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Trypanosoma cruzi contains two galactokinases; molecular and biochemical characterization

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ABSTRACT

Two different putative galactokinase genes, found in the genome database of *Trypanosoma cruzi* were cloned and sequenced. Expression of the genes in *Escherichia coli* resulted for *TcGALK*-1 in the synthesis of a soluble and active enzyme, and in the case of *TcGALK*-2 gene a less soluble protein, with predicted molecular masses of 51.9 kDa and 51.3 kDa, respectively. The K_m values determined for the recombinant proteins were for galactose 0.108 mM (*Tc*GALK-1) and 0.091 mM (*Tc*GALK-2) and for ATP 0.36 mM (*Tc*GALK-1) and 0.1 mM (*Tc*GALK-2). Substrate inhibition by ATP (K_i 0.414 mM) was only observed for *Tc*GALK-2. Gel-filtration chromatography showed that natural *Tc*GALKs and recombinant *Tc*GALK-1 are monomeric. In agreement with the possession of a type-1 peroxisome-targeting signal by both *Tc*GALKs, they were found to be present inside glycosomes using two different methods of subcellular fractionation in conjunction with mass spectrometry. Both genes are expressed in epimastigote and trypomastigote stages since the respective proteins were immunodetected by western blotting. The *T. cruzi* galactokinases present their highest (52–47%) sequence identity with their counterpart from *Leishmania* spp., followed by prokaryotic galactokinases such as those from *E. coli* and *Lactococcus lactis* (26–23%). In a phylogenetic analysis, the trypanosomatid galactokinases form a separate cluster, showing an affiliation with bacteria. Epimastigotes of *T. cruzi* can grow in glucose-depleted LIT-medium supplemented with 20 mM of galactose, suggesting that this hexose, upon phosphorylation by a *Tc*GALK, could be used in the synthesis of UDP-galactose and also as a possible carbon and energy source.

1. Introduction

Trypanosoma cruzi, the causative agent of Chagas' disease, is a parasite belonging to the protistan Trypanosomatidae family. It undergoes multiple morphological and metabolic changes during its complex life cycle involving two hosts, a vertebrate and an insect [1]. The transition between the different developmental stages is accompanied by changes in the carbohydrate composition of the macromolecules on the cell surface, coated with a dense glycocalyx composed mainly of glycoconjugates, some of which play essential roles in parasite survival, infectivity and virulence [2].

The monosaccharides that make up these glycoconjugates are D-mannose (Man), D-N-acetylglucosamine (GlcNAc), D-glucosamine (GlcN), D-glucose (Glc), D-xylose (Xyl), L-rhamnopyranose (Rha), L-fucose (Fuc), D-galactose (D-Gal: D-galactopyranose and D-galactofuranose), with this latter sugar being particularly abundant in all trypanosomatids [3]. The glycoconjugates are synthesized by specific glycosyltransferases using nucleotide-sugars (NDP-sugars) as glycosyl donors [2].

Formation of nucleotide-sugars occurs via two distinct pathways, i) the *de novo* synthesis or interconversion of pre-existing NDP-sugars, and ii) a salvage pathway where free sugars are taken up from the environment by a specific sugar transporter or generated intracellularly upon the degradation of polysaccharides, glycoproteins and glycolipids. These free sugars can be phosphorylated at position C-1 by a specific kinase to form sugar 1-phosphates. Subsequently, a nucleotide diphosphate (NDP)-sugar pyrophosphorylase (USP) transfers a nucleotidyl residue to form the NDP-sugar [3]. It has been suggested that in trypanosomatids much of the synthesis of the nucleotide-sugars occurs inside glycosomes, based on the finding that some enzymes of these pathways contain a peroxisome-targeting signal (PTS-1 or PTS-2) [4], and for some of them localization in these organelles has been experimentally demonstrated [5,6].

Abbreviations: *Tc*GALKs, (*Tc*GALK-1, *Tc*GALK-2) galactokinases from *T. cruzi*; HK, hexokinase; GEF, glycosome-enriched fraction; IMAC, immobilized metal affinity chromatography; PTS-1, peroxisome-targeting signal type 1; ORF, open-reading frame.

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The nucleotide sugar UDP-galactose (UDP-Gal) is essential for the biosynthesis of several abundant glycoconjugates that form the glycocalyx surface of *T. cruzi*. In this parasite the synthesis of UDP-Gal has been proposed to occur only by the direct conversion of UDP-Glc to UDP-Gal by UDP-glucose-4-epimerase (GALE) [7]. Galactose cannot be obtained from the environment as it is not recognized by the hexose transporter (*Tcr*HT1) [8]. Moreover, it was found that, at least for the epimastigote stage, GALE is essential for the biosynthesis of UDP-Gal and derived glycoconjugates, and hence for the parasite's survival [9]. However, the genomes of *T. cruzi*[10] and *Leishmania* spp. [11], but not of *Trypanosomabrucei* [12] encode the entire enzymatic machinery for the salvage route, the Isselbacher pathway, to synthesize UDP-Gal [13]. In *Leishmaniamajor* both pathways are active and contribute to the UDP-Gal synthesis [14,15]. In contrast, the *T. brucei* genome lacks a homologue of the USP gene and it has a pseudogene of GALK [12]. Indeed, in this parasite the UDP-Gal synthesis occurs only by the *de novo* pathway [16].

Isselbacher route enzymes encoded in the *T. cruzi* genome are: aldose-1-epimerase (TcCLB.509331.180), galactokinase (GALK) (two genes, TcCLB.507001.110 and TcCLB.510667.120), and UDP-sugar pyrophosphorylase (USP) (TcCLB.511761.10); this latter enzyme has been recently characterized in *T. cruzi* and *Leishmania*[3,15]. In order to contribute to the elucidation of this pathway and its role in the UDP-Gal formation in *T. cruzi*, we aimed to study the enzyme galactokinase (EC 2.7.1.6), which catalyzes the ATP-dependent phosphorylation of α-D-galactose to produce α-D-galactose 1-phosphate (Gal-1-P). GALK plays an important role not only because it is involved in the synthesis of UDP-Gal, but the enzyme is also part of the Leloir pathway in species ranging from *Escherichiacoli* to human [17]. This latter pathway comprises galactose mutarotase, galactokinase (GALK), galactose-1-P uridyltransferase (GALT), and UDP-galactose 4′-epimerase (GALE), which together mediate the transformation of β-D-galactose into α-D-glucose-1-P [17,18]. Finally, α-D-glucose-1-P is transformed into α-D-glucose-6-P by phosphoglucomutase (PGM). In this way the carbons from galactose can enter into the glycolytic pathway and be used as carbon and energy source.

