An Insight into the Constitutive Proteome Throughout *Leishmania donovani* Promastigote Growth and Differentiation

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Abstract

Anthroponotic visceral leishmaniasis is a life-threatening disease caused by *Leishmania donovani* (Kinetoplastida: Trypanosomatidae) in East Africa and the Indian subcontinent. Unlike promastigote growth and differentiation in the sand fly gut or in axenic culture, *L. donovani* promastigote-into-amastigote development has been studied by high-throughput gene expression profiling. In this study, we have identified abundant constitutive proteins in axenically cultured promastigotes by two-dimension electrophoresis and matrix-assisted laser desorption-ionization tandem time-of-flight (MALDI-TOF/TOF) mass spectrometry. Most proteins involved in the trypanothione-based redox antioxidant system are expressed constitutively throughout axenic *L. donovani* promastigote growth and differentiation (tryparedoxin, trypanothione peroxidase, generic peroxidoxin, iron-superoxide dismutase, and elongation factor 1 β). These findings are in agreement with previous data on other Old World species (i.e., *L. major* and *L. infantum*), whereas New World species (i.e., *L. amazonensis* and *L. pifanoi*) and *Crithidia fasciculata* show different expression patterns.

Keywords *Leishmania donovani* · Constitutive proteins · 2DE · MALDI-TOF/TOF · Elongation factor 1 β · Peroxiredoxins · Tryparedoxin peroxidase · Iron superoxide dismutase

Introduction

Visceral leishmaniasis (VL) or Kala-azar is a life-threatening disease caused by parasites from the genus *Leishmania*

(Kinetoplastida: Trypanosomatidae). *L. donovani* is the etiological agent of anthroponotic visceral leishmaniasis (AVL), which is restricted to East Africa and the Indian subcontinent, where death rates are high (Desjeux 2001; WHO 2010). Post-

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Kala-azar dermal leishmaniasis (PKDL) is an AVL sequela which contributes to disease dissemination (Desjeux 2001).

In the *Leishmania* spp. life cycle, two stages alternate in two different hosts. Extracellular promastigotes undergo a differentiation process in the sand fly vector gut (Psychodidae: Phlebotominae). As a result, they become more infective. This process is mimicked routinely in axenic culture. When the sand fly feeds from blood of a mammalian host, promastigotes are injected in the dermis. Once internalized by phagocytic cells, they differentiate into non-motile amastigotes. The main life cycle stages of several Old World and New World species causing cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), or VL have been compared using high-throughput gene expression analysis (e.g., Akopyants et al. 2004; Alcolea et al. 2010; Holzer et al. 2006; Magalhaes et al. 2014; Saxena et al. 2007). Most comparisons are focused on promastigote-into-amastigote development. Regarding *L. donovani*, high-throughput transcriptome and proteome analysis of promastigote-into-amastigote differentiation have been performed (Depledge et al. 2009; Rosenzweig et al. 2008; Saxena et al. 2007; Srividya et al. 2007). However, gene expression profiles throughout promastigote growth and differentiation in culture mimicking development within the sand fly gut have not been studied yet. With the aim of finding abundant constitutive proteins, we have screened the axenic *L. donovani* promastigote proteome by two-dimension electrophoresis analysis (2DE). These proteins have been identified by matrix-assisted laser desorption-ionization tandem time-of-flight mass spectrometry (MALDI-TOF/TOF).

Trypanothione is a trypanosomatid-specific molecule which consists of two glutathione residues coupled by spermidine. Trypanosomatid genomes also retain the ubiquitous glutathione-based antioxidant system (e.g., LdBPK_140970.1, glutathione synthetase, putative; LdBPK_260780.1, glutathione peroxidase-like protein, putative; LdBPK_363160.1, glutathione peroxidase, putative; and LdBPK_141580.1, glutathione-S-transferase/glutaredoxin, putative). Indeed, these genes have been annotated in the respective genome sequences (GeneDB, TriTrypDB) and their expression has been experimentally confirmed (Lahav et al. 2011; Rastrojo et al. 2013; Rochette et al. 2009; Tsigankov et al. 2013). This study reveals that most trypanothione-based antioxidant system proteins are abundant and constitutive during *L. donovani* promastigote growth and development, which is discussed herein, including comparison with *L. infantum*, a related species belonging to the VL-causing “*L. donovani* complex.”

