1 *Leishmania major* phosphoglycerate kinase transcript and protein stability contributes to differences
2 in isoform expression levels

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16 **ABSTRACT**

17

18 *Leishmania* contains two phosphoglycerate kinase (PGK) genes, *PGKB* and *PGKC*, which code for
19 the cytosolic and glycosomal isoforms of the enzyme, respectively. Although differences in PGKB
20 and PGKC transcript and protein levels and isoform activities have been well documented, the
21 mechanisms of control of both transcript and protein abundance have not been described to date. To
22 better understand the regulation of *Leishmania* PGK expression, we investigated the stabilities of
23 both PGK transcripts using reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)
24 in combination with transcription and *trans*-splicing inhibitors. Cells were treated with sinefungin
25 and actinomycin D, and RNA decay kinetics were assessed. In addition, immunoblotting and
26 protein synthesis inhibition by cycloheximide were employed to evaluate protein steady states and
27 degradation. We observed increased stabilities of both PGKB mRNA and protein compared with
28 the glycosomal isoform (PGKC). Our results indicate that both post-transcriptional and post-
29 translational events contribute to the distinct expression levels of the PGKB and PGKC isoforms in
30 *L. major*.

31

32 **Keywords:** *Leishmania*; phosphoglycerate kinase; untranslated regions; gene expression, glycosome
33 turnover.

34 1. Introduction

35

36 Organisms of the genus *Leishmania* are heteroxenous protozoan parasites from the
37 Trypanosomatidae family. Twenty species of this genus are important human pathogens (Ashford,
38 2000) because they cause leishmaniasis, a spectral disease with several clinical manifestations that
39 is widespread in Central and South America, Africa, Asia and the Mediterranean. Approximately 12
40 million people worldwide are currently infected with this parasite (Kedzierski, et al., 2009). In
41 addition to their relevance to human health, *Leishmania* are good model organisms given their
42 position in the evolutionary tree. Trypanosomatids undergo several molecular processes that are
43 unusual for eukaryotes, such as *trans*-splicing, wherein the RNA splicing mechanism engages exons
44 from different genomic origins (Walder, et al., 1986). Transcription occurs in long polycistronic
45 units (Johnson, et al., 1987, Martínez-Calvillo, et al., 2003). Accumulating data indicate that
46 sequences present in the 3'-untranslated regions (3'-UTRs) of trypanosomatid mRNAs are key
47 elements in the control of gene expression. The 3'-UTRs of mRNAs are involved in regulatory
48 mechanisms either by modulating mRNA stability or translation efficiency (Clayton and Shapira,
49 2007, Haanstra, et al., 2008). Additionally, in trypanosomatids, various metabolic pathways are
50 uniquely compartmentalized in organelles, such as glycosomes, which are peroxisome-like
51 organelles that contain most enzymes of the glycolytic pathways (Opperdoes and Borst, 1977).

52 In *Trypanosoma brucei*, phosphoglycerate kinase (PGK) is an enzyme involved in the
53 glycolytic pathway for which the following three isoforms have been reported: PGKA, which codes
54 for a variant glycosomal form that is expressed throughout the parasite life cycle, but only at very
55 low levels (Alexander and Parsons, 1991); PGKB, which codes for a cytosolic enzyme that is
56 detected exclusively during the insect stage (procyclics); and PGKC, which codes for an enzyme
57 that is compartmentalized in the glycosome and is expressed exclusively during the mammalian
58 stage (bloodstream forms) (Blattner and Clayton, 1995). The strict control of PGK expression

59 during the life cycle of parasites is essential for their survival because bloodstream trypanosomes
60 are killed following the expression of cytosolic phosphoglycerate kinase (PGKB) (Blattner, et al.,
61 1998).

62 In contrast to *T. brucei*, *Leishmania* multiplies intracellularly as amastigotes within the
63 phagolysosomes of macrophages in mammalian hosts. All *Leishmania* species investigated to date
64 contain orthologs of the *PGKB* and *PGKC* genes, which code for proteins that are located in the
65 cytosol and glycosome, respectively. During mammalian (amastigote) and insect (promastigote)
66 parasite stages, both PGKB and PGKC transcripts and proteins are expressed at a ratio of
67 approximately 4:1 (Adjé, et al., 1997, McKoy, et al., 1997). In fact, these differences may result in
68 divergent control mechanisms governing the glycolytic pathway in these trypanosomatids.
69 Therefore, we investigated the level at which the control of PGKB and PGKC abundance occurs in
70 *L. major*.