The enzyme GALK has been isolated from different sources such as bacteria [19], yeast [20], mammalian liver [21], plants [22], and human [23]. In man, mutations in the *GALK* gene lead to the disease state referred as Type II galactosemia. Patients with this disorder exhibit neonatal cataracts that self-resolve upon dietary restriction of galactose [23]. On the basis of sequence comparisons, it was concluded that galactokinases belong to a unique class of ATP-dependent enzymes known as the GHMP superfamily (galactokinase, homoserine kinase, mevalonate kinase, and phosphomevalonate kinase) [24]. All these proteins contain three common structural/functional motifs, the second one being the highly conserved Pro-XXX-Gly-Leu-X-Ser-Ser-Ala motif that is involved in ATP binding [23,24]. In this paper, we report the amplification and cloning of the two *TcGALK* genes of *T. cruzi*. The expressed proteins of the two genes TcCLB.507001.110 (*TcGALK*-1) and TcCLB.510667.120 (*TcGALK*-2) were soluble and able to phosphorylate α-D-galactose using ATP as phosphoryl donor, thus confirming them as GALKs. Both *TcGALK* genes are expressed in epimastigotes and trypomastigotes. Furthermore, galactokinase activity was detected in the epimastigote form of the parasite, and located within the glycosomes. In addition, we found that epimastigotes of *T. cruzi* are able to grow in LIT medium supplemented with galactose, supporting the notion that this sugar could be used as carbon and energy source.

2. Materials and methods

2.1. Parasites and growth of T. cruzi epimastigotes and bloodstream trypomastigotes

Epimastigotes of *T. cruzi* strain EP were cultured axenically at 28 °C, with constant shaking, in liver infusion-tryptose (LIT) medium supplemented with 5% heat-inactivated fetal bovine serum [25]. Bloodstream trypomastigotes were obtained from infected Vero cells as described by Bertelli et al. [26].

Growth curves of *T. cruzi* epimastigotes were determined in three different conditions: i) low glucose, which is a partially depleted medium (1.5 mM D-glucose), supplemented with 5% heat-inactivated fetal bovine serum dialyzed by tangential flow filtration against 0.15 M NaCl (Life Technologies, D-glucose concentration < 0.0278 μM). When required, this medium was supplemented with either ii) 20 mM of D-glucose (high glucose) or iii) 20 mM of α-D-galactose.All cultures (100 ml) were started with 5×10^6 cells/ml and incubated at 28 °C. Growth was then followed up by optical density ($OD_{600\ nm}$), measurement and samples were collected every 24 h until the culture reached the stationary growth phase. All fractions were centrifuged at 3000 ×*g* for 15 min to harvest parasites from the culture medium. Subsequently, the pellets were washed four times with normal buffer (140 mM NaCl, 11 mM KCl and 75 mM Tris, pH 7.4) and stored at −80 °C before measuring the enzymatic activity of galactokinase. Supernatants were also frozen for later determination of glucose, galactose and ammonium concentrations in the culture medium.

2.2. Cloning of galactokinase genes

Sequences of the *T. cruzi GALK* genes (accession codes TcCLB.507001.110 and TcCLB.510667.120) were retrieved from the GeneDB database (http://www.Genedb.org/). Specific primers were designed in order to amplify by PCR both full-length genes: for *TcGALK*-1 the forward primer 5′-CG**CATATG**AATCCCCTCAGCTACAC-3′ (*Nde*I site in bold) and the reverse primer 5′-GC**GGATCC**CTACAGGTTGCTATCGGGC-3′ (*BamH*I site in bold), for *TcGALK*-2 as forward primer 5′-CG**CATATG**CCGAG CTACTCAGACAA-3′ (*Nde*I site in bold) and as reverse primer 5′-CG**GGATCC**TCATAGCTTTAACACCC-3′ (*BamH*I site in bold). PCR was performed using the above-mentioned primers, Platinum *Taq* DNA polymerase (Promega), and genomic DNA from *T. cruzi* (EP strain). In both cases, the PCR product was ligated into the pGEM-T-easy vector (Promega), and sequenced using the T7 and SP6 universal primers in an automated sequencer. Subsequently, each full-length gene was transferred to the pET28a vector (Novagen) and used to transform *E. coli* strains BL21(DE3) pLys (Novagen) and BL21 Star (DE3) (Life Technologies). The expression systems yielded recombinant proteins contained a N-terminal 20-residues long extension having a $(His)_6$-tag plus a cleavage site for thrombin protease.

2.3. Phylogenetic analysis

Sequences of GALKs were retrieved from the Interpro database (www.ebi.ac.uk/interpro/) using the identifier code IPR019539. In addition, we performed searches in the TriTryp and GeneDB databases to find galactokinases from other species of trypanosomatids like *Leishmania* (*Leishmania mexicana*, *braziliensis* and *infantum*) and African trypanosomes (*T. brucei brucei* TREU927, *T. brucei gambiense*, *T. congolense*, *T. vivax*). Two galactokinase-like genes, one pseudogene (TriTryp accession code: Tb927.5.287) and the other, a galactokinase degenerate sequence (TriTryp accession code: Tb927.5.290), were found in *T. b. brucei* TREU927; these were not considered further in this study. In *T. b. gambiense* an incomplete gene (TriTryp accession code: Tbg972.5.60) that codes for a product of 286 amino acids, and lacks residues involved in catalysis, was used for the phylogenetic analysis. In *T. congolense* and *T. vivax* the *GALK* gene is absent, and for the species of the *Leishmania* genus only one *GALK* gene was found (TriTryp accession codes: LbrM.34.365, LdBPK 352790.1, LinJ.35.279, LmjF.35.2740, LmexM.34.2740), although *L. braziliensis* presents a second galactokinase-like sequence (TriTryp accession code: LbrM.34.264) which was further considered in this study. The phylogenetic tree was constructed with 40 galactokinase amino-acid sequences using the Neighbor-Joining method. Numbers at the individual nodes represent

bootstrap support (1000 replicates). The evolutionary distances were computed using the Poisson correction method [27]. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 1004 positions in the final dataset. Phylogenetic analyses were performed in MEGA4 [28].

