Beaverton, OR 97006, USA

^cDepartment of Molecular Microbiology, Washington University School of Medicine, St Louis, MO 63110, USA

^dMolecular Biology Unit, National Center for Cell Science, University of Pune, Ganeshkhind, Pune 411007, India

^eProteomics Shared Resource, Oregon Health & Science University, Portland, OR 97239

^fInstitute of Parasitology, McGill University, Ste. Anne de Bellevue, Quebec H9X 3V9, Canada

Abstract

Glucose is a major nutrient in the insect vector stage of *Leishmania* parasites. Glucose transporter null mutants of *L. mexicana* exhibit profound phenotypic changes in both insect stage promastigotes and mammalian host stage amastigotes that reside within phagolysosomes of host macrophages. Some of these phenotypic changes could be either mediated or attenuated by changes in gene expression that accompany deletion of the glucose transporter genes. To search for changes in protein expression, the profile of proteins detected on two-dimensional gels was compared for wild type and glucose transporter null mutant promastigotes. A total of 50 spots whose intensities changed significantly and consistently in multiple experiments were detected, suggesting that a cohort of proteins is altered in expression levels in the null mutant parasites. Following identification of proteins by mass spectrometry, 3 such regulated proteins were chosen for more detailed analysis: mitochondrial aldehyde dehydrogenase, ribokinase, and hexokinase. Immunoblots employing antisera against these enzymes confirmed that their levels were upregulated, both in glucose transporter null mutants and in wild type parasites starved for glucose. Quantitative reverse transcriptase PCR (qRT-PCR) revealed that the levels of mRNAs encoding these enzymes were also enhanced. Global expression profiling using microarrays revealed a limited number of additional changes, although the sensitivity of the microarrays to detect modest changes in amplitude was less than that of two-dimensional gels. Hence, there is likely to be a network of proteins whose expression levels are altered by genetic ablation of glucose transporters, and much of this regulation may be

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*Corresponding author. Tel: +1 503-494 2426; fax: +1 503 494 6862. landfear@ohsu.edu.

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reflected by changes in the levels of the cognate mRNAs. Some of these changes in protein expression may reflect an adaptive response of the parasites to limitation of glucose.

Keywords

Leishmania mexicana; Glucose transporter null mutant; Glucose starvation; Regulation of protein expression

1. Introduction

Leishmania are parasitic protozoa that exhibit two principal life cycle stages: promastigotes that live in the gut of the sand fly insect vector and amastigotes that live inside phagolysosomal vesicles of vertebrate host macrophages. These two life cycle stages exhibit dramatically different morphologies, promastigotes being spindle shaped, flagellated organisms that are highly motile while amastigotes are oval shaped, non-motile forms that possess only a residual flagellum. These two developmental forms live in very distinct physiological environments. Promastigotes are extracellular, reside at the ambient temperature of the sand fly and at a pH that is close to neutrality [1], and are exposed to varying but often high levels of sugars in the sand fly gut [2]. Amastigotes are intracellular, are exposed to the more elevated temperature of the vertebrate and the acidic environment of the phagolysosome [3], and are thought to prevail in a relatively carbohydrate-poor environment [4]. Both life cycle stages are exposed to changes in their milieu, both during propagation and upon the developmental transformation between the two stages. Thus sugars are high in the sand fly gut following a plant nectar meal but drop to much lower levels following digestion of the sugar meal [2]. Similarly, amastigotes may experience changes in the level of carbohydrates available when they are released from the macrophage into the extracellular space following lysis of the host cell, especially in glucose rich serum, and they could be exposed to changes in available metabolites during residency within the macrophage. One fundamental but poorly understood aspect of parasite physiology concerns how these microorganisms adapt to changes in their environments and what molecular mechanisms they employ to mediate these adaptations.

Hexose uptake in *L. mexicana* and related *Leishmania* species is mediated by 3 hexose or 'glucose' transporters designated GT1, GT2, and GT3 that are encoded by a cluster of linked genes [5]. A null mutant, generated by targeted gene replacement of the *GT1-GT3* gene cluster and designated Δlmg t [5], was deficient in measurable uptake of glucose, fructose, mannose, and galactose [6]. In addition, this glucose transporter null mutant exhibited profound metabolic and cellular changes, including substantially reduced levels of the mannose polymer and storage carbohydrate β -mannan [7], decreased cell size and protein content, and increased susceptibility to nutrient starvation, elevated temperature, and oxidative stress [6]. These alterations may explain in part why the null mutants did not survive following infection of murine primary macrophages or as culture form amastigotes [5,8].

Since the glucose transporter null mutants exhibit many phenotypic changes, one intriguing question is whether these genetically altered parasites also experience alterations in expression of mRNAs or proteins compared to wild type parasites. Such potential changes could represent either mechanisms for adaption to low hexose environments or they could play a role in the observed increased susceptibility to environmental stresses noted above. To determine whether there are changes in mRNA or protein expression in glucose transporter null mutants compared to wild type promastigotes, both parasite lines were subjected to protein profiling by two-dimensional gel analysis and mRNA profiling employing an oligonucleotide microarray. Significant changes in the levels of individual proteins and mRNAs have been detected.

2. Materials and methods

2.1 Parasite culture

Promastigotes of *L. mexicana* wild type (strain MNYZ/BZ/62/M379) or glucose transporter null mutant ($\Delta lmg1$) [5] derived from this wild type strain were cultured at 26°C in RPMI 1640 medium containing either 11 mM or no glucose (glucose-limited) and 10% heat-inactivated fetal bovine serum (FBS), which contained ~5 mM glucose before dilution. For many experiments, parasites were pelleted and washed in Dulbecco's Phosphate Buffered Saline (PBS) (GIBCO Invitrogen). Stationary phase was achieved at a density of $\sim 5 \times 10^7$ and $\sim 2.5 \times 10^7$ cells ml⁻¹ for wild type and $\Delta lmg1$ parasites respectively. The concentration of glucose in glucose-limited medium was measured using the BioVision Glucose Assay Kit.

2.2 Two-dimensional gel electrophoresis

Parasites were grown to log phase ($\sim 1 \times 10^7$ cells ml⁻¹) and cells ($\sim 1 \times 10^9$) were harvested by centrifugation at 2500 × g for 12 min, washed twice at 4°C in Tris buffer (10 mM Tris-HCl, pH 7.0; 25 mM sorbitol), and resuspended in cell lysis buffer (8 M urea, 2 M thiourea, 2% CHAPS, 5% Amberlite IRN-150L, 1 mM protease inhibitor cocktail, 65 mM dithiothreitol). Lysis was allowed to proceed for 2 h at room temperature with vortexing every 15 min, and samples were centrifuged to remove insoluble material. Protein concentration was assayed using the 2D Quant Kit (GE Healthcare). Total cell extracts were separated into aliquots and stored at -80°C. For gels analyzed by silver staining, 100 µg of protein were loaded leading to identification of ~1500 spots. For gels to be analyzed by Coomassie brilliant blue staining, 600 µg were loaded leading to identification of ~550 spots.