Materials and methods

L. donovani promastigote axenic culture

The *L. donovani* Karthoum isolate MHOM/SD/43/124 used in this study was kindly provided by A. Toraño and M.

Dominguez (Service of Immunology, Centro Nacional de Microbiología, Virología e Inmunología Sanitarias, Instituto de Salud Carlos III, Majadahonda, Spain). Promastigotes were cultured at 27 °C in RPMI 1640 supplemented with L-glutamine (Life Technologies, Carlsbad, CA), 10% heat-inactivated fetal bovine serum (Lonza, Basel, Switzerland), and 100 µg/ml streptomycin – 100 IU/ml penicillin (Life Technologies), pH 7.2. The initial cell density was 2×10^6 /ml. Growth was registered daily using a Neubauer chamber at the light microscope, and 10^8 promastigote samples were harvested at 2000 g for 10 min.

Total protein extraction

The samples were washed in PBS and incubated at 4 °C under mild rotation for 30 min in 150 µl lysis buffer (8.4 M urea, 2.4 M thiourea, 5% CHAPS, 50 mM DTT, 1% Triton X-100, 50 µg/ml DNase, and Mini EDTA-free Protease Inhibitor Cocktail according to the manufacturer’s instructions—Roche, Mannheim, Germany). The lysate was immediately centrifuged at 8000g for 10 min and the supernatant was recovered. Protein precipitation was carried out with methanol/chloroform (Wessel and Flugge 1984). Finally, the samples were dried at room temperature for 5 min and resuspended in 2× rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.003% bromophenol blue). Protein concentration was measured using RC DC Protein Assay Kit (BioRad) following the manufacturer’s recommendations and by SDS-PAGE.

2DE protein analysis

Total protein extracts (50 µg per sample) were diluted to a final volume of 140 µl using 2× isoelectrofocusing (IEF) buffer (18.2 mM DTT and 0.5% IPG buffer solution pH 3–10, BioRad). IEF was performed on 7-cm pH 3–10 nonlinear gradient IPG strips (BioRad) in a Protean IEF Cell system (BioRad) as follows: 50 V for 12 h, 250 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 2000 V for 1 h, linear ramp to 8000 V for 1 h, and 8000 V up to 3500 V·h. More than 12,000 V h was reached in each step. Next, 12% SDS-PAGE was performed in a pre-cooled MiniProtean 3 Dodeca Cell system (BioRad) at 0.5 W/gel for 30 min and then at 1.5 W. The run was stopped 5 min after the dye-front reached the bottom edge. The gels were stained with SYPRO Ruby (BioRad), and the images were acquired using EXQuest Spot Cutter (BioRad) following the manufacturer’s instructions.

PDQuest 2D Advanced 8.0.1 software was used to detect and analyze protein levels (BioRad). First, the gel images were cropped and the settings adjusted (three biological replicates per condition). Then, spots were automatically detected in all individual member gel images and in the master gel

composite image. Automatically undetected spots were highlighted and accounted using the software's "Add a spot at cursor" option in the "Edit Spot Tools" menu. Pairwise recognition of each spot between gels was enhanced using the landmark tool. All spots were manually checked using the 3D density graph viewer and single spot quantitation histograms. The raw data were normalized using the *Total Quantity in Valid Spots* algorithm. Spots showing ≤ 1.5 -fold levels at days 4 and 6 compared to day 2, and p values ≥ 0.05 (Student's t test, $\alpha = 0.05$), were considered constitutive during promastigote growth and differentiation.