71

72 **2. Materials and methods**

73

74 **2.1. *Leishmania* strain and culture conditions**

75 The *L. major* LT252 (MHOM/IR/83/LT252) CC1 clonal line used in this study was
76 previously described (Beverley, 1988). Promastigotes were cultured at 26°C in M199 medium
77 (Gibco BRL, NY, USA), which was supplemented as recommended (Kapler, et al., 1990).

78

79 **2.2. Characterization of PGK transcripts**

80 The PGK locus sequence is available at TriTrypDB (<http://tritrypdb.org/tritrypdb/>). Using
81 RNASeq data that were also available at TriTrypDB, we determined the boundaries of the mature
82 PGKB (LmjF.10.0110) and PGKC (LmjF.10.0100) transcripts. Based on the PGKB 5'-UTR, CDS,
83 and 3'-UTR sequence sizes (169, 1254, and 743 bp, respectively), the PGKB transcript is at least

2166 bp (excluding the poly-A tail). Similarly, considering the PGKC 5'-UTR, CDS, and 3'-UTR sequence sizes (238, 1440, and 1628 bp, respectively), the PGKC transcript is at least 3306 bp. Both predicted transcript sizes are compatible with those that have been previously determined (McKoy, et al., 1997).

88

2.3. RNA extraction and reverse transcriptase-quantitative polymerase chain reaction

For RNA stability assays, we added 2 µg/mL sinefungin (Sigma-Aldrich, MO, USA) to the promastigote log phase culture 10 minutes before the addition of 10 µg/mL actinomycin D (ActD; Sigma-Aldrich) to inhibit *trans*-splicing and mRNA synthesis, respectively (Wilson, et al., 1993). Total RNA was then isolated at different time points, and RNA decay was analyzed by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR).

Total RNA was extracted from axenic promastigotes with TRIZOL (Life Technologies, CA, USA) according to the manufacturer's instructions. RNA concentration and purity were determined by measuring absorbance at A260 and A280, and the samples were stored at -70°C. RNA samples (2 µg) were treated with 2 U of DNase I (Life Technologies). The reaction was incubated at 25°C for 15 minutes and stopped by incubation with 1 µL of 25 mM EDTA at 65°C for 10 minutes. cDNA synthesis was performed with 2 µg of RNA in a total volume of 20 µL using random primers under the reaction conditions recommended by the High Capacity cDNA Reverse Transcription Kit instructions (Applied Biosystems, CA, USA). The reaction was incubated at 25°C for 10 minutes and at 37°C for 2 hours and stopped by incubation at 55°C for 5 min.

Quantitative PCR was performed (ABI Prism 7500 DNA Sequence Detection System) using SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, CA, USA) with 4 pmol of each primer specifically designed for quantitative PCR and cDNA diluted 1:100. Because the PGKB and PGKC coding sequences are highly conserved, primer pairs for PGKB (forward: TTATGCCAGTCCCACGGTAT, and reverse: CCACAGGTAAGGAGGCGATA) and PGKC

109 (forward: TCGCCTCGTTTTTGGTATTC, and reverse: CATCCTCCCATCTCATTGCT) were
 110 designed to amplify divergent sequences at the 3'-UTRs of each transcript. Control G6PD primers
 111 (forward: ACCGCATTGACCACTACCTC and reverse: GATGTTGTTCGAGTTCCAC) were
 112 obtained from the coding DNA sequence of the gene (Accession number XM_001686045). The
 113 samples were incubated for 2 minutes at 50°C and 10 minutes at 95°C followed by 40 cycles at 95°C
 114 for 15 seconds and 60°C for 1 minute. The amplification efficiency for each primer pair was
 115 evaluated at a 1:10 serial dilution and was adequate for RT-qPCR analysis. Data regarding primer
 116 characteristics, amplification efficiency, and product length and GC content are presented (Table 1).
 117 To evaluate PGKB and PGKC transcript levels, three replicate analyses were performed, and the
 118 amount of target RNA was normalized to glucose 6-phosphate dehydrogenase (G6PD) RNA. To
 119 determine PGKB and PGKC transcript half-lives, quantification cycles (Ct) for each transcript were
 120 compared to the respective Ct of untreated cells at the presented time points after
 121 actinomycin/sinefungin treatment. Data were expressed according to the $2^{-\Delta\Delta C_t}$ method using the
 122 mean value of the ΔC_t of the control group as the calibrator (CC1 at 0 hours with actinomycin D).
 123 The mRNA half-life ($t_{1/2}$) was calculated according to the equation $(t_{1/2}) = \ln(2)/k_{\text{decay}}$, where k_{decay} is
 124 the mRNA decay constant (Chen, et al., 2008). The rate constant for mRNA decay is the slope of a
 125 semi-logarithmic plot of the mRNA concentration as a function of time and was determined by
 126 nonlinear regression (least-square) analysis of three biological replicate samples fitted using the
 127 first-order decay model (GraphPad Prism 6.0).