2.4. Expression of T. cruzi galactokinases in E. coli and their purification

To express the two galactokinases in *E. coli*, cells harboring the expression plasmids were grown at 28 °C for 24 h in ZYM-5052 autoinduction medium [29], supplemented with 33 μg/ml kanamycin and 34 μg/ml chloramphenicol. *Tc*GALK-1 was purified under native conditions. For this purpose, pelleted cells were resuspended in 8 ml of lysis buffer (40 mM Tris-HCl, pH 7.8, 300 mM KCl) and disrupted by sonication on ice. The lysate was then centrifuged at 12,000 ×*g* for 15 min at 4 °C and the supernatant was loaded onto a ProBond resin (Invitrogen), pre-equilibrated with twenty volumes of lysis buffer. Subsequently the column was washed with twenty volumes of washing buffer (40 mM Tris-HCl, pH 7.8, 300 mM KCl, 75 mM imidazole), and the recombinant protein eluted using elution buffer (40 mM Tris-HCl, pH 7.8, 300 mM KCl, 150 mM imidazole).The *Tc*GALK-1 pure was used to raise a polyclonal antiserum.

*Tc*GALK-2 was expressed non-soluble form and was purified under denaturing conditions. Cells were harvested by centrifugation at 12,000 ×*g* for 10 min at 4 °C, the pelleted cells were resuspended in 8 ml of lysis buffer (10 mM Tris-HCl, pH 7.8), and disrupted by sonication on ice. The inclusion bodies were obtained as a pellet by centrifugation at 12,000 ×*g* for 10 min at 4 °C. This pellet was resuspended in denaturing lysis buffer (6 M guanidine-hydrochloride, 20 mM sodium phosphate, pH 7.8, 500 mM NaCl) and mixed by inversion for 20 min. The lysate was then centrifuged at 12,000 ×*g* for 15 min at 4 °C and the supernatant loaded onto a ProBond resin (Invitrogen), pre-equilibrated with seven volumes of denaturing binding buffer (8 M urea, 20 mM sodium phosphate, pH 7.8, 500 mM NaCl). Subsequent purification steps were performed as recommended by the resin manufacturer. The recombinant protein was eluted using denaturing elution buffer (8 M urea, 20 mM sodium phosphate, pH 4.0, 500 mM NaCl). The *Tc*GALK-2 pure was used to raise a polyclonal antiserum.

Another strain of *E. coli* BL21 Star (DE3) was used to express *Tc*GALK-2. The optimal growth conditions allowed to express it in a soluble form, although only 10% of the total recombinant protein. Cells were incubated for 12 h at room temperature in ZYM-5052 autoinduction medium. After that, harvested cells were resuspended in 5 ml of lysis buffer (40 mM Tris-HCl, pH 7.8, 300 mM KCl) and disrupted by sonication on ice. The lysate was treated in the same way as described for *Tc*GALK-1 and the procedure for soluble recombinant protein purification was also performed. However, it was unsuccessful in the purification of the recombinant *Tc*GALK-2 protein, because its weak interaction with the ProBond resin caused it to be eluted in the wash steps (Supplementary Figure S1). The clarified cell extract was used to assay the galactokinase activity, even when the enzyme was not pure.

The purity of both proteins (*Tc*GALK-1 and *Tc*GALK-2) was assessed by SDS-PAGE, and the protein concentration determined by the method of Lowry et al. using BSA as standard [30].

2.5. Production of rabbit and mouse polyclonal antisera against recombinant galactokinases

Polyclonal antisera against purified recombinant *Tc*GALKs were raised in mice (*Tc*GALK-1) and rabbit (*Tc*GALK-2) as described by Cáceres et al. [31].Both antisera were stored at −20 °C. The antibody anti-*Tc*GALK-1 showed no cross-reactivity with the recombinant *Tc*GALK-2 (Supplementary Figure S2), while the other antibody raised in rabbit (anti-*Tc*GALK-2) did show cross-reactivity with the recombinant *Tc*GALK-1.

In order to remove the cross-reactivity from the polyclonal anti-*Tc*GALK-2 with the *Tc*GALK-1 protein a negative affinity purification was performed, using an Affi-gel 10 resin (BIO-RAD) with *Tc*GALK-1 as ligand, then the anti-*Tc*GALK-2 serum was added, to absorb the cross-reacting antibodies. The recombinant protein (*Tc*GALK-1) was purified as described in Section 2.4, and finally precipitated with ammonium sulfate at 80%. The precipitate was resuspended in 2 ml of 0.1 M MOPS buffer pH 7.5, and then desalted through a PD-10 column. Subsequently, the *Tc*GALK-1 (3 mg) was mixed with the activated Affi-gel 10 resin (1 ml), and incubated by stirring for 4 h. The *Tc*GALK-1 ligated Affi-gel was then washed 5 times with 4 ml of 0.1 M MOPS buffer pH 7.5, and blocked with 0.5 M ethanolamine-HCl pH 8.0 for 30 min under shaking. Finally, the resin was washed 9 times with 8 ml of 0.1 M PBS buffer, and stored at 5 °C in PBS plus 0.02% sodium azide.

On other hand, the anti-*Tc*GALK-2 serum (3.5 ml) was precipitated with 10–50% of ammonium sulfate, and the pellet was resuspended in 2 ml of PBS buffer. The sample was desalted using a PD-10 column, and the eluted fraction mixed with the *Tc*GALK-1 ligated Affi-gel-10 resin previously equilibrated with 10 volumes of PBS buffer. The antibodies and the resin were incubated overnight at 4 °C with gently shaking. Then, the preparation was incubated for 1 h at room temperature, and anti-*Tc*GALK-2 antibodies were obtained in the flow-through fraction. In order to corroborate the specificity of the purified antibodies, they were assayed against the recombinant *Tc*GALK-1 (Supplementary Figure S2). Finally, the specific anti-*Tc*GALK-2 was stabilized in 10% glycerol and used in all immunodetection assays.

2.6. Enzymatic assays

Galactokinase activity was measured by coupling it, via its production of ADP, to the reactions catalyzed by pyruvate kinase (PYK) and L-lactate dehydrogenase (LDH). PYK uses ADP plus phospho*enol*pyruvate (PEP) to produce pyruvate and ATP, LDH takes the pyruvate produced and in the presence of NADH catalyzes the formation of L-lactate and NAD^+. The concomitant oxidation of NADH was followed spectrophotometrically at 340 nm and room temperature [32]. The assay was performed in a 1-ml cuvette containing 50 mM Tris-HCl, pH 8.6, 1.5 mM $MgCl_2$, 50 mM KCl, 2.5 mM ATP, 20 mM NaF, 2 mM PEP, 0.2 mM NADH, 5 mM α-D-galactose, 0.7 U/ml of PYK (rabbit muscle; EC 2.7.1.40; Sigma) and 0.9 U/ml of L-LDH (rabbit muscle; EC 1.1.1.27; Sigma).