Protein identification by MALDI-TOF/TOF

The selected spots were excised with EXQuest Spot Cutter (BioRad). In-gel digestion was carried out with porcine trypsin and prepared for MALDI-TOF/TOF mass-spectrometry as described (Alcolea et al. 2011b). Each digest (0.8 μ l) was deposited in a well of an OptiTOF™ Plate (Life Technologies) containing 0.8 μ l of 3 μ g/ μ l α -cyano-4-hydroxycinnamic acid (Sigma). The mixture was allowed to dry at room temperature. Then, mass spectrometry was performed in an ABI 4800 MALDI-TOF/TOF instrument (Life Technologies) in positive reflector mode at 25 kV for MS and 1 kV for MS/MS. The data obtained were processed with ABI 4000 Series Explorer Software 3.6 (Life Technologies). Peptide mass fingerprinting (PMF) and MS/MS fragment ion spectra were smoothed and corrected to zero baseline using routines embedded in ABI 4000 Series Explorer Software v3.6. Each PMF spectrum was internally calibrated with the trypsin autolysis ion mass signals to reach typical mass measurement accuracy of < 25 ppm. Known trypsin and keratin mass signals and potential sodium and potassium adducts (+ 21 Da and + 39 Da) were removed from the peak list. Protein identifications were performed with MASCOT 2.1 using Global Protein Server Explorer 4.9 (Life Technologies). The settings were: enzyme, trypsin; allowed missed cleavages, 1; carbamidomethyl cysteine as fixed modification due to treatment with iodoacetamide; variable modifications, methionine oxidation; and mass tolerance, ± 50 ppm for precursors, and ± 0.3 Da for MS/MS fragment ions. The confidence interval for protein identification was set to $\geq 95\%$ ($p < 0.05$), and only peptides with an individual ion score above the identity threshold (52) were considered correctly identified. The reference genome sequence for MASCOT identifications can be retrieved from TriTrypDB (<http://tritrypdb.org/common/downloads/release-9.0/LdonovaniBPK282A1/fasta/data/>). The identifications are available in the PRIDE repository of the ProteomeXchange Consortium (Vizcaino et al. 2014) with the accession number PXD009011.

Results and discussion

Screening of constitutive proteins in cultured *L. donovani* promastigotes by 2DE-MALDI-TOF/TOF

Proteome profiles throughout *L. donovani* promastigote growth and differentiation processes were analyzed using daily prepared protein extracts from cultures maintained for 7 days. Promastigotes reached the stationary phase at day 5. Protein concentration was between 4 and 5 mg/ml. Once 2DE was performed, the gel images were analyzed using PDQuest. In total, 36 constitutive abundant proteins were identified using MALDI-TOF/TOF (MASCOT score > 52 ; Figs. 1, 2, 3 and Table 1).

Gene expression regulation and proteolysis

The ribonucleoprotein p18 mitochondrial precursor (pRNP18) is constitutive in *L. donovani* promastigotes. Differential pRNP18 gene regulation at the protein or transcript levels was not found in the analogous *L. infantum* stage (another "*L. donovani* complex" species), in culture, or in the sand fly gut (e.g., Alcolea et al. 2010, 2014b). However, the protein increases in early-logarithmic phase promastigotes of the New World species *L. amazonensis* and *L. pifanoi* (Alcolea et al. 2016b), which belong to the "*L. mexicana* complex." pRNP18 is one of the proteins forming a complex that is involved in the trypanosomatid-specific uridine insertion/deletion mitochondrial transcript pan-editing process (Aphasizhev et al. 2003; Panigrahi et al. 2001). As a result, genes encoding respiratory chain proteins are edited through this unique mechanism. However, the biological consequences are unknown, and the aforementioned gene regulation differences between *Leishmania* species cannot be explained with the information available.

Translation elongation factor 1 β (EF1 β) levels are high and constitutive in *L. donovani* promastigotes. EF1 β was shown to increase under stress induced by cadmium in *L. infantum* promastigotes (Alcolea et al. 2011a). This translation factor is also involved in redox homeostasis in these parasites. The implications of being constitutively expressed in *L. donovani* promastigotes are discussed at the end of the "[Redox homeostasis](#)" section.

Cyclophilin LdBPK_060120 is constitutive in *L. donovani* promastigotes. The cyclophilin family is involved in protein folding through peptidyl-prolyl cis-trans isomerase activity. In general, specific biological functions of this family have been poorly studied in *Leishmania* spp. However, it is known that LdBPK_060120 specifically binds to cyclosporin A (CpsA). Compartmentalization of this CpsA-binding protein into secretory pathway organelles and associated vesicles is a possible cause of *L. donovani* resistance to CspA (Dutta et al. 2001). Therefore, cyclophilin levels are maintained during

As for proteolysis, mitochondrial processing peptidase subunit β (metallo-peptidase, Clan ME, Family M16), ubiquitin-conjugating enzyme (UbCE), calpain-like cysteine peptidase, and ATP-dependent HslVU complex proteolytic subunit (threonine peptidase, Clan T(1), family T1B) levels are constitutive. Some genes encoding for ubiquitin-proteasome system proteins are differentially regulated at the transcript and/or protein levels in *L. infantum* promastigotes in culture and in the sand fly vector (Alcolea et al. 2010, 2014b), but UbCE is not among them. Therefore, UbCE is probably constitutive in both *L. donovani* and *L. infantum* promastigotes. Heme uptake is relevant in these parasites due to their heme-group auxotrophy. Even though biological roles of ubiquitin conjugation have been scarcely studied in *Leishmania* spp., relation with heme uptake through early endosomes has been recently