First dimension isoelectric focusing (IEF) was performed at 20°C using 18 cm IPG strips (GE Healthcare) applying pH ranges from 3–10, 4–7, 5–6, and 6–11. Protein extracts were purified using 2D Clean-up Kit (GE Healthcare), redissolved in 360 µl Destreak solution (GE Healthcare) containing 0.5% IPG buffer of the respective pH gradient, and loaded into 18 cm Immobiline Dry-strips in a ceramic strip holder. The maximum current setting was 30 µA per strip using the IPGphor Isoelectric Focusing unit (GE Healthcare). The strips were rehydrated for a minimum of 12 h at 20°C. After rehydration, the IEF run was carried out using the following conditions: 500 V for 500 Vhr, 1000 V for 1000 Vhr, 5000 V for 5000 Vhr, 6500 V for 9900 Vhr, 8000 V for 80,000 Vhr.

Strips were equilibrated in equilibration buffer (50 mM Tris-HCl, pH 8.8; 6 M urea, 30% glycerol, 2% SDS, 0.002% bromphenol blue) containing 65 mM dithiothreitol for 15 min and then in equilibration buffer containing 260 mM iodoacetamide for 15 min. Strips were then sealed on the top of 8 – 16% gradient acrylamide gel using 0.5% agarose in standard Tris-glycine electrophoresis buffer containing 0.002% bromphenol blue. Second dimension SDS-PAGE was run in a Hoefer DALT apparatus (GE Healthcare) at 14 °C and constant voltage of 100 V until the tracking dye reached the bottom of the gel. Gels were fixed and stained with AgNO₃ or colloidal Coomassie brilliant blue G250 (Boehringer Mannheim).

Stained gels were scanned with a Fuji FLA-5000 scanner (Fujitsu Computer Products of America), and the images were analyzed with Phoretix 2D Evolution software (Nonlinear Dynamics Ltd.). Two-dimensional gels of 8 independent samples per strain (wild type or $\Delta lmg1$ null mutant) were averaged and compared.

2.3 Mass spectrometry of tryptic peptides

To identify proteins present in spots showing significant regulation between wild type and glucose transporter null mutant parasites, 21 regulated spots were excised using a One Touch Spot Picker (The Gel Company, San Francisco, CA), digested with trypsin, extracted, and

submitted to the Oregon National Primate Research Center Proteomics Core Facility for peptide mass finger printing.

Tryptic digests were analyzed by electrospray ionization tandem mass spectrometry (LC-MS/MS) using a LCQ Deca XP Plus ion trap mass spectrometer (ThermoElectron). Peptide analysis utilized a 10 cm × 180 µm capillary column packed with Biobasic-C18 resin (Thermo Hypersil Keystone). Samples were applied to the column directly from the Surveyor autosampler (ThermoElectron) using a Surveyor sample pump at a flow rate of 1 µl per min. The peptides were eluted from the column using a mobile phase of 0.1% formic acid in water and a 30 min 0–50% acetonitrile gradient. Peptide ions were analyzed using data dependent scanning, where the MS was set to trigger data dependent MS/MS acquisition of the 3 most intense ions detected during the MS survey scan. Protein identifications were obtained using TurboSEQUENT software (ThermoElectron) by searching for matching peptide mass fingerprints in a protein database generated from the *L. mexicana* genome sequence (www.tritrypdb.org/tritryp). All peptides were identified with greater than 95% probability [9], and all proteins were assigned with greater than 99% probability [10] using Scaffold software (Version 2_06_00, Proteome Software), with an additional requirement that there were two or more unique peptides matched to each protein.

2.4 Antibodies

The open reading frames (ORFs) of mALDH and RK were amplified from *L. mexicana* genomic DNA using the polymerase chain reaction employing Pfu Taq polymerase (Stratagene) and oligonucleotide primers designed against the NH₂- and COOH-terminal sequences of the corresponding ORFs from *L. major* (LmjF25.1120 and LmjF27.0420, respectively). Amplified ORFs were cloned into the pET200 or PET30b(+) vectors for generation of 6x histidine-tagged fusion proteins, and recombinant proteins were purified by nickel column chromatography using Ni-NTA resin (Qiagen) according to the manufacturer's instructions. Purified fusion proteins were sent to Cocalico Biologicals, Inc and employed to raise antisera in rabbits using the CBI standard protocol.

The anti-mALDH and anti-HK antibodies each produced a single band of ~55 kDa, that comigrated with the purified recombinant protein, on immunoblots of lysates of *L. mexicana* promastigotes when employed at a dilution of 1:10,000. Hence, these antisera were employed at these or higher dilutions without further purification. The anti-RK antiserum was affinity purified employing recombinant RK coupled to a Affi-Gel 10 (Bio Rad Laboratories) column, as described [11]. The affinity purified antibody produced a single band of ~34 kDa in immunoblots when employed at a 1:500 dilution.

2.5 Immunoblots

Promastigotes (~2 × 10⁶) were lysed in Laemmli sample buffer [12], separated on a 10% SDS-polyacrylamide gel, and transferred onto a nitrocellulose membrane. Membranes were blocked with Infrared Imaging System Blocking Buffer (LI-COR Biosciences) for 1 h or overnight, incubated with crude serum of anti-mALDH or anti-HK (1:10,000 dilution) or affinity purified serum of anti-RK (1:500 dilution) in blocking solution for 1 h, washed 3 times with PBS containing 0.02% Tween-20, and then incubated with secondary antibody (goat anti-mouse IRDye 680 and goat anti-rabbit IRDye 800CW, LI-COR Biosciences) at a 1:20,000 dilution in Blocking Buffer followed by washing with PBS plus 0.02% Tween-20. Membranes were analyzed using a LI-COR Odyssey Infrared Imaging System with excitation at 685 nm and 785 nm and detection at 720 nm and 820 nm. Images (16-bit TIFF files) were quantified using the software provided with the Imaging System.