128

129 2.4. Protein extraction and decay analyses

130 Mid-log phase promastigote cultures (6.0 to 8.0×10^6 promastigotes/mL) from CC1 cells
 131 were treated with cycloheximide ($50 \mu\text{g/mL}$; Sigma-Aldrich, USA) to inhibit translation (Carrillo,
 132 et al., 2000). The cultures were collected at the following time points: 1, 2, 3 and 4 hours. In
 133 addition, prior to drug treatment (time 0), the cultures were collected to evaluate cytosolic and

glycosomal protein levels. The cell pellet was harvested by centrifugation at 2000 x g for 10 minutes at 4°C, and a lysis solution containing 1 mM Tris, 0.1% sodium dodecyl sulfate (SDS) and Protease Inhibitor Cocktail (Roche, IN, USA) was added. The samples were subjected to five freeze-thaw cycles. The lysate was treated with 2 mg/mL DNase, 1 mg/mL RNase and 100 mM MgCl₂. Proteins were collected from the supernatant fraction by centrifugation at 12000 x g for 1 minute at room temperature. The proteins were quantified, and 10 µg of protein was loaded into a 10% polyacrylamide gel for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

142

2.5. Immunoblotting

Total protein extracts from the *Leishmania* strain (CC1) under analysis were size-fractionated by SDS-PAGE (Sambrook, et al., 1989) and transferred to a nitrocellulose membrane as previously described (Harlow and Lane, 1999). After electroblotting, the nitrocellulose membrane was blocked by incubation in TBS-T buffer (20 mM Tris-HCl, pH 7.6; 137 mM NaCl; 0.1% Tween 20 containing 5% non-fat milk). The blots were incubated with a rabbit polyclonal anti-recombinant *Trypanosoma brucei* PGK antibody (1:10,000) purified from *Escherichia coli* (kindly provided by Paul Michels, Institute of Structural and Molecular Biology, School of Biological Sciences, University of Edinburgh, UK). This antibody recognizes both the PGKB and PGKC isoforms with similar avidity because their amino acid sequences are 99% identical, except for a C-terminal extension of 62 residues in PGKC (Adjé, et al., 1997, McKoy, et al., 1997). The blots were then incubated with an anti-rabbit secondary antibody coupled with peroxidase at a dilution of 1:5,000. The membrane was stained with Ponceau S solution (Sigma-Aldrich) and used as a control for protein transfer as well as loading. The resulting bands were quantified with ImageJ software based on pixel intensity. Decay curves were plotted against time, and nonlinear regression (least-square) analysis of two biological replicate samples was fitted using first-order decay kinetics

159 (GraphPad Prism 6) to determine the rate of decay. The half-lives were estimated using the equation
160 $(t_{1/2}) = \ln(2)/k_{\text{decay}}$.

161

162 **3. Results**

163

164 3.1 *PGKB* and *PGKC* transcript levels and stability

165 *PGKC* and *PGKB* transcript levels in *L. major* promastigotes were determined by RT-qPCR.
166 Relative quantification revealed that the *PGKB* transcript was approximately three-fold more
167 abundant than the *PGKC* transcript (Fig. 1A).

168 Subsequently, we investigated *PGKB* and *PGKC* stability by determining the transcript half-
169 lives in cells treated with transcription (actinomycin D) and *trans*-splicing (sinefungin) inhibitors.
170 Analysis of the relative quantification data (Fig. 1B and 1C) revealed that the *PGKC* and *PGKB*
171 transcript half-lives were approximately 108 minutes and 212 minutes, respectively. The data
172 indicated that the *PGKC* transcript was less stable than the *PGKB* transcript.

173

174 3.2 Protein levels and stabilities of PGKB and PGKC isoforms

175 Next, we investigated PGK isoform levels in the glycosome and cytosol. To estimate the
176 levels of each PGK isoform as well as the decay kinetics of the enzymes, immunoblotting
177 experiments were conducted with an anti-PGK polyclonal antibody from *T. brucei*. Total protein
178 was extracted from the *Leishmania* CC1 line. We observed bands with the expected molecular
179 weights (51 kDa for PGKC and 45 kDa for PGKB, as previously described). The levels of the
180 PGKC and PGKB-proteins were compatible with the expected 1:4 ratio in *L. major* (Fig. 2).