The effect of pH on activity was studied using a poly-buffer: 10 mM acetate, 10 mM imidazole, 10 mM Tris and 10 mM lysine, plus 1 mM $MgCl_2$. Assays to evaluate the effect of ionic strength were done using different concentrations of NaCl and KCl between 0–500 mM. The K_m for galactose was determined by varying the sugar concentration between 0.01 and 5 mM, whereas the K_m for ATP was measured with concentrations of the nucleotide varying between 0.05 and 2.5 mM. The galactokinase activity was measured by the standard assay mentioned above. Apparent K_m ($K_{m.app}$) values were calculated from the Michaelis-Menten equation-derived curve, fitted to the experimental data by using a non-linear least-squares regression program (SigmaPlot 8.0, SPSS). One unit of galactokinase activity is defined as the amount of the enzyme that catalyzes the production of 1 μmol of ADP per minute. The activity of other enzymes was assayed as described previously: hexokinase (HK, EC 2.7.1.1), pyruvate kinase (PYK, EC 2.7.1.40) and L-glutamate dehydrogenase (GlDH-NAD^+, EC 1.1.1.3) [32].

Glucose, galactose and ammonium concentrations in the culture media were determined by enzymatic methods [32]. Galactose concentration in the medium was measured using the purified recombinant *Tc*GALK-1 (0.3 U/ml). Glucose concentration was determined using a commercial peroxidase-glucose oxidase assay system (Wiener lab). Ammonium concentration was determined using α-ketoglutaric acid in the presence of L-glutamate dehydrogenase (GlDH-NAD^+) with a commercial system (Sigma), following the instructions of the manufacturer.

2.7. Gel filtration chromatography and ELISA

The subunit molecular mass of the recombinant and natural enzymes was determined by SDS-PAGE. The native molecular mass was determined by gel-filtration chromatography on a Superdex 200 10/300 GL column (Tricorn™-Amersham Biosciences) equilibrated with 25 mM Tris-HCl, pH 7.9, 150 mM KCl, and calibrated with molecular markers (Amersham Biosciences). For natural enzymes and the recombinant *Tc*GALK-1 a volume of 0.5 ml of a glycosome-enriched fraction previously solubilized with 0.1% Triton X-100 and 0.3 M KCl (GEF, 4.75 mg/ml) and the purified recombinant protein (2 mg/ml), were applied onto the column, respectively. The elution was monitored at 280 nm and fractions were collected every 60 s. In the case of recombinant *Tc*GALK-1, fractions containing enzymatic activity were registered, whereas eluted fractions of the GEF were collected and immune-probed for *Tc*GALKs by ELISA. Briefly, the ELISA was carried out in a 96-well plate coated with 100 μl of each fraction plus 100 μl of binding buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and incubated overnight at 4 °C. The plate was subsequently washed with PBS (15 mM Na_2HPO_4, 5 mM KH_2PO_4, pH 7.2, 130 mM NaCl) containing 0.05% Tween-20 and then blocked with PBS containing 2% non-fat milk and 0.05% Tween-20 for 1.5 h at 37 °C. After two washes, 100 μl of the polyclonal anti-*Tc*GALK-1 (1:1000) or anti-*Tc*GALK-2 (1:300) was added in PBS with 2% non-fat milk, and incubated for 1 h at 37 °C. Then, after washing, 100 μl of the secondary anti-mouse (1:20,000) or anti-rabbit (1:5000) IgG antibody coupled with horse radish peroxidase was added, and incubated for 1 h at 37 °C. The wells were washed and the amount of bound peroxidase determined by its reaction with the substrate 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) (DIAGEN C.A.). The reaction was stopped with 50 μl of 0.5 M HCl and the colored product measured at 450 nm using an automated microplate ELISA reader (Thermo). The data obtained were plotted using the package SigmaPlot 8.0, SPSS.

2.8. SDS-PAGE and immunoblotting

SDS-PAGE was performed in 12% (w/v) polyacrylamide gels in the presence of 0.1% SDS according to Laemmli [33]. Staining was performed with Coomassie brilliant blue R-250.For western blots, proteins separated by SDS-PAGE were electro-transferred to a nitrocellulose membrane by using a semi-dry system (Amersham Biosciences) and incubated with either the polyclonal anti-*Tc*GALK-1 (1:1000), anti-*Tc*GALK-2 (1:300), anti-*Tc*HK (1:1000) or anti-His antibody (1:3000; Amersham Biosciences).The secondary antibody was a peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin (Sigma), diluted 1:4000. Peroxidase-labeled proteins on the immunoblots were revealed using 0.5 mg/ml diaminobenzidine (Sigma), 0.0025% $CoCl_2$ and 0.2% H_2O_2.

2.9. Subcellular localization

The subcellular localization of *Tc*GALKs was determined by two different methods: differential and isopycnic centrifugation. The homogenate of *T. cruzi* epimastigotes was prepared by grinding washed cells with silicon carbide (200 mesh), and fractionated by differential and isopycnic centrifugation as described by Concepción et al. [34]. In addition, mass spectrometry was performed on a glycosomal fraction purified by double isopycnic centrifugation and treated with an osmotic shock as described by Quiñones et al. [35]. Briefly, proteins were digested with trypsin using the FASP protocol as described by Wiśniewski et al. [36]. Peptides were solubilized in 2% acetonitrile with 0.1% trifluoroacetic acid and fractionated on a nanoflow uHPLC system (Thermo Scientific RSLCnano) before online analysis by electrospray ionisation (ESI) mass spectrometry on an Amazon Speed ion trap MS/MS (Bruker Daltonics). Peptide separation was performed on a Pepmap C18 reversed phase column (Thermo Scientific). Peptides were desalted and concentrated for 4 min on a C18 trap column followed by an acetonitrile gradient (in 0.1% v/v formic acid) (3.2–32% v/v 4–27 min, 32% to 80% v/v 27–36 min, held at 80% v/v 36–41 min and re-equilibrium at 3.2%) for a total time of 45 min. A fixed solvent flow rate of 0.3 μl/min was used for the analytical column. The trap column solvent flow was 25 μl/min of 2% acetonitrile with 0.1% v/v trifluoroacetic acid. Eluate was analyzed by LC-MS using an Orbitrap Elite MS (Thermo Scientific), acquiring a continuous duty cycle of a high resolution precursor scan at 60,000 RP (at 400 *m/z*), while simultaneously acquiring the top 20 precursors subjected to CID fragmentation in the linear ion trap. Singly charged ions were excluded from selection, while selected precursors are added to a dynamic exclusion list for 120 s. Protein identifications were assigned using the Mascot search engine (v2.5.1) to interrogate *T cruzi* CL Brener protein coding sequences in the NCBI database, allowing a mass tolerance of 10 ppm for the precursor and 0.6 Da MS/MS matching.