2.6 Pulse-labeling and immunoprecipitation

Wild type *L. mexicana* and glucose transporter null mutant promastigotes were grown to early logarithmic phase ($4-8 \times 10^6$ cells ml^{-1}) in RPMI 1640 medium supplemented with 10% heat-inactivated FBS. Parasites (1.5×10^8 promastigotes) were washed twice with Dulbecco Modified Eagle Medium containing high glucose but deficient for methionine and cysteine (GIBCO Invitrogen), resuspend in 1 ml of this medium, and incubated at 26°C for 1 h. [^{35}S]methionine plus [^{35}S]cysteine (400 μCi , Tran ^{35}S -Label, MP Biomedicals) was added and the cell suspension was incubated at 26°C for 2.5 h. Subsequently cells were washed 3 times with PBS and the cell pellet was resuspended in 1 ml Lysis Buffer (PBS containing 1.0% Triton X-100 and Protease Inhibitor Cocktail Tablets, Roche Diagnostics Corp), incubated on ice for 30 min with occasional vortexing, and centrifuged in a microcentrifuge at 13,000 rpm for 15 min at 4°C . Pre-immune serum was added to the cleared lysate followed by incubation at 4°C with rotation for 1 h. Pre-washed protein-A sepharose (Invitrogen) was added and incubated overnight to absorb background material followed by centrifugation at 13,000 rpm for 3 min. Anti-mALDH antiserum (10 μl undiluted) was added and incubated at 4°C for 4 h followed by incubation with pre-washed protein-A sepharose at 4°C overnight. Finally the beads were washed 3 times with Lysis Buffer. A 60 μl aliquot of 2 x Laemmli sample buffer was added to the pellet and samples were heated at 97°C for 10 min. Equal amounts of radioactive material from each immunoprecipitation were loaded onto a 10% SDS-polyacrylamide gel and separated by electrophoresis. Gels were fixed, dried, and exposed on a PhosphorImager S1 (Molecular Dynamics) for 5 days. Images were recorded and quantified using Quantity One Software (Bio-Rad Laboratories).

2.7 Protein half-life experiments

Cycloheximide ($10 \mu\text{g ml}^{-1}$, a concentration that inhibited protein synthesis by $>90\%$) was added to cultures of wild type and glucose transporter null mutant promastigotes ($\sim 1 \times 10^7$ cells ml^{-1}) in RPMI 1640 plus 10% FBS. Aliquots were removed at the indicated time points and fractions representing $\sim 2 \times 10^6$ cells analyzed by immunoblot as described above. Control experiments revealed that parasites treated with cycloheximide for 0, 5, 24, and 48 h had 0, 0, 2.1, and 5.0 % Trypan blue positive cells respectively. Cell number did not decrease significantly during this time period, and parasites maintained motility throughout a 48 h treatment.

2.8 Microarray experiments

A DNA microarray (N. Akopyants, E. Kruvand, and S.M. Beverley, manuscript in preparation and [8]), consisting of 12,841 oligonucleotides of length 68–72 nucleotides derived from the *L. major* genome, was designed and printed by the Washington University Microarray Facility. RNA was prepared from 4 biological replicates of wild type and glucose transporter null mutant promastigotes cultured at a density of $\sim 4-6 \times 10^6$ cells ml^{-1} . Total RNA was prepared using TRIzol and the instructions from the manufacturer (Invitrogen) followed by treatment with DNase (Qiagen DNase Kit) and further purification using the RNeasy Kit (Qiagen). Hybridizations and data analysis were performed by the Washington University Microarray Facility as described [8,13] using a posthybridization labeling method that significantly reduced dye swap effects. For each pair of biological replicates, two slides were hybridized, one in which the wild type RNA had been labeled with the dye Cy3 and null mutant RNA with the dye Cy5 and another in which the dye labeling was swapped. For scanned slides, the data were spot found, normalized, and fused using BlueFuse software (BlueGnome). Data with a confidence of <0.2 were filtered out prior to final analysis.

2.9 Quantitative Real Time PCR (qRT-PCR)

qRT-PCR was performed as described in reference [14].

3. Results

3.1 Glucose transporter null mutants alter the expression of various proteins

To determine whether deletion of glucose transporter genes in *L. mexicana* resulted in global changes in protein expression, the constellation of cellular proteins observed on silver stained two-dimensional gels was compared for wild type and glucose transporter null mutant ($\Delta lmg1$) promastigotes. Figure 1A displays a composite electronic image, generated using Phoretix 2D software, in which spots from the wild type lysate are colored green and those from the glucose transporter null mutant are colored magenta. Thus spots that emerge as magenta in the composite image are upregulated and spots that emerge as green are downregulated when the null mutant parasites are compared to wild type parasites. In Fig. 1A, gels were analyzed from 8 independent pairs of wild type and null mutant parasites to identify spots that were consistently up- or downregulated in the deletion mutants. Quantitative analysis of relative spot intensities using Phoretix 2D software, in which gels were normalized to the total staining intensity of each gel, revealed 31 spots that were consistently upregulated in null mutants (6 of these spots were not detected in wild type parasites), and 19 spots that were consistently downregulated in null mutants. Quantification of the levels of changes in intensity for 21 of these spots (Table S1) revealed upregulation between 2–14-fold and downregulation between 2–6-fold. Graphic representations of 3 relevant regions (I–III) of these gels (Fig. 1B) display topographic illustrations of the spot intensities for wild type and null mutant lysates. The green numbers in each region designate spots whose intensity is consistently higher (all numbered spots except spot 1 in Box III) or lower (spot 1 in Box III) in null mutants compared to wild type parasites (identities of proteins in each spot are listed in the legend to Fig. 1). In these studies, a broad range pH dimension was employed to capture the largest number of potentially regulated proteins. However, less extensive analysis of gels covering pH ranges of 4–7, 5–6, 5.5–6.7, and 6–11 (not shown) also exhibited significant differences in spot profiles between wild type and null mutant lysates. Such changes in spot intensities could represent increases or decreases in the levels of individual proteins or changes in post-translational modifications (PTMs) that shift spot positions in one or both dimensions. Analysis of 3 regulated proteins discussed below reveal that these polypeptides are regulated at the level of protein and mRNA expression and, in once case, also by differential PTMs.

3.2 Identification of proteins whose expression levels change in glucose transporter null mutants

To obtain molecular identities for some of the spots with modified intensities, similar two-dimensional gels were run and stained with colloidal Coomassie brilliant blue, and selected spots were excised from gels (see supplemental Fig. S1 for examples) and analyzed by mass spectrometry. Several spots appeared to contain one predominant protein that contributes all or most of the peptides identified by LC-MS/MS (Supplemental Table S1): mitochondrial aldehyde dehydrogenase (3 spots, one absent in wild type and the others 2-fold or +2, and 7-fold or +7 upregulated in the null mutant as measured by relative Coomassie blue staining intensity), ribokinase (+7), and T complex binding protein β subunit (6-fold downregulated in null mutants, or –6). Another spot containing peptides from both D-lactate dehydrogenase and ribokinase showed especially strong upregulation (+14) in glucose transporter null mutants.