Metabolism

The following enzymes involved in catabolism are constitutive throughout *L. donovani* promastigote growth:

Table 1 Constitutive proteins in *L. donovani* promastigotes. The following parameters are shown: spot numbers; TriTrypDB identifiers; protein names (italicized entries, unequivocally identified constitutive proteins; non-italicized entries, more than one protein match for the same spot); MASCOt protein identification scores (significant over 52); estimated MW and pI (physical map in Fig. 1); protein abundance fold changes (*F*) with respect to day 2; and Student's *t* test outcome ($\alpha = 0.05$). Three biological replicates were performed. The null hypothesis is absence of differential gene expression regulation (accepted whenever $p \geq 0.05$). Fold change threshold values: $F = 1.5$ or $F = -1.5$. Please give the significance of the italicized entries in Table 1 in the form of a table note. Otherwise, amend if necessary.

Spot	TriTrypDB Id.	Protein	MASCOt score	MW (kDa)	pI	<i>F</i> (d4:d2)	<i>F</i> (d6:d2)	<i>p</i> (d4:d2)	<i>p</i> (d6:d2)
LdK.0101	LdBPK_241560.1	<i>IgE-dependent histamine-releasing factor, putative</i>	333	23.2	3.2	1.02	1.07	0.936	0.729
LdK.1301	LdBPK_361490.1	<i>Translation elongation factor 1-beta, putative</i>	251	30.9	3.4	1.50	-1.20	0.585	0.059
LdK.1601	LdBPK_250780.1	<i>Protein phosphatase, putative</i>	394	45.0	3.5	1.13	1.31	0.063	0.050
LdK.1909	LdBPK_290790.1	<i>Glucose regulated protein 94 (GRP94, LPG3)</i>	529	122.3	4.5	1.50	1.10	0.077	0.856
LdK.2001	LdBPK_201350.1	<i>Calpain-like cysteine peptidase, putative</i>	184	15.0	4.7	-1.35	-1.06	0.064	0.602
LdK.2003	LdBPK_290520.1	<i>Cofilin-like protein</i>	79	15.0	5.2	1.07	-1.23	0.136	0.577
LdK.2101	LdBPK_291250.1	<i>Tryparedoxin, putative (TXN)</i>	353	15.5	4.9	1.37	1.30	0.404	0.589
LdK.2201	LdBPK_230050.1	<i>Peroxidoxin</i>	501	23.7	5	1.20	1.20	0.475	0.298
LdK.2301	LdBPK_364180.1	<i>HsIVU complex proteolytic subunit, threonine peptidase, Clan Tr(1), family T1B, ATP-dependent protease subunit HsIV, putative (HsIV)</i>	219	32.8	4.9	-1.25	-1.44	0.621	0.343
LdK.2303	LdBPK_362070.1	<i>Phosphonannomulase, putative</i>	362	32.9	5.2	1.23	1.23	0.494	0.667
LdK.2501	LdBPK_302740.1/LdBPK_364070.1	TPR domain protein, conserved/eukaryotic translation initiation factor 3 subunit, putative	281/275	42.3	4.7	1.44	1.01	0.125	0.965
LdK.2502	LdBPK_120620.1	<i>Cytochrome c oxidase subunit IV</i>	435	40.5	5.1	1.39	1.21	0.384	0.491
LdK.3101	LdBPK_321910.1/20.1	<i>Iron superoxide dismutase, putative</i>	720/328	20.7	5.4	1.39	1.49	0.184	0.021
LdK.3201	LdBPK_181360.1	<i>Pyruvate dehydrogenase E1 component alpha subunit, putative</i>	289	24.7	5.2	1.09	1.11	0.718	0.417
LdK.3501	LdBPK_070710.1	<i>Hypothetical protein, conserved</i>	534	40.2	5.3	-1.05	-1.49	0.963	0.454
LdK.3602	LdBPK_041250.1	<i>Actin</i>	467	46.8	5.3	1.42	-1.03	0.570	0.823
LdK.4101	LdBPK_150330.1	<i>Ribonucleoprotein p18, mitochondrial precursor, putative</i>	249	18.5	5.6	-1.33	-1.15	0.272	0.401
LdK.4102	LdBPK_131320.1	<i>Ubiquitin-conjugating enzyme, putative</i>	510	16.1	5.6	1.39	1.25	0.096	0.204
LdK.4301	LdBPK_252090.1	<i>Hypothetical protein, conserved</i>	322	29.0	5.7	-1.45	1.02	0.231	0.929
LdK.4401	LdBPK_363970.1/LdBPK_312210.1	Hypothetical protein, conserved/prostaglandin f2-alpha synthase/D-arabinose dehydrogenase	454/289	34.2	5.8	-1.36	-1.33	0.613	0.511
LdK.4701	LdBPK_364100.1/LdBPK_271610.1	S-adenosylhomocysteine hydrolase/eukaryotic translation release factor, putative	561/115	56.4	4.7	-1.02	1.28	0.941	0.383
LdK.5201	LdBPK_251460.1	<i>GTP-binding protein, putative</i>	593	25.9	5.8	-1.41	-1.12	0.473	0.812
LdK.5301	LdBPK_262290.1	<i>Nirilase, putative</i>	348	29.1	5.8	-1.03	1.01	0.909	0.967
LdK.5302	LdBPK_312210.1	<i>Prostaglandin f2-alpha synthase, D-arabinose dehydrogenase</i>	602	26.8	6.1	-1.20	-1.16	0.485	0.382
LdK.5502	LdBPK_363100.1	<i>Succinyl-CoA ligase [GDP-forming] beta-chain, putative</i>	750	42.0	6	-1.36	-1.14	0.416	0.632
LdK.5504	LdBPK_230410.1	<i>NADP-dependent alcohol dehydrogenase, putative</i>	536	39.4	6.1	-1.43	-1.27	0.284	0.573
LdK.5705	LdBPK_242200.1/LdBPK_010670.1	3-hydroxy-3-methylglutaryl-CoA synthase, putative (HMGS)/metallo-peptidase, Clan ME, Family M16, mitochondrial-processing peptidase subunit beta, putative (MPPB)	286/251	55.3	6	-1.50	-1.37	0.676	0.724
LdK.6101	LdBPK_151120.1/40.1	<i>Tryparedoxin peroxidase</i>	320/310	22.4	6.3	-1.04	1.15	0.573	0.742