2.10. RNA purification and RT-PCR

The expression of both *T. cruzi GALK* genes was studied by RT-PCR. Total RNA from the parasite was purified by TRIZOL (Invitrogen) method, from 1×10^8 cells in two different developmental stages (epimastigotes and bloodstream trypomastigotes) and the reverse transcription step (RT) was performed as described by Cáceres et al. [37], with the following modification, anoligo-(dT) instead of random primers was used. For PCR 0.2 μg of cDNA or plasmid DNA harboring the respective *TcGALK* gene were used as template with 0.2 mM of a dNTPs mix, 10 pmol of forward and reverse primers and 1.5 U of Go*Taq* DNA polymerase (Promega). The PCR was performed using two forward internal primers; for *TcGALK*-1 5′-CTACAACGTCACCGAGCAG-3′ and for *TcGALK*-2 5′-CATCTTTAACATTGCCGAGCAA-3′, and the reverse primers described in Section 2.2, which were specific to amplify a fragment of 400 bp of each *TcGALK* gene. PCR conditions used were as described in Section 2.2.

3. Results

3.1. Cloning and sequence analysis of the Trypanosoma cruzi galactokinase genes

Putative *TcGALK* genes of *T. cruzi* were identified in the GeneDB database (TcCLB.507001.110 and TcCLB.510667.120) and amplified by PCR using genomic DNA from the parasite (strain EP) and cloned. The putative *TcGALK*-1 gene is a 1407-bp long open-reading frame (ORF) for a polypeptide of 468 amino acids with a molecular mass of 51.9 kDa. The putative *TcGALK*-2 gene is a 1392-bp ORF that predicts a polypeptide of 463 amino acids with a molecular mass of 51.3 kDa. The predicted molecular weight of these proteins is very similar to that of species of the *Leishmania* genus: *L. mexicana*, *L. infantum*, *L. major* and *L. braziliensis*, and those reported for others members of the galactokinase family [38]. An analysis using the amino-acid sequences of galactokinases from organisms such as bacteria (*Escherichia coli*, *Lactococcus lactis*, *Vibrio cholera*), yeast (*Saccharomyces cerevisiae* Gal1 and Gal3), *Arabidopsis thaliana*, *Homo sapiens* and trypanosomatids (*T. cruzi* GALK-1 and GALK-2, *L. major*, *L. mexicana*, *L. braziliensis*, *L. infantum*) showed that those enzymes exhibited a range of isoelectric point (pI) between 5.28 (*E. coli*) and 6.99 (*Tc*GALK-1), with the exception of *Tc*GALK-2 whose pI is 8.1.

The *Tc*GALKs show 53% amino-acid sequence identity to each other. A comparison with homologous sequences identified by BLASTP using as queries *T. cruzi* GALKs showed the following degrees of identity: the highest values were found with GALKs from other trypanosomatids such as *T. gambiense* (49% *Tc*GALK-1 and 72% *Tc*GALK-2) and species of the *Leishmania* genus (52% *Tc*GALK-1 and 47% *Tc*GALK-2), followed by their counterparts in prokaryotes, *Lactococcus lactis* (26% for both *Tc*GALKs) and *E. coli* (25% *Tc*GALK-1 and 23% *Tc*GALK-2), and with other eukaryotic organisms such as *Arabidopsis thaliana* (23% *Tc*GALK-1 and 24% *Tc*GALK-2), *Saccharomyces cerevisiae* (19% *Tc*GALK-1 and 22% *Tc*GALK-2), and *Homo sapiens* (21% *Tc*GALK-1 and 23% *Tc*GALK-2).

The amino-acid sequences of the putative *T. cruzi* GALKs were aligned with those of *L. major*, *L. infantum*, *L. braziliensis*, and *Lactococcus lactis*, all proteins belonging to the galactokinase family (Pfam PF10509) and superfamily GHMP (Galactokinase, Homoserine kinase, Mevalonate kinase and Phosphomevalonate kinase) (Pfam PF08544). As shown in Supplementary Figure S3, three motifs are well conserved in the enzymes belonging to this superfamily. The first motif, PGRVNLIG(AE)HXDY, a signature of the galactokinase family, is located in the N-terminal region of the protein, and contains all residues associated with the galactose-binding site. The second motif, having the typical sequence Pro-XXX-**Gly**-Leu-X-**Ser**-**Ser**-**Ala** (conserved Gly, Ser, Ser and Ala in bold), is involved in the ATP-binding site.The third motif, GAGXG, located in the C-terminal part, is involved in catalysis and transfer of the γ phosphate from ATP to the sugar [23,24,39]. In the *Tc*GALK sequences, the residues associated with the galactose-binding site correspond to R45, E51, D54, D216, Y272 for *Tc*GALK-1, and R43, E49, D52, D214, Y270 for *Tc*GALK-2, which have been functionally identified in the crystal structure of *L. lactis* GALK [39]. The second motif, also present in both *Tc*GALK sequences, involves residues E144, G148, M149, S150, S151, A152 for *Tc*GALK-1 and P142, G144, M147, S148, S150, A151 for *Tc*GALK-2. The third motif, located in the C-terminal region of those proteins, comprises the following amino acids G407, G408, G409, G411 for *Tc*GALK-1 and G404, G405, G406, G408 for *Tc*GALK-2. All trypanosomatid GALKs present a type 1 peroxisome-targeting signal (PTS-1) (Supplementary Figure S3), the C-terminal tripeptide SKL or a variation thereof, a signal found in the majority of glycosomal enzymes [40].

3.2. Phylogenetic analysis

Sequences to be used for the multiple alignment and subsequent phylogenetic analysis were selected from the InterPro database, based on their classification as galactokinases and their degree of similarity to trypanosomatid GALKs (see Section 2.3). Fig. 1 shows the consensus tree obtained by the Neighbor-Joining method for 40 sequences and 1004 amino-acid positions. The branches of the tree represent clusters of sequences from different phyla: 8 from Euglenozoa, 1 from Stramenopiles, 3 from Firmicutes, 2 from Proteobacteria, 1 from Actinobacteria, 1 from Acidobacteria, 1 from Apicomplexa, 6 from Ascomycota, 2 from Ciliophora, 2 from Magnoliophyta, 2 from Nematoda, 1from Platyhelmithes, 2 from Arthropoda and 8 from Chordata. All retrieved sequences belong to members of the galactokinase family and the GHMP superfamily, as defined in the InterPro database (www.ebi.ac.uk/interpro/) by code IPR019539. As outgroup was used the human enolase. The phylogenetic tree reveals the relationship among the galactokinases of *T. cruzi* and several other organisms from different domains of life. Trypanosomatid sequences do not cluster with galactokinases from other eukaryotes, and branched off as an independent clade supported by high bootstrap values. The trypanosomatid cluster itself is subdivided into two clades; one clade includes the *Tc*GALK-1 and the GALKs sequences from *Leishmania* spp., while the second clade comprises the *Tc*GALK-2 and a relatively short sequence of 286 amino acids for a putative galactokinase of *T. b. gambiense*, whereas the galactokinase 2 from *L. braziliensis* branched off away from all the other trypanosomatid galactokinases. The cluster that involves representatives from all trypanosomatids is most related to Eubacteria, while only a limited similarity is shared with other eukaryotic galactokinases. Furthermore, the analysis shows that there is a high degree of conservation among galactokinases from mammals.