181 To investigate the potential protection of compartmentalized proteins from proteolytic
182 pathways, we measured the decay kinetics of PGK proteins in the cytosolic and glycosomal
183 fractions. Promastigotes were treated with cycloheximide to inhibit translation elongation, and the

184 kinetics of protein decay were evaluated in two independent experiments. Cytosolic and glycosomal
185 proteins were fractionated by SDS-PAGE, and an anti-PGK polyclonal antibody was used to assess
186 protein stability. In addition to differences in the protein levels of each isoform at “time 0”
187 (PGKB/PGKC ratio 4:1), the glycosomal and cytosolic isoforms exhibited different rates of
188 degradation after cycloheximide treatment (Fig. 2A; 1-4 hour time points). Quantitative analysis
189 permitted the estimation of the PGK half-lives; at approximately 104 minutes for PGKC and 296
190 minutes for PGKB, these isoforms had reached 50% of the original amounts (Fig. 2B).

191

192 **4. Discussion**

193 Our research on the regulation of cytosolic (PGKB) and glycosomal (PGKC)
194 phosphoglycerate kinase gene expression in *Leishmania* suggests that elements that are unique to
195 the PGKC locus are responsible for the regulation of expression at the posttranscriptional level via
196 multiple mechanisms. In the present study, the *PGKC* transcript level was found to be ~3-fold
197 reduced compared with the *PGKB* transcript, which is consistent with previous data reported by
198 other groups (Adjé, et al., 1997, McKoy, et al., 1997). Additionally, we demonstrated that the
199 stability of the *PGKC* transcript was reduced (~108 minute half-life) compared with the *PGKB*
200 transcript (~212 minute half-life). A PGKC 3'-UTR cis-element appears to be involved in PGKC
201 transcript decay in procyclic forms of *T. brucei*, as demonstrated by gene reporter experiments
202 (Colasante, et al., 2007). In addition, we searched *in silico* for conserved *cis*-elements shared by the
203 glycosomal protein genes of *L. major* and found nothing but short (10 nucleotides long) repeat
204 elements (such as GCGCs or CACAs).

205 The degradation kinetics of the glycosomal enzyme (PGKC) were faster compared with
206 PGKB and correlated well with the transcript degradation profile. The mechanism of protein
207 degradation may differ between the isoforms. As a cytosolic protein, PGKB is probably degraded
208 after its ubiquitination via bulk autophagy of the cytosol. In contrast, PGKC degradation may be

209 associated with glycosomal turnover. Indeed, it has been recently shown in *L. major* that glycosome
210 turnover occurs via autophagy, which is upregulated at different life cycle stages (Cull, et al., 2014)
211 and is not restricted to the differentiation period, as has been previously demonstrated for *T. brucei*
212 glycosomes (Herman, et al., 2008).

213 These differences may contribute to the distinct PGKC half-lives in *L. major* (104 minutes)
214 and in *T. brucei* bloodstream forms, in which no PGKC degradation was observed over an eight-
215 hour chase period in [³⁵S]methionine pulse-chase experiments (Haanstra, et al., 2008). Similar
216 results were previously obtained for aldolase in *T. brucei* procyclic forms (Clayton, 1988). In
217 support of the hypothesis that PGKC degradation in *L. major* is linked to glycosome turnover,
218 rather than an isolated phenomenon or related to degradation of an individual glycosomal matrix
219 protein, we found comparable half-life values for PGKC (104 minutes) and another glycosomal
220 enzyme, glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 105.5 minutes, Supp. Fig. 1), in *L.*
221 *major*. Although different approaches to analyze protein stability were utilized, it is unlikely that
222 cycloheximide would be the cause of the differences observed compared to *T. brucei*. In fact, pulse-
223 chase and cycloheximide treatment provided similar results in terms of decay for the NT1
224 adenosine/pyrimidine nucleotide transporter protein in *L. major* (Ortiz, et al., 2010). The differences
225 between *Leishmania* and *T. brucei* glycolytic enzyme stability suggest that glycosome turnover
226 control differs in these parasites, and further investigation is required to fully understand the
227 mechanisms.

228 Therefore, it is likely that the mRNA stability and translation initiation rates in this case are
229 influenced by elements present in the 5'-UTR and/or the 3'-UTR. Although it is improbable, we
230 cannot exclude the participation of the nucleotide sequence coding for the 62-amino acid extension
231 at the C-terminus of PGKC, which targets the protein to the glycosome according to previous
232 reports. Most likely, *PGKB* and *PGKC* divergent sequences within the untranslated regions are
233 involved in the control of transcript stability.