Table 1 (continued)

Spot	TriTrypDB Id.	Protein	MASCOT score	MW (kDa)	pI	F (d4:d2)	F (d6:d2)	p (d4:d2)	p (d6:d2)
LdK.6102	LdBPK_060120.1	<i>Cyclophilin</i>	276	17.6	6.3	1.31	-1.12	0.173	0.706
LdK.6105	LdBPK_151140.120.1	<i>Tryparedoxin peroxidase</i>	389/362	22.6	6.4	1.02	1.19	0.755	0.593
LdK.6301	LdBPK_311070.1	<i>Biotin/lipoate protein ligase-like protein</i>	408	27.6	6.2	-1.49	-1.35	0.155	0.223
LdK.6402	LdBPK_181570.1/LdBPK_365010.1	<i>Nonspecific nucleoside hydrolase/hypothetical protein, conserved</i>	327/139	37.0	6.2	-1.09	-1.01	0.669	0.974
LdK.6404	LdBPK_081010.1	<i>Hypothetical protein, conserved</i>	572	39.4	6.4	-1.43	-1.25	0.164	0.489
LdK.6702	LdBPK_242200.1	<i>3-hydroxy-3-methylglutaryl-CoA synthase, putative (HMGs)</i>	739	54.8	6.2	-1.23	1.00	0.412	0.992
LdK.7602	LdBPK_351390.1	<i>Mitochondrial processing peptidase, beta subunit, putative, metallo-peptidase, Clan ME, Family M16</i>	888	50.1	6.8	-1.50	-1.22	0.316	0.603
LdK.8101	LdBPK_080300.1	<i>Iron superoxide dismutase</i>	328	22.7	7.5	1.23	1.01	0.416	0.944