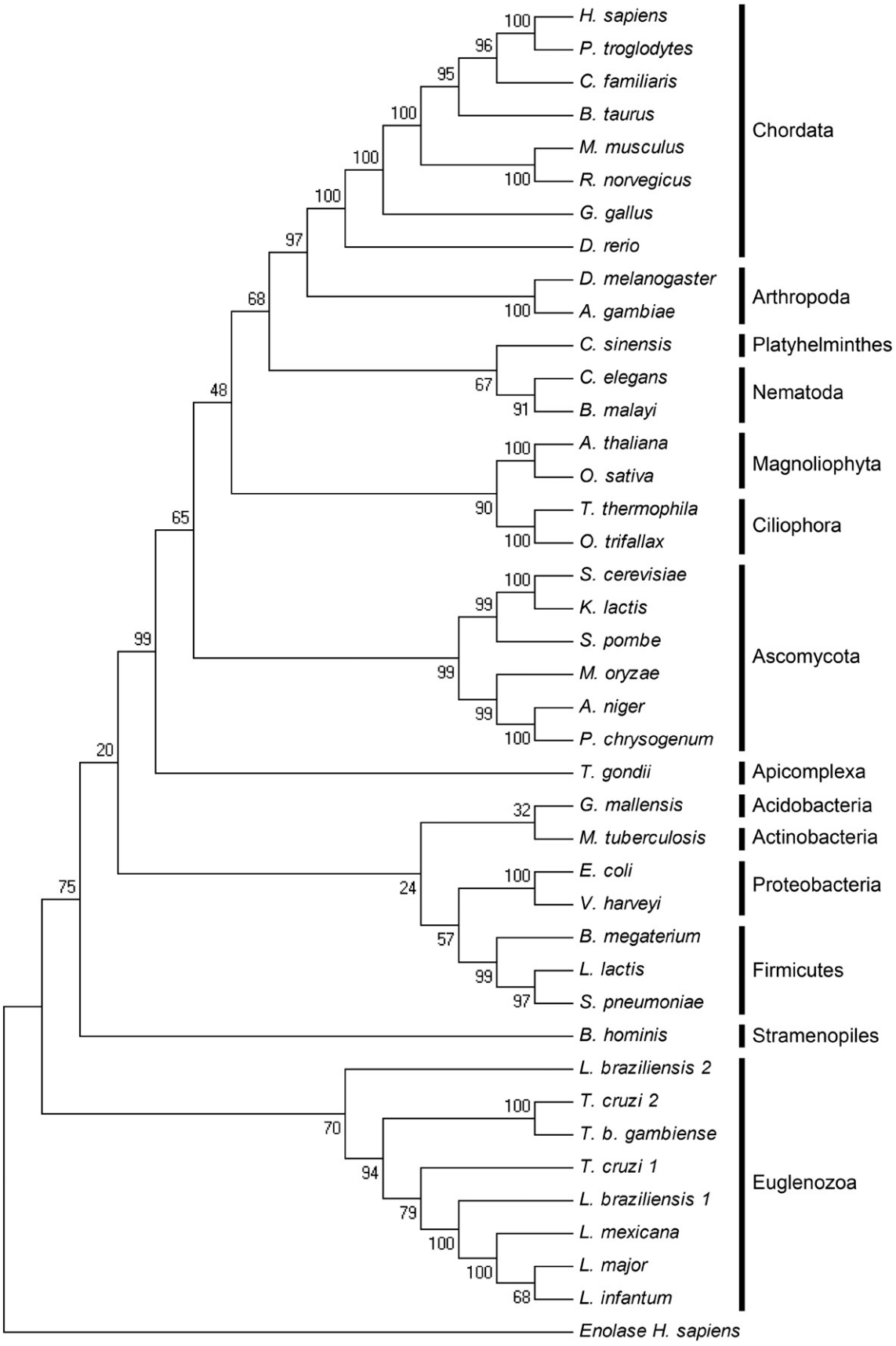
3.3. Expression of *T. cruzi* galactokinases in Escherichia coli and their purification

The *Tc*GALKs were expressed in *E. coli* strain BL21 (DE3) in order to investigate their biochemical and kinetic properties. The coding sequences were ligated to the expression vector pET28a to produce a fusion protein with an amino terminal $(His)_6$-tag. The bacteria were grown in autoinduction medium, an expression system that allowed us to produce large amounts of recombinant protein. The recombinant *Tc*GALK-1 was expressed in soluble form and eluted during purification by IMAC with 150 mM imidazole: the yield of the purification was 2.9 mg of GALK-1 per 30 mg protein of bacterial cell-free extract. The recombinant *Tc*GALK-2 expressed in the strain BL21-pLys was always insoluble, in the form of inclusion bodies; nevertheless, this recombinant protein was purified under denaturing conditions. Both purified proteins were used to raise specific antisera (Fig. 2). In addition, *Tc*GALK-2 was expressed in the strain BL21Star (DE3), where it was mostly insoluble (90%) and with 10% in a soluble form. However, this soluble recombinant protein could not be purified under native conditions due to its weak binding to the ProBond resin, causing it to elute always in the wash step (10 mM imidazole). Furthermore, we observed that *Tc*GALK-2 became degraded very fast, even in the presence of a proteases inhibitor cocktail; degradation products appeared as bands around 30 and 15 kDa upon PAGE of the lysate and the wash fractions, and were recognized by the monoclonal antibody anti-His (Supplementary Figure S1).