234 Accumulating evidence indicates that the regulation of gene expression in trypanosomatids
235 is determined at the posttranscriptional level, and several studies have demonstrated that 3'-UTRs
236 play major roles in the control of mRNA stability and the protein translation rate (Abanades, et al.,
237 2009, Blattner and Clayton, 1995, De Gaudenzi, et al., 2013, Pérez-Díaz, et al., 2013, Suganuma, et
238 al., 2012). Fewer reports are available that indicate that 5'-UTR elements and in some cases
239 intergenic regions (IR) also play roles in posttranscriptional mechanisms of gene expression
240 regulation (Jaeger and Brandao, 2011, Mahmood and Ray, 1998).

241 In conclusion, we have demonstrated that PGKB and PGKC protein levels are maintained as
242 a result of multiple levels of control, similar to previous reports (Clayton and Hotz, 1996, Folgueira,
243 et al., 2007, McNicoll, et al., 2005). We postulate that RNA stability is influenced by regulatory *cis*-
244 elements and that these control elements are involved in the maintenance of reduced PGKC levels
245 compared with PGKB throughout the life cycle of these parasites.

246

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1 **Figure legends**

2 **Fig. 1: Relative quantification and determination of the transcript half-lives of *PGKB* and**

3 ***PGKC*.** (A) Total RNA was obtained from *L. major* promastigotes, and the levels of *PGKB* and
4 *PGKC* transcripts were quantified by RT-qPCR using the $2^{-\Delta\Delta C_t}$ method. The values correspond to
5 the mean $\pm$ s.d. of three separate experiments performed in triplicate. (B) and (C) *L. major*
6 promastigotes were treated with 2 μ g/mL sinefungin and actinomycin D (see Materials and
7 Methods for details), and samples were removed at the indicated times. Total RNA was extracted,
8 and *PGKB* and *PGKC* transcript levels were quantified by RT-qPCR using the 2^{-ddC_t} method. The
9 values correspond to the mean $\pm$ s.d. of three separate experiments performed in triplicate. mRNA
10 decay curves were plotted against time, and nonlinear regression (least-square) was fitted using
11 first-order decay kinetics (GraphPad Prism 6) to determine the rate of decay (k_{decay}). The half-lives
12 were estimated using the equation $(t_{1/2}) = \ln(2)/k_{\text{decay}}$. The *PGKB* and *PGKC* transcript half-lives
13 were approximately 212 and 108 minutes, respectively.

15 **Fig. 2: Analysis of PGK isoform half-lives.** Wild-type promastigotes were treated with

16 cycloheximide. (A) Protein fractions were collected at 0, 1, 2, 3 and 4 hours, fractionated by SDS-
17 PAGE and transferred to a membrane. The membrane was incubated with an anti-PGK primary
18 antibody. The lower panel depicts the Ponceau S-stained membrane, which was used as a control
19 for protein transfer and integrity. One of the proteins from the ladder is shown on the right side of
20 the panel in kDa. (B) *PGKB* and *PGKC* decay rates as determined by two independent experiments.
21 The data were plotted against time, and nonlinear regression (least-square) was fitted using first-
22 order decay kinetics (GraphPad Prism 6) to determine the rate of decay. The *PGKB* and *PGKC*
23 protein half-lives were estimated as 296 and 104 minutes, respectively. These values were estimated
24 using the equation $(t_{1/2}) = \ln(2)/k_{\text{decay}}$.

Figure 1

Figure 2

1 Table 1. PCR primers and product features and amplification efficiency for G6PD, PGKC and
2 PGKB.

Target (region) ^a	Primer Tm (°C)		Amplification efficiency (%) ^b	Product size (bp)	Product GC content (%)
	Forward	Reverse			
G6PD (CDS)	60	54	96	101	53,5
PGKB (3'-UTR)	60	60	97	113	57,5
PGKC (3'-UTR)	60	60	91	122	52,5

3 ^aRegion amplified of the target gene (CDS: coding DNA sequence; 3'-UTR: 3'-untranslated region).
4 G6PD, glucose-6-phosphate dehydrogenase; PGKB, cytosolic phosphoglycerate kinase; PGKC,
5 glycosomal phosphoglycerate kinase.

6 ^bAmplification efficiencies were determined from the slope of standard curves. G6PD (slope = -
7 3.41); PGKB (slope = -3.39); PGKC (slope = -3.57).

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