The two-dimensional gel analysis represents an initial screen for proteins potentially regulated in glucose transporter null mutants. To firmly establish the existence of regulated proteins, and to analyze mechanisms of regulation, several strongly regulated (7–14-fold upregulated in $\Delta lmg1$ null mutants) candidates were selected for more detailed analysis. Mitochondrial aldehyde dehydrogenase (mALDH) and ribokinase (RK) represented the two most strongly upregulated proteins with predominant protein identifications by mass spectrometry. Concurrently, preliminary work by two of the co-authors (P.K.U. and A.J.) using an antibody

against the glycolytic enzyme hexokinase (HK) indicated that the levels of this protein were upregulated by glucose starvation of *L. major* and *L. donovani* parasites and hence might represent an additional marker for proteins whose expression is enhanced in the null mutants. Consequently, these 3 proteins (mALDH, RK, and HK) were selected for more detailed studies to examine regulation of protein expression in glucose transporter null mutants. D-LDH was also investigated but was not regulated in glucose transporter null mutants compared to wild type parasites, as determined by immunoblot analysis (not shown).

3.3 Confirmation of regulation using specific antibodies

Antibodies generated against recombinant mALDH, RK, and HK all recognized a single predominant band of the predicted molecular weight on immunoblots of *L. mexicana* lysates (Fig. 2A). Furthermore, an immunoblot from parasites expressing a mALDH-GFP fusion protein reacted with both the ~54 kDa mALDH and the ~84 kDa mALDH-GFP band, further confirming specificity of this antiserum (Fig. 2A, left panel). Immunoblots employing these antisera (Fig. 2B) revealed that mALDH, RK, and HK are upregulated 7.4 ± 3.3 ($n = 4$, $p < 0.001$), 6.7 ± 3.3 ($n = 8$, $p < 0.001$), and 2.0 ± 0.4 ($n = 6$, $p < 0.01$) -fold in glucose transporter null mutants compared to wild type promastigotes. Furthermore, complementation of the null mutant with an episomal expression vector encompassing the *L. mexicana* *GT2* gene reduced expression of these proteins close to wild type levels. These experiments confirm i) that expression levels of these proteins are regulated in null mutants compared to wild type parasites, as suggested from the two-dimensional gel data, and ii) that this regulation is linked to the genetic ablation of the glucose transporter genes and not to some other adventitious genetic alteration that occurred during the process of targeted gene replacement.

In addition, localization of mALDH-GFP by fluorescence microscopy revealed that this fusion protein colocalized extensively with the mitochondrial-specific dye MitoTracker® (data not shown), confirming that this enzyme is the mitochondrial isoform of ALDH.

3.4 Upregulation of mALDH, RK, and HK correlates with increased mRNA levels

To determine whether upregulation of these 3 proteins was the result of increased levels of their cognate mRNAs, the mRNA levels in wild type and glucose transporter null promastigotes were quantified by qRT-PCR using 4 biologically independent preparations of RNA from wild type and $\Delta lmg1$ null mutants. Table 1 reveals that the mRNAs encoding these 3 enzymes were indeed increased in glucose transporter null mutants (4.1 ± 0.5 , $p < 0.001$; 2.2 ± 0.8 , $p < 0.01$; 1.7 ± 0.6 , $p < 0.01$ -fold respectively). In addition, the levels of mRNAs for several other enzymes of the glycolytic (glucokinase, phosphofructokinase and pyruvate kinase) and gluconeogenic (fructose 1,6-bisphosphatase, phosphoenolpyruvate carboxykinase, pyruvate phosphate dikinase) pathways were measured to determine whether they might respond to genetic ablation of the glucose transporter genes. No significant differences were noted in the levels of these mRNAs with the exception of pyruvate phosphate dikinase that was upregulated ~2-fold ($p < 0.01$). These results indicate that the regulation of mALDH, RK, HK, and likely other proteins in the glucose transporter null mutants, occurs at least in part at the level of mRNA abundance.

3.5 mRNA expression profiling of wild type and glucose transporter null mutants

To analyze at a global level the possible changes in mRNA levels that might accompany ablation of the glucose transporter genes, mRNA expression profiling was performed on wild type and null mutants employing an oligonucleotide microarray containing sequences designed from the genome of the closely related parasite *L. major*. This microarray [8] provides ~99% coverage of the predicted open reading frames. The use of a heterologous microarray is justified both by the high degree of sequence identity between open reading frames of distinct *Leishmania* species [15] and by the successful use of this microarray in comparative genomic

hybridization studies utilizing genomic DNAs from different mutant lines of *L. mexicana* [8]. Hybridizations were performed with 4 sets of biological replicates each from wild type and null mutant promastigotes. Table 2 reports mRNAs whose levels were either upregulated or downregulated 2-fold or more, in 3 of the 4 replicates, in null mutants compared to wild type parasites. The first striking result is that among the ~8,300 protein-coding and ~700 RNA-coding genes in the *Leishmania* genome [16], very few RNAs were detectably regulated: 22 were upregulated and 3 were downregulated 2-fold or more. One of the most strongly regulated mRNAs was that encoding the cytosolic isoform of serine hydroxymethyltransferase (SHMT, LmjF 14.1320), which experienced an upregulation in the null mutant with a $\log_2$ value of ~2.6. Most of these RNAs were altered in level by a small degree, typically a $\log_2$ value of ~1.5 or less, and only 5 RNAs are altered by a $\log_2$ value of 2 or more. Among the 21 upregulated RNAs, 11 are derived from a segment of chromosome 29 that is amplified 7.45 ± 0.88 -fold ($n = 7$) in the glucose transporter null mutant [8]. Thus, these 11 mRNAs are upregulated (from $\log_2$ values of 1.21 for LmjF29.2780 to 2.81 for LmjF29.2850) as a consequence of a gene amplification, which represents a secondary genetic alteration in the null mutant accompanying replacement of the glucose transporter genes. As expected for transcripts that are not present in the Δlmg t line, mRNAs from the *GT1-3* glucose transporter locus, LmjF36.6280–6300, were strongly downregulated ($\log_2$ of -3.14) in the null mutant. The detection of a finite ratio of signal for *GT* mRNAs when wild type and null mutants are compared is consistent with results from other null mutant hybridizations employing these arrays (Kruvand, E. and Beverley, S.M., unpublished observations) and is indicative of a residual background signal for hybridization to the relevant oligonucleotide in the absence of the corresponding mRNA. In addition, the mRNAs for the major surface glycoprotein GP63 experienced a small (maximum $\log_2$ of -1.12) reduction. However, it appears that the majority of proteins whose expression levels are altered in the null mutant did not exhibit a detectable alteration in the levels of their mRNAs when monitored by microarray analysis. In particular, this method did not detect alterations in the levels of mRNAs encoding proteins identified by two-dimensional gel analysis (Table S1). These effects probably reflect ‘compression’ of the microarray signal that often decreases differential signal intensity in microarray hybridizations [17] and imply that the microarray experiments were less sensitive in their ability to detect modest changes in mRNA levels than is qRT-PCR. To provide an overview, Table 3 summarizes the alterations detected for several proteins, employing immunoblots, and their mRNAs as determined both by qRT-PCR and microarrays.