Cytoskeleton, signal transduction, and other functions

Actin levels are constitutive in *L. donovani* promastigotes, as well as actin-interacting cofilin-like protein levels (Table 1), as in many eukaryotic organisms and cell types. Two proteins involved in signaling have been found at constitutive levels: a protein phosphatase and a GTP-binding protein. The IgE-dependent histamine-releasing factor, which is constitutive in *L. donovani*-cultured promastigotes, was proposed as a tool in serodiagnosis of canine visceral leishmaniasis and human tegumentary leishmaniasis (Coelho et al. 2016). This protein is a mammalian cytokine ortholog released by basophils in inflammatory processes (MacDonald et al. 1991) and its function in the parasite is unknown. Expression of the encoding gene has been demonstrated in promastigotes and amastigotes (Nirujogi et al. 2014; Pawar et al. 2012), but no hypothetical roles at the first infection stages have been tested. Regarding surface molecule biosynthesis, the glucose-regulated protein 94 (GRP94, LPG3) is involved in lypophosphoglycan (LPG) biosynthesis. Gene LdBPK290790.1 (TriTrypDB) encodes for this molecule, which is related to parasite virulence, but not viability (Descoteaux et al. 2002). The gene product is incorrectly annotated as heat shock protein 90. A possible explanation for LPG3 constitutive levels may be the need for LPG molecules throughout the entire promastigote growth and differentiation process, as well as metabolic modulation by glucose as a regulation mechanism distinct from steady-state protein level control.

Redox homeostasis

Peroxiredoxins or peroxidoxins are ubiquitous cysteine-dependent peroxidases acting in the first step of the antioxidant system reaction cascades. The genomes of the VL agents *L. donovani* and *L. infantum* (GeneDB, TriTrypDB) contain three and four peroxidoxin genes, respectively. The *L. donovani* genes LdBPK_151120.1 and LdBPK_151140.1 and their *L. infantum* orthologs LinJ.15.1100, LinJ.15.1120, and LinJ.11.1140 are tryparedoxin peroxidases (TryP), whereas LdBPK_230050.1 and LinJ.23.0050 are generic peroxidoxins (Prx). TryP orthologs are identical, except for the lack of the first 77 amino acids in the LdBPK_151120.1 gene-encoded protein N-terminal region, probably due to an annotation error (Fig. 4). We suggest that the generic Prxs are also TryPs, as in other trypanosomatids (TriTrypDB), but we maintain generic names in this study because their sequences are substantially different to the TryP genes cited above (Fig. 4). In this study, we have observed constitutive TryP and Prx levels in *L. donovani* promastigotes (Fig. 5, Table 1). Differential TryP and Prx regulation at the transcript and/or protein level was not detected in *L. infantum* promastigotes throughout differentiation in culture or in *Phlebotomus perniciosus* sand fly's midgut (e.g., Alcolea