3.4. Kinetic characterization of recombinant enzymes

In order to determine if the genes identified indeed code for GALKs, the specificity of the recombinant proteins for the sugar substrate was assayed. For *Tc*GALK-1, the enzyme purified to homogeneity was used, while for *Tc*GALK-2 the *E. coli* cell extract was employed, as was possible since the strain BL21Star (DE3) is deficient for *gal* gene. Similar to other galactokinases from different organisms, the trypanosomatid enzymes were able to phosphorylate only α-D-galactose, using ATP as phosphoryl donor. No activity was observed with D-glucose, N-acetyl-D-glucosamine or glucosamine. *Tc*GALK-1 and *Tc*GALK-2 follow classical Michaelis-Menten kinetics with K_m values for galactose of 108 μM and 91 μM, while the values for ATP were 358 μM and 100 μM, respectively. Interestingly, *Tc*GALK-2 exhibited substrate inhibition by ATP, with a K_i of 414 μM (See Table 1). A more detailed kinetic analysis was done for *Tc*GALK-1, because the enzyme was purified to homogeneity. We tested PPi as a possible inhibitor of *Tc*GALK-1 activity, since it has been reported to inhibit hexokinase from *T. cruzi* [41], but did not find any effect on

Fig. 1. Phylogenetic analysis of galactokinases. The phylogenetic tree was constructed with 40 galactokinase amino acid sequences using the Neighbor-Joining method. Numbers at the individual nodes represent bootstrap support (1000 replicates). The evolutionary distances were computed using the Poisson correction method [3]. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). The following sequences listed with their NCBI accession code were used. *H. sapiens*, *Homo sapiens* (AAP36276.1); *P. troglodytes*, *Pan troglodytes* (XP_001167197.1); *C. familiaris*, *Canis familiaris* (XP_544673.2); *B. taurus*, *Bos taurus* (NP_001033259.1); *M. musculus*, *Mus musculus* (NP_780363.1), *R. norvegicus*, *Rattus norvegicus* (NP_001013941.1); *G. gallus*, *Gallus gallus* (NP_001025728.1); *D. rerio*, *Danio rerio* (NP_001007433.2); *D. melanogaster*, *Drosophila melanogaster* (NP_648276.1); *A. gambiae*, *Anopheles gambiae* (XP_315119.4); *C. sinensis*, *Clonorchis sinensis* (GAA52165.1); *C. elegans*, *Caenorhabditis elegans* (NP_490909.2); *B. malayi*, *Brugia malayi* (EDP36398.1); *A. thaliana*,*Arabidopsis thaliana* (NP_187310.1), *O. sativa*, *Oryza sativa* (NP_001051803.1); *T. thermophila*, *Tetrahymena thermophila* (XP_001011166.1); *O. trifallax*, *Oxytricha trifallax* (EJY68642.1); *S. cerevisiae*, *Saccharomyces cerevisiae* (NP_009576.1); *K. lactis*, *Kluyveromyces lactis* (XP_455461.1); *S. pombe*, *Schizosaccharomyces pombe* (NP_596859.1); *M. oryzae*, *Magnaporthe oryzae* (XP_003710300.1); *A. niger*, *Aspergillus niger* (XP_001397727.1); *P. chrysogenum*, *Penicillium chrysogenum* (XP_002559436.1); *T. gondii*, *Toxoplasma gondii* (EPR64197.1); *G. mallensis*, *Granulicella mallensis* (WP_014264824.1); *M. tuberculosis*, *Mycobacterium tuberculosis* (AJP78627.1); *E. coli*, *Escherichia coli* (NP_308812.1); *V. harveyi*, *Vibrio harveyi* (YP_001446502.1); *B. megaterium*, *Bacillus megaterium* (YP_003595903.1); *L. lactis*, *Lactococcus lactis* (YP_005876867.1); *S. pneumoniae*, *Streptococcus pneumoniae* (YP_001695220.1); *B. hominis*, *Blastocystis hominis* (CBH10868.1); *L. braziliensis 2*, *Leishmania braziliensis-2* (XP_003723233.1); *T. cruzi 2*, *Trypanosoma cruzi*-2 (XP_813139.1); *T. b. gambiense*, *Trypanosoma brucei gambiense* (CBH10868.1); *T. cruzi 1*, *Trypanosoma cruzi*-1 (XP_812977.1); *L. braziliensis 1*, *Leishmania braziliensis-1* (XP_001568371.1); *L. mexicana*, *Leishmania mexicana* (XP_003879270.1); *L. major*, *Leishmania major* (XP_003722668.1); *L. infantum*, *Leishmania infantum* (XP_001469061.1); *Enolase H. sapiens*, Enolase from *Homo sapiens* (outgroup) (CAA47179.1). There were a total of 1004 positions in the final dataset. Phylogenetic analyses were performed in MEGA4 [4].


H. sapiens
P. troglodytes
C. familiaris
B. taurus
M. musculus
R. norvegicus
G. gallus
D. rerio
Chordata
D. melanogaster
A. gambiae
Arthropoda
C. sinensis
Platyhelminthes
C. elegans
B. malayi
Nematoda
A. thaliana
O. sativa
Magnoliophyta
T. thermophila
O. trifallax
Ciliophora
S. cerevisiae
K. lactis
S. pombe
M. oryzae
A. niger
P. chrysogenum
Ascomycota
T. gondii
Apicomplexa
G. mallensis
Acidobacteria
M. tuberculosis
Actinobacteria
E. coli
V. harveyi
Proteobacteria
B. megaterium
L. lactis
S. pneumoniae
Firmicutes
B. hominis
Stramenopiles
L. braziliensis 2
T. cruzi 2
T. b. gambiense
T. cruzi 1
L. braziliensis 1
L. mexicana
L. major
L. infantum
Euglenozoa
Enolase H. sapiens

the recombinant enzyme. The *Tc*GALK-1 exhibited an optimal pH for activity at values between 7.5 and 9.5 (Table 1). As all other kinases, the enzyme required a divalent cation for activity; $MgCl_2$ was the most effective followed by $MnCl_2$, which could provide 62% of the activity found with $MgCl_2$. Furthermore, we observed that the galactokinase activity is sensitive to both the ionic strength and the type of salt used. In the presence of NaCl (50–500 mM) the enzymatic activity decreased approximately 40%–60%, respectively. In contrast, when KCl was used (50–100 mM), a positive effect was observed, with an increase in activity around 80%. At higher KCl concentrations (150–500 mM) the positive effect began to reverse concomitantly. Nevertheless, the effect was always positive with a basal increase of 50% in the enzymatic activity (data not shown).

3.5. Molecular mass determination of recombinant and natural galactokinases

The molecular mass of both recombinant enzymes as determined by SDS-PAGE was about 50 kDa, in agreement with their predicted molecular weights, 51.9 kDa (*Tc*GALK-1) and 51.3 kDa (*Tc*GALK-2). The native molecular masses of the natural *Tc*GALKs were determined by gel filtration (Fig. 3); their elution profile as assessed by ELISA showed that these are monomeric enzymes, with a molecular weight of approximately 50 kDa. Furthermore, the elution profile of the recombinant *Tc*GALK-1, followed by enzymatic activity, was in agreement with a monomeric enzyme (data not shown).