3.6 Regulation of mALDH, RK, and HK by glucose starvation

It is possible that many of the changes in protein expression observed in the glucose transporter null mutant are the result of glucose limitation within parasites that can no longer import this nutrient. To determine whether mALDH, RK, and HK are upregulated by glucose starvation, we quantified the levels of these proteins in wild type parasites that had been transferred from glucose-replete to glucose-limited medium (RPMI medium plus 10% FBS with or without 11 mM glucose). In glucose-limited medium, the initial measured glucose was ~1 mM at the beginning (day 0) of the experiment, but glucose had decreased to an undetectable level by day 2. Figure 3A shows expression of mALDH in wild type *L. mexicana* promastigotes that were inoculated into glucose-replete or glucose-limited medium. Glucose-replete parasites maintained the initial level of mALDH expression, whereas parasites inoculated into glucose-limited medium experienced an ~8-fold upregulation of mALDH by day 4. The absolute degree of mALDH induction varied from one experiment to another from ~3-fold to ~8-fold for reasons that were not clear, but a pronounced increase of this protein was always observed. Figures 3B and 3C show similar experiments for RK and HK and confirm that expression of these proteins is enhanced by glucose starvation as well.

Transport of other substrates by the *L. mexicana* glucose transporters, such as fructose, mannose, galactose [6] and ribose [18], is eliminated in the Δlmg t null mutant, and as such the decreased levels of these nutrients in the null mutant could trigger induction of some of the regulated proteins. Thus the level of RK, a ribose-metabolizing enzyme, might in principle be upregulated by reduction of intracellular ribose in the Δlmg t parasites. However, the induction of mALDH, RK, and HK specifically by glucose limitation (Fig. 3 A–C) confirms that this sugar is a principal determinant for regulation of these proteins.

3.7 Comparison of protein synthesis and protein turnover in wild type and glucose transporter null mutants

The preceding results indicate that the level of expression of mALDH, RK, and HK are regulated significantly by glucose limitation at the mRNA level, although smaller effects of protein stability or translational control could be operative. To investigate these possibilities, the relative rate of protein turnover in wild type and glucose transporter null mutants was examined by inhibiting translation by >90% with cycloheximide and following the level of each protein by immunoblotting. For each of the 3 proteins, degradation was very limited within a time course covering the first 24 hours following drug treatment, and no significant differences were observed between wild type and null mutant cells (data not shown). Within the limitations of any drug treatment experiment, these data suggest that regulation of these 3 proteins by glucose availability does not occur at the level of protein degradation.

Relative rates of protein synthesis can be approximated by labeling with [³⁵S]methionine/[³⁵S]cysteine followed by quantification of label incorporated into each protein by immunoprecipitation. These experiments were attempted for mALDH and RK, the two proteins with the largest level of induction. Figure 4 shows the results for an [³⁵S] labeling – immunoprecipitation experiment employing the anti-mALDH antibody and equivalent amounts of radiolabeled lysates from wild type and glucose transporter null mutant promastigotes. Two such experiments revealed a 4.5- and a 6.8-fold induction in the band intensity of the ~54 kDa radiolabeled band, indicating the mALDH is upregulated at the level of protein synthesis to approximately the same degree that its mRNA level is upregulated. Similar experiments using the anti-RK antiserum did not produce a clear pattern of immunoprecipitation, preventing quantitative measurement of synthesis for this protein.

3.8 Probable post-translational modifications of RK in the Δlmg t null mutant

In the initial evaluation of protein expression in wild type and Δlmg t parasites employing two-dimensional gels (Fig. 1), RK was detected in two distinct spots (K1 and K2, Supplementary Table S1) whose intensities increased considerably in the glucose transporter null mutant. These results raised the possibility that RK might experience PTMs that occur differentially between wild type and null mutant parasites. To investigate this possibility, lysates from wild type, Δlmg t, and Δlmg t[pGT2] parasites were separated on two-dimensional gels, blotted onto nitrocellulose filters, and probed with the anti-RK antibody. As illustrated in Fig. 5, RK was represented by two major spots and several weak spots in blots from wild type parasites but by 6 strong spots in blots from the Δlmg t null mutants. All 6 spots migrated as a train at the same apparent molecular weight, but most of the spots that appeared selectively in the null mutants focused at a more basic pI compared to those present in the wild type lysates. Furthermore, complementation of the null mutants with an episomal copy of the GT2 gene resulted in a large decrease in the intensity of the spots that were selectively increased in the null mutant, indicating that the induction of these new species indeed resulted from deletion of the glucose transporters.

The preceding observations suggest that RK is probably differentially modified at the post-translational level in glucose transporter null mutants. Currently we do not know the nature of

these PTMs, but ultimately it may be possible to identify them by mass spectrometric analysis. Similar immunoblots probed with the anti-mALDH antibody resulted in multiple spots in lysates from both wild type and null mutant parasites, but all spots were upregulated to a similar degree in null mutants compared to wild type parasites (not shown) suggesting that there is no differential regulation of PTMs for this protein.

4. Discussion

Leishmania parasites are exposed to pronounced changes in environment during their life cycle, including changes in the availability of central nutrients such as glucose, and responding to such changes appropriately is necessary for survival and successful transformation between developmental stages. Nonetheless, how these or related kinetoplastid parasites sense and respond to their environment is currently largely a mystery. The existence of a glucose transporter null mutant for *L. mexicana* provided an opportunity to probe how these parasites respond to reduction of glucose availability via genetic ablation of hexose permeases. To examine alterations in the glucose transporter null mutants, we monitored global changes in expression of proteins by two-dimensional gels and, more comprehensively, mRNAs by microarray analysis.