TryPs & Prx

LinJ.23.0050	MLRRLLPTSCFLKRSQFRGFAATSPLLNLDYQMYRTATVREAAPQFSG-QAVVNGAIKDIN
LdBPK_230050.1	MLRRLLPTSCFLKRSQFRGFAATSPLLNLDYQMYRTATVREAAPQFSG-QAVVNGAIKEIN
LinJ.15.1120	-----MSCGDAKINCPAPPFEEVALMPNGSFKKIS
LdBPK_151120.1	-----MSCGDAKINCPAPPFEEVALMPNGSFKKIS
LinJ.15.1100	-----MSCGDAKINCPAPPFEEVALMPNGSFKKIS
LinJ.15.1140	-----MSCGDAKINCPAPPFEEVALMPNGSFKKIS
LdBPK_151140.1	-----MSCGDAKINCPAPPFEEVALMPNGSFKKIS
LinJ.23.0050	MNDYKGKYIVLFFYPMDFTFVCPTIIAFSDRHADFELNLTQWVAVSCDSVYSHLAWVNT
LdBPK_230050.1	MNDYKGKYIVLFFYPMDFTFVCPTIIAFSDRHADFELNLTQWVAVSCDSVYSHLAWVNT
LinJ.15.1120	LAAYKGKVVVLFYPLDFTFVCPTIIQFSENISRFNENLCEVLACSMDEYAHQLQWTLQ
LdBPK_151120.1	-----MDSEYAHQLQWTLQ
LinJ.15.1100	LAAYKGKVVVLFYPLDFTFVCPTIIAFSENVSFRFENLCEVLACSMDEYAHQLQWTLQ
LinJ.15.1140	LAAYKGKVVVLFYPLDFTFVCPTIIAFSENVSFRFENLCEVLACSMDEYAHQLQWTLQ
LdBPK_151140.1	LAAYKGKVVVLFYPLDFTFVCPTIIAFSENVSFRFENLCEVLACSMDEYAHQLQWTLQ
	** * : * * *
LinJ.23.0050	PRKKGGLGEMHIPVLADKSMIARDYGVLIIEESGIALRGLFIIDKKGILRHSTINDLPVG
LdBPK_230050.1	PRKKGGLGEMHIPVLADKSMIARDYGVLIIEESGIALRGLFIIDKKGILRHSTINDLPVG
LinJ.15.1120	DRKKGGLGAMAIPMLADKTKSIARAYGVLEEKQGVAYRGLFIIDPNGMVRQITVNDMPVG
LdBPK_151120.1	DRKKGGLGAMAIPMLADKTKSIARAYGVLEEKQGVAYRGLFIIDPNGMVRQITVNDMPVG
LinJ.15.1100	DRKKGGLGAMAIPMLADKTKSIARAYGVLEEKQGVAYRGLFIIDPNGMVRQITVNDMPVG
LinJ.15.1140	DRKKGGLGAMAIPMLADKTKSIARAYGVLEEKQGVAYRGLFIIDPNGMVRQITVNDMPVG
LdBPK_151140.1	DRKKGGLGAMAIPMLADKTKSIARAYGVLEEKQGVAYRGLFIIDPNGMVRQITVNDMPVG
	***** * * : * * * : * * * * * : * : * * * * * : * : * : * : * : * : *
LinJ.23.0050	RNVDEALRVLEAFQYADENGDAIPCGWKPQPTLDTTKAG---EFFEKNM
LdBPK_230050.1	RNVDEALRVLEAFQYADENGDAIPCGWKPQPTLDTTKAG---EFFEKNM
LinJ.15.1120	RNVEEVLRLLEAFQFVEKHGEVCPANWKKGDPGLKVDHINK-----
LdBPK_151120.1	RNVEEVLRLLEAFQFVEKHGEVCPANWKKGDPGLKVDHINK-----
LinJ.15.1100	RNVEEVLRLLEAFQFVEKHGEVCPANWKKGAPTMKPEPKASVEGYFSKL-
LinJ.15.1140	RNVEEVLRLLEAFQFVEKHGEVCPANWKKGAPTMKPEPKASVEGYFSKQ-
LdBPK_151140.1	RNVEEVLRLLEAFQFVEKHGEVCPANWKKGAPTMKPEPKASVEGYFSKQ-
	*** : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *

Fig. 4 Primary structure of the *L. donovani* and *L. infantum* peroxiredoxins. *L. donovani* and *L. infantum* contain 3 and 4 peroxiredoxin genes, respectively. LdBPK_151120.1 and LdBPK_151140.1 are TryP ortholog genes of LinJ.15.1100, LinJ.15.1120, and LinJ.11.1140. LinJ.23.0050 is an LdBPK_

230050.1 ortholog. LdBPK_151120.1 lacks the first 77 amino acids of the N-terminal region but is identical to LdBPK_151140.1, which suggests a 5'-end annotation error

et al. 2010; 2011b; Leifso et al. 2007; Levick et al. 1998). As an exception, the increase of a thiol-dependent thioredoxin-like

antioxidant protein (later known as the TryP LinJ.15.1120) was found in *L. infantum* promastigotes in logarithmic phase of

Fig. 5 Trypanothione-based antioxidant system in *L. donovani* promastigotes. The steady-state levels of the trypanothione peroxidases/peroxiredoxins (TryP/Pxn), trypanothione (Txn), elongation factor 1β

(EF1β), and iron superoxide dismutase (FeSODA) are constitutive throughout the *L. donovani* promastigote growth curve.