3.6. Galactokinase expression in T. cruzi

The expression of both galactokinase genes was studied by probing the presence of the mature transcripts and the respective proteins. The two mRNAs (*TcGALK*-1 and *TcGALK*-2) were detected in both developmental stages; epimastigote (the multiplicative form in the insect vector) and trypomastigote (the non-multiplicative, infective form present in the bloodstream of the mammalian host) (Fig. 4A). The possibility of cross amplification of the two genes with both pairs of primers was excluded by performing also control PCR with DNA from each of the recombinant plasmids (pGEM*Tc*GALK-1 and pGEM*Tc*GALK-2) (Supplementary Figure S4).

Protein probing was done by immunodetection with the specific anti-*Tc*GALK-1 and anti-*Tc*GALK-2. Using the homogenates from epimastigote and trypomastigote-bloodstream forms, only one band of 50 kDa was detected with each of the antisera (Fig. 4B). These results confirm that both galactokinases are present in epimastigotes and trypomastigotes from *T. cruzi*. Importantly, additional assays demonstrated two peaks with galactokinase activity when a GEF from epimastigotes was separated by an anionic exchange chromatography (Supplementary Figure S5).

3.7. Subcellular localization of galactokinases in epimastigotes of T. cruzi

In order to confirm that the PTS-1 identified at the C-terminus of the *Tc*GALKs sequences, sort these proteins to the glycosomes, a subcellular fractionation of *T. cruzi* epimastigotes homogenate was performed by differential and isopycnic centrifugation. The GALK activity was measured in the different fractions obtained after differential centrifugation. We found that GALK activity was enriched in the small granular fraction or GEF as did the glycosomal hexokinase marker (Fig. 5A). This result is in agreement with the signal obtained by western blot analysis using the anti-*Tc*GALK-1 antibody, with the fractions of the same experiment (Fig. 5B). The glycosomal location of *Tc*GALK-2 was addressed by subcellular fractionation in a sucrose gradient followed by western blot analysis of the recovered fractions (Fig 5C). We detected *Tc*GALK-2, using the specific antibody anti-*Tc*GALK-2, in the same fractions that contain hexokinase. Furthermore, to corroborate that both *Tc*GALKs were expressed in glycosome, at least in epimastigotes, a mass spectrometry analysis was performed on the soluble fraction of glycosomes purified by an isopycnic gradient. The analysis indeed identified peptides matching with high confidence to both galactokinases: 26 peptide fragmentation spectra were matched to *Tc*GALK-1 and 20 were matched to *Tc*GALK-2, giving Mascot significance scores of 779 and 487, respectively. Together, these results support the simultaneous glycosomal localization of both galactokinases in *T. cruzi*.

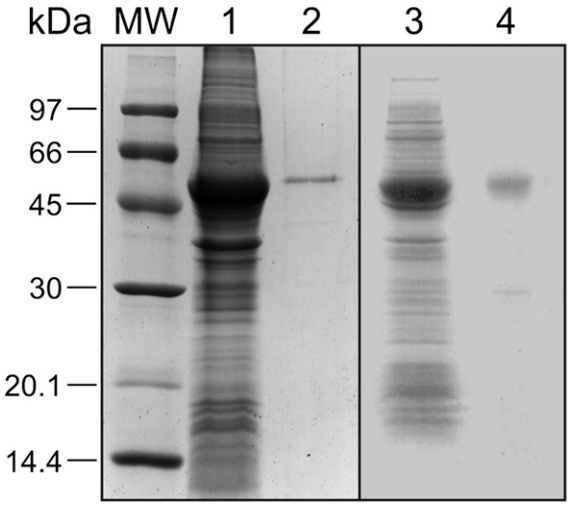


Fig. 2. SDS- PAGE showing the purification of recombinant *T. cruzi* GALKs expressed in *E. coli*. Cells harboring the expression plasmids were grown at 28 °C for 24 h in ZYM-5052 autoinduction medium. *Tc*GALK-1 was purified under native conditions. For this purpose pelleted cells were resuspended in lysis buffer and disrupted by sonication on ice. The supernatant of the lysate was loaded onto a ProBond resin (Invitrogen), and the recombinant protein was eluted using elution buffer plus 150 mM imidazole.In contrast, *Tc*GALK-2 was expressed insoluble and was purified under denaturing conditions. The inclusion bodies were resuspended in denaturing lysis buffer. The lysate was clarified by centrifugation, and the supernatant loaded onto a ProBond resin. The recombinant protein was eluted using denaturing elution buffer pH 4.0. These fractions were analyzed by SDS-PAGE. MW, molecular weight markers; lane 1, cell extract *Tc*GALK-1; lane 2, purified *Tc*GALK-1; lane 3, cell extract *Tc*GALK-2; lane 4, purified *Tc*GALK-2.

3.8. Growth of T. cruzi epimastigotes on glucose, galactose and depleted-glucose media

To determine if *T. cruzi* epimastigotes (EP strain) can grow on galactose, this developmental stage was grown in modified LIT medium. This involved partially glucose-depleted LIT medium (low glucose), which in some cases was supplemented with 20 mM of either galactose or glucose (high glucose). Fig. 6A shows the growth curves of epimastigotes in the presence of different carbon sources. The cultures supplemented with glucose or galactose exhibited a similar behavior, until they reached the end of the exponential phase of growth. Subsequently, for the trypanosomes in the galactose-based medium a decrease in the growth rate was observed, and when the growth stopped in the stationary phase, a lower cellular density (33×10^7 cell/ml) was reached when compared with trypanosomes cultured in glucose-based medium (42×10^7 cells/ml). In contrast, the culture in low glucose medium exhibited poor growth rate throughout the entire period, reaching the stationary phase after approximately 3 days, when glucose was exhausted from the medium, and with a much lower cellular density (13×10^7 cells/ml). In our culture conditions, the epimastigotes of *T. cruzi* (EP strain) consumed glucose, galactose and amino acids, with the catabolism of the latter substrates determined by measuring ammonium excretion. In each case, exhaustion of exogenous glucose or galactose occurred concomitantly with the progress of the parasites into the stationary phase (Fig. 6B), while the time course of ammonium excretion (Fig. 6C) varied between the cultures. When parasites are growing in galactose or in low glucose-based media, they began to consume amino acids earlier (at the

Table 1
Kinetic characteristics of *Trypanosoma cruzi* recombinant galactokinases. ND: non determined, *:no inhibition.

Enzyme	Activity pH optimum	Kinetic constants					
		K_m (mM)		K_i (mM)	Vmax (μmol·min^{-1}·mg^{-1})	K_{cat} (min^{-1})	K_{cat}/K_m (min^{-1})
		Galactose	ATP	ATP		Galactose