Analysis of protein expression in wild type and glucose transporter null mutants by two-dimensional gels allowed the observation of a significant number of silver stained spots whose intensities changed between wild type and null mutant parasites. This observation implies that there exists a network of proteins whose expression levels are altered by ablation of the glucose transporter genes. Identification of proteins in such spots by mass spectrometry allowed the selection of 3 glucose-regulated proteins (GRPs) that were studied in greater detail. Monospecific antibodies were raised against these 3 GRPs to confirm their regulation both by transporter gene ablation and by glucose limitation in wild type parasites. While some of the changes observed on two-dimensional gels could represent alterations in PTMs, the pronounced changes in protein expression levels detected on immunoblots for mALDH, RK, and HK (Fig. 2) indicate that these enzymes are indeed regulated at the level of protein abundance. Quantification by qRT-PCR revealed that the cognate mRNAs were upregulated in glucose transporter null mutants to a similar degree as the proteins. Hence, at least for these GRPs, much of the regulation appears to occur at the level of mRNA abundance. Comparison of the mRNA sequences for mALDH, RK, and SHMT, the 3 most strongly regulated mRNAs, using the MEME search algorithm [19,20] revealed 2 sequences in the 5' flanks (shared motifs of 29 and 32 nts within 250 nucleotides upstream from each ORF), 3 sequences within the ORFs (shared motifs of 8, 13, and 18 nts), and 3 sequences within the 3'-flanks (shared motifs of 18, 22, and 44 nts, all located within 1.8 kb downstream of the ORF) that were conserved among all 3 mRNAs. Future studies will be required to determine whether any of these sequences serve functions in glucose-regulated expression of these mRNAs.

Previously reported [8] comparative genomic hybridization experiments revealed several unanticipated changes in chromosomal and subchromosomal copy number between wild type and $\Delta lmg1$ parasites. In addition to the aforementioned amplification of a subregion of chromosome 29 encompassing 15 genes, the intact chromosomes 3, 16, 23, and 27 experienced an increase and chromosome 31 a decrease in copy number in the null mutants compared to wild type parasites, although the absolute changes in levels of individual chromosomes were small possibly representing loss or amplification of a single chromosome copy. These latter alterations in ploidy could also induce changes in the levels of mRNAs and proteins encoded within these aneuploid chromosomes, and it is possible that some of the modest changes in protein expression observed on two-dimensional gels could be explained by these genetic alterations. Nonetheless, the ability of the complemented *GT2* gene to reverse changes in expression of the 3 GRPs studied here, as well as the capacity of glucose limitation of wild

type parasites to induce similar changes to those seen in the null mutants, indicates that significant changes in protein expression are directly linked to the absence of glucose transporter activity.

Microarrays were employed to measure global differences in mRNA levels between wild type parasites and glucose transporter null mutants. Although a limited number of changes were detected using this approach, the microarray experiments are useful in two regards. First, they did detect several mRNAs that were significantly upregulated ($\log_2$ values of 1.03 – 2.59, Table 2) in the $\Delta lmg1$ null mutants, most notably those for SHMT, cyclin, and several hypothetical conserved proteins. Second, the global nature of microarray analysis implies that there is not a large body of mRNAs that experience increases of ~4-fold or greater in their mRNA levels in the null mutants. Although the microarrays are less sensitive in their ability to detect modest changes in expression compared to two-dimensional gels or qRT-PCR, they are much more comprehensive in their ability to interrogate the entire transcriptome for more robust changes in mRNA expression.

The observation of multiple differentially regulated spots that react with the anti-RK antibody on two-dimensional immunoblots (Fig. 5) suggests that these parasites may selectively modify various proteins under conditions of glucose limitation. A potentially large role for PTMs in differential function of gene products is raised by the diminished role that transcriptional regulation plays in *Leishmania* and related kinetoplastid protozoa [21] and the consequent need for other mechanisms to take over as modulators of gene function. Hence, a more comprehensive examination of differential PTMs in glucose-limited parasites is warranted.

In mammalian cells a family of GRPs, whose expression levels were strongly upregulated by glucose starvation, was identified many years ago [22]. These proteins are regulated as part of a more general mammalian stress response and include endoplasmic reticulum chaperones such as Grp78, also referred to as BiP [23]. Western blot analysis of lysates from wild type and $\Delta lmg1$ parasites, using an antibody directed against BiP from *Trypanosoma brucei* [24], revealed no enhancement of *L. mexicana* BiP expression in the null mutants (data not shown). Hence, BiP does not appear to be a GRP in *Leishmania* parasites.

It is appropriate to speculate about the possible reasons for regulation of protein expression in parasites exposed to limiting glucose. Upregulation of the 3 proteins that are the focus of this study could represent a compensatory response of the parasite to the consequences of glucose deprivation. One likely result of glucose limitation is a decrease in synthesis of ribose 5'-phosphate that is synthesized from glucose via the pentose phosphate pathway [25]. Ribokinase could provide an alternate route for synthesis of this metabolite via phosphorylation of the free sugar. Upregulation of hexokinase could represent a way for the parasite to utilize limiting glucose more efficiently by increasing the level of the first enzyme in the glycolytic pathway. We have shown previously that $\Delta lmg1$ null mutants are more sensitive than wild type parasites to oxidative stress, possibly due to decreased synthesis of NADPH via the pentose phosphate pathway [6]. In several mammalian systems, increased mALDH activity protected against oxidative stress [26], and decreased activity increased susceptibility to oxidative stress [27, 28]. Hence, increased expression of mALDH might protect *Leishmania* parasites against oxidative stress during glucose limitation. SMHT converts glycine to serine, a glucogenic amino acid (<http://themedicalbiochemistrypage.org/amino-acid-metabolism.html>). Hence, the observed enhancement in *SMHT* mRNA (Tables 1 and 2) could enable increased utilization of glycine for gluconeogenesis during glucose limitation. While these suggestions are speculative, they raise the possibility that a network of proteins regulated in response to glucose limitation could represent a strategy, on the part of this parasite, to accommodate inevitable fluctuations in nutrient availability and the metabolic and cellular consequences of such limitation. However, the reasons for altered expression of other proteins (e.g., the T-complex component,

Supplementary Table S1) or RNAs (e.g. two snRNAs, Table 2) in Δlmg null mutants are not clear.