axenic culture (Alcolea et al. 2011b). The TryP LdBPK_151120.1 and the *L. donovani* Prx decrease in amastigotes compared to stationary phase promastigotes (Lahav et al. 2011; Rosenzweig et al. 2008). The same expression patterns were observed when promastigotes were obtained from the stomodeal valve of the sand fly gut (Alcolea et al. 2014b). In summary, these findings support TryP and Prx constitutive levels in promastigotes and decrease in amastigotes of both “*L. donovani* complex” species. On the contrary, TryPs were reported to be constitutive in all CL agent *L. major* life cycle stages, including amastigotes (Levick et al. 1998). Therefore, Old World *Leishmania* species do not change TryP and Prx levels during growth and differentiation in culture, whereas at least some increase in VL-causing species amastigotes only. Substantially different results have been found in the New World species *L. amazonensis* and *L. pifanoi* (Alcolea et al. 2016b), which belong to the “*L. mexicana* complex” and are responsible for CL. These studies support that at least a TryP is considerably upregulated (> 5-fold) in stationary phase promastigotes in both species. Different variants of the same TryP are also differentially regulated in other *L. amazonensis* promastigote growth phases (early- and mid-logarithmic phase). The TryP CfaC1_10_1430 (NCBI: gi385150) is also differentially abundant in the motile *Crithidia fasciculata* choanomastigote stage (Alcolea et al. 2014a), a trypanosomatid not pathogenic to mammals. In this organism, a 12.9-kDa variant of the protein product is more abundant in early-logarithmic phase choanomastigotes, whereas heavier variants are more abundant in mid-logarithmic phase promastigotes (24.1 kDa, pI 5.4) and the peanut lectin non-agglutinating subpopulation within the population in stationary phase (20.46 and 22.36 kDa, pI 5.7 and 5.4). Taken as a whole, these data suggest that certain New World *Leishmania* species and *C. fasciculata* are prone to change TryP abundance, whereas Old World species do not generally vary the TryP expression levels throughout promastigote development in culture. Prx gene expression is generally invariant in these organisms at the transcript and protein levels.

The iron superoxide dismutase LdBPK_080300.1 (FeSODA) is also constitutive in *L. donovani* promastigotes in culture (Fig. 5, Table 1), as a difference with *C. fasciculata* choanomastigotes (Alcolea et al. 2014a). This enzyme catalyzes the reduction of superoxide anions generated in reactions catalyzed by enzymes like the ribonucleotide reductase (RNR). The Prx and the TryPs are able to reduce these anions and other reactive oxygen species (ROS) in the presence of reduced trypanothione (TXN[SH₂]), which is oxidized (TXN[S]) during this reaction. TXN[SH₂] is regenerated by reduced trypanothione (T[SH₂]), which is recycled by the NADPH-dependent enzyme trypanothione reductase (TryR) (Day 2009; Flohe et al. 1999; Gretes et al. 2012). In this study, the TXN1 has been detected as constitutively expressed in *L. donovani* promastigotes in culture, in agreement with the TXN1 of *L. amazonensis* (Alcolea et al. 2016b). So far, no TXN gene has been found to be differentially regulated in

L. infantum, *L. donovani*, *L. major*, *L. pifanoi*, *L. amazonensis* promastigotes, or *C. fasciculata* choanomastigotes.

Finally, the aforementioned EF1β is constitutive in *L. donovani* promastigotes in culture (Fig. 5, Table 1), and differential expression at the transcript or protein level was not detected in *L. infantum* promastigotes either (Alcolea et al. 2010, 2011b). However, this protein is upregulated in early-logarithmic phase *L. pifanoi* promastigotes (Alcolea et al. 2016b) and *C. fasciculata* choanomastigotes (Alcolea et al. 2014a). The EF1β is not just a translation factor. It also bears trypanothione S-transferase activity (Vickers and Fairlamb 2004; Vickers et al. 2004). An interesting observation is that the EF1β together with the TryPs might have a role in the disseminated MCL form caused by *L. (Viannia) guyanensis* (Walker et al. 2006). Therefore, these proteins may have important biological roles in the parasite, in agreement with their high expression levels. Indeed, these proteins are among the most abundant constitutive proteins of the *L. donovani* promastigote proteome (Fig. 5, Table 1). Both are probably highly abundant and constitutive in other Old World species. Interestingly, EF1β and TR transcripts were found to be increased in promastigotes exposed to cadmium ion stress (Alcolea et al. 2011a). This suggests that EF1β is expressed more actively in certain stress situations than in standard conditions. The EF1β and TryP levels also tend to change throughout promastigote growth and differentiation in New World species belonging to the “*L. mexicana* complex”.

Conclusions

The EF1β, FESODA, TryPs, Prx, and TXN are abundant and constitutive during *L. donovani* promastigote growth and differentiation in culture. The Old World species *L. major* and *L. infantum* also tend to maintain constitutive levels of proteins involved in the trypanothione-based antioxidant system, whereas New World *Leishmania* species modulate these protein levels in certain cases. Interestingly, the entire system is differentially regulated throughout growth of *C. fasciculata*, which is not able to infect mammals. Consequently, these data suggest that the particular expression patterns of genes involved in redox homeostasis are important for specific adaptations throughout the different life cycles they develop.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest

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