In summary, the expression of multiple proteins is up- or downregulated by glucose limitation in *Leishmania* parasites. Detailed analysis of 3 characteristic GRPs revealed that significant regulation is exerted at the level of abundance of the cognate mRNAs. Differential post-translational modification also likely occurs in response to limited glucose availability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

qRT-PCR	quantitative real time polymerase chain reaction
PBS	phosphate buffered saline
IEF	isoelectric focusing
MS	mass spectrometry
mALDH	mitochondrial aldehyde dehydrogenase
RK	ribokinase
HK	hexokinase
D-LDH	D-lactate dehydrogenase
FBS	fetal bovine serum
ORF	open reading frame
LC	liquid chromatography
GRP	glucose-regulated protein
kDa	kilodaltons
PTM	post-translational modification
SHMT	serine hydroxymethyltransferase
UTR	untranslated region

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Fig. 1.

Two-dimensional gels from lysates of wild type (green) and $\Delta lmgf$ glucose transporter null mutants (magenta). A. Lysates were separated on non-linear isoelectric focusing gels in the first dimension and gradient SDS-polyacrylamide gels in the second dimension. Numbers at the left indicate the positions of protein molecular weight standards, designated in kDa; numbers at the bottom indicate the pH of the first dimension. The figure represents a composite electronic image of the 8 gels of wild type lysates and 8 gels of $\Delta lmgf$ glucose transporter null mutant lysates that were silver stained. B. Three regions of the gels, indicated by boxes I–III in part A, are presented as topographic images, where peak heights represent staining intensities. Images from the $\Delta lmgf$ glucose transporter null mutant are shown at the left and wild type images are at the right. Blue outlines around peaks represent individual spots, as determined by Phoretix 2D software, and the numbers in green indicate peaks of interest within each box whose height differed significantly between $\Delta lmgf$ and wild type parasites. The following proteins were identified by mass spectrometry in each spot: Box I: spot 1 – mALDH; Box II: spot 1 – D-LDH and RK, spot 2 – RK, spot 4 – mALDH; Box III: spot 1, nucleoside diphosphate kinase, spot 2 – mALDH, spot 3 – cyclophilin.

Fig. 2.

Quantification of expression of mALDH, ribokinase, and hexokinase in wild type and $\Delta lmgT$ null mutants with monospecific antibodies. A. Characterization of antisera by immunoblotting. The mALDH antibody (left panel) was used to probe an immunoblot of lysates from wild type parasites (mALDH) and lysates from parasites expressing a mALDH-GFP fusion protein from an episomal expression vector. Ribokinase (middle) and hexokinase (right) antibodies were employed to probe blots of lysates from $\Delta lmgT$ parasites. B. Relative expression levels of mALDH, ribokinase, and hexokinase were quantified in 1, wild type (WT); 2, $\Delta lmgT$ null mutants; and 3, $\Delta lmgT[pGT2]$ complemented null mutants by immunoblot analysis. Blots (top) were probed with each of the 3 antibodies and with anti- α -tubulin antibody as a normalization control, and the normalized values were plotted (bottom) setting the relative level of expression in wild type parasites to 1.0. Values represent the mean and standard deviation of n independent measurements (mALDH, $n = 4$; ribokinase, $n = 8$; hexokinase, $n = 6$). The statistical significance of the difference in protein expression compared to the $\Delta lmgT$ null mutant was determined using Student's t -test (**, $p < 0.01$; ***, $p < 0.001$).

Fig. 3.

Quantification of induction of mALDH (A), ribokinase (B), and hexokinase (C) in wild type parasites cultured in glucose-replete (11 mM glucose, 10% FBS) or glucose-limited (no added glucose, 10% FBS) RPMI medium. Immunoblots were probed with each antibody and with anti- α -tubulin antibody, the signals were normalized to that of α -tubulin, and the relative levels were plotted. The amount of each protein present at day 0 was set to a value of 1.0. Data represent mean and standard deviation of n independent measurements (mALDH, $n = 4$; ribokinase, $n = 4$; hexokinase, $n = 3$).

Fig. 4.

Relative rates of synthesis of mALDH were determined in wild type (WT) and $\Delta lmg1$ null mutants by pulse labeling followed by immunoprecipitation. mALDH is the band at ~55 kDa (arrow), and the doublet between ~36–40 kDa represents non-specifically precipitated proteins that serve as controls for synthesis and loading. Numbers at the left represent protein molecular weight markers in kDa.

Fig. 5. Immunoblots of two-dimensional gels probed with anti-ribokinase antibody. Lysates were prepared from wild type, $\Delta lmg1$, and $\Delta lmg1[pGT2]$ parasites (indicated at the right). The position of a 35 kDa molecular weight marker is indicated at the left, and the approximate pH of the isoelectric focusing dimension is indicated by the numbers at the top. These results were reproduced in 3 independent experiments.

Table 1

Quantification of relative mRNA levels (average (Av) $\pm$ standard deviation (SD)) by qRT-PCR for various genes in Δlmg t null mutants compared to wild type (WT) *L. mexicana* promastigotes. Four biological replicates of RNA were prepared from WT and Δlmg t parasites (Rep 1–4). Numbers in the penultimate column represent the average and standard deviations from Reps 1–4. Systematic ID refers to the gene designation in the *L. major* genome database (www.genedb.org). For analysis of GP63 mRNAs, one primer set (GP63*) represents sequences that are identical in all 5 alleles present in the GP63 gene cluster on chromosome 10 of *L. mexicana* (LmxM 10.0390, LmxM 10.0405, LmxM 10.0460, LmxM 10.0465, LmxM 10.0470, which correspond to the LmjF genes in the chromosome 10 cluster shown in the table), and the second primer set (GP63**) represents sequences unique to a GP63 gene on chromosome 28 (LmxM28.0570). To determine p values for multiple qRT-PCR measurements of a particular mRNA, ΔC_t values (C_t , threshold cycle, of reference rRNA gene minus C_t of target gene) were calculated for each of the 4 qRT-PCR measurements for both WT and Δlmg t RNAs. These 4 ΔC_t values were then analyzed using an unpaired Student's *t*-test. 'ns' means not significant.

Gene	Systematic ID	Av $\pm$ SD Reps 1–4 Δlmg t/WT	p Values
mALDH	LmjF25.1120	4.1 $\pm$ 0.5	p<0.001
Ribokinase	LmjF27.0420	2.2 $\pm$ 0.8	p<0.01
Hexokinase	LmjF21.0240 LmjF21.0250	1.7 $\pm$ 0.6	p<0.05
Glucokinase	LmjF36.2320	1.1 $\pm$ 0.1	ns
Phosphofructokinase	LmjF29.2510	1.0 $\pm$ 0.1	ns
Pyruvate kinase	LmjF35.0020 LmjF35.0030	−1.5 $\pm$ 0.4	ns
Fructose 1,6-biphosphatase	LmjF04.1160	1.3 $\pm$ 0.1	ns
PEPCK	LmjF27.1810	1.3 $\pm$ 0.5	ns
PPDK	LmjF11.1000	2.0 $\pm$ 0.3	p<0.01
Serine hydroxymethyltransferase	LmjF14.1320	7.7 $\pm$ 3.0	p<0.01
Hypothetical protein – Chr 29	LmjF29.2800	4.5 $\pm$ 2.4	p<0.05
Hypothetical protein – Chr 29	LmjF29.2870	8.9 $\pm$ 3.0	p<0.001
GP63*(Chr10 cluster)	LmjF10.0460 LmjF10.0465 LmjF10.0470 LmjF10.0480	−3.7 $\pm$ 1.3	p<0.05
GP63** (LmxM28.0570)	LmjF28.0570	−1.5 $\pm$ 0.5	p<0.05

Table 2

Results of microarray experiments employing RNA from wild type (WT) and Δlmg t null mutants. Genes are shown in which the average Δlmg t/WT signal intensity was upregulated (A) or downregulated (B) by 2-fold ($\log_2$ of 1.0) or more in at least 3 out of 4 replicates performed with the same 4 distinct biological preparations of RNA employed in Table 1. $\log_2$ values are reported as the average (Av) $\pm$ standard deviation (SD) for the 4 replicates. Name/ID refers to the oligonucleotide employed in hybridization for each gene, Systematic ID is the GeneDB (www.genedb.org) designation for the *L. major* gene corresponding to the oligonucleotide probe, and the Annotation refers to the gene function or identity as catalogued in TriTrypDB (tritrypdb.org) version 2.2.

A) Up by 2-fold or more in Δlmg t/WT in 3 out of 4 replicates			
Name/ID	Systematic ID	Annotation TriTrypDB v1.3	($\log_2$ values) Av $\pm$ SD Reps 1–4 Δlmg t/WT
LMFV1.359	LmjF14.1320	serine hydroxymethyltransferase, putative (cytosolic)	2.59 $\pm$ 0.90
LMFV1.9362	LmjF23.snRNA.02	small nuclear RNA, U3 snRNA	1.79 $\pm$ 0.96
LMFV1.1230	LmjF25.1470	cyclin	1.92 $\pm$ 0.96
LMFV1.5560	LmjF27.0800	hypothetical protein, conserved	1.03 $\pm$ 0.11
LMFV1.429	LmjF29.1210	hypothetical protein, conserved	1.33 $\pm$ 0.35
LMFV1.4979	LmjF32.0240	hypothetical protein, conserved	1.18 $\pm$ 0.05
LMFV1.9696	LmjF33.2360	hypothetical protein, conserved	2.29 $\pm$ 0.13
LMFV1.3951	LmjF34.2780	40S ribosomal protein S19 protein, putative	1.44 $\pm$ 0.18
LMFV1.9804	LmjF35.0680	hypothetical protein, conserved	1.52 $\pm$ 0.41
LMFV1.6558	LmjF36.5760	hypothetical protein, conserved	1.15 $\pm$ 0.41
LMFV1.10977	LmjF36.snRNA.01	small nuclear RNA, U4 snRNA	1.40 $\pm$ 0.35
Genes on Chromosome 29 Amplicon			
LMFV1.10828	LmjF29.2740	actin-like protein, putative	1.49 $\pm$ 0.44
LMFV1.10276	LmjF29.2760	hypothetical protein, conserved	1.53 $\pm$ 0.35
LMFV1.11311	LmjF29.2770	hypothetical protein, conserved	1.56 $\pm$ 0.32
LMFV1.10575	LmjF29.2780	mitochondrial carrier-like protein	1.21 $\pm$ 0.17
LMFV1.9373	LmjF29.2800	inosine-adenosine-guanosine-nucleoside hydrolase, putative	1.22 $\pm$ 0.34
LMFV1.11158	LmjF29.2820	hypothetical protein, conserved	1.24 $\pm$ 0.26
LMFV1.9852	LmjF29.2830	hypothetical protein, conserved	1.25 $\pm$ 0.07
LMFV1.10563	LmjF29.2840	hypothetical protein, conserved	1.88 $\pm$ 0.22
LMFV1.9954	LmjF29.2850	hypothetical protein, conserved	2.81 $\pm$ 0.27
LMFV1.10159	LmjF29.2860	40S ribosomal protein S19-like protein	1.59 $\pm$ 0.25
LMFV1.10869	LmjF29.2870	hypothetical protein, conserved	2.66 $\pm$ 0.37
B) Down by 2-fold or more in Δlmg t/WT			
LMFV1.6722	LmjF10.0480	GP63, leishmanolysin	-1.10 $\pm$ 0.23
LMFV1.6792	LmjF10.0480	GP63, leishmanolysin	-1.12 $\pm$ 0.22

A) Up by 2-fold or more in Δlmg /WT in 3 out of 4 replicates			
Name/ID	Systematic ID	Annotation TriTrypDB v1.3	(log ₂ values) Av $\pm$ SD Reps 1–4 Δlmg /WT
LMFV1.6821	LmjF10.0480	GP63, leishmanolysin	-1.07 ± 0.21
LMFV1.8603	LmjF35.snoRNA.0049	LM35Cs4H1	-0.97 ± 0.15
LMFV1.6628	LmjF36.6280, LmjF36.6290, LmjF36.6300	LmjGT1, LmjGT2, LmjGT3 glucose transporters	-3.14 ± 0.30

Table 3

Comparison of increases in levels of proteins, measured by immunoblots, and/or mRNAs, quantified by qRT-PCR and microarray analysis. Data were assembled from Tables 1, 2, and Supplementary Table S1 and are reported as the average $\pm$ standard deviation. ND in the immunoblot column indicates 'not determined' since no antibodies are available against these proteins. NCD in the microarray column designates 'no change detected'.

Protein Name	Systematic ID	Protein Increase Immunoblot	mRNA Increase qRT-PCR	mRNA Increase Microarray
mALDH	LmjF25.1120	7.4 ± 3.3	4.1 ± 0.5	NCD
Ribokinase	Lmj27.0420	6.7 ± 3.3	2.2 ± 0.8	NCD
Hexokinase	LmjF21.0240 LmjF21.0250	2.0 ± 0.4	1.7 ± 0.6	NCD
Serine hydroxymethyltransferase (cytosolic isoform)	LmjF14.1320	ND	7.7 ± 3.0	6.8 ± 3.5
Hypothetical protein – Chr 29	LmjF29.2800	ND	4.5 ± 2.4	2.4 ± 0.54
Hypothetical protein – Chr 29	LmjF29.2870	ND	8.9 ± 3.0	6.5 ± 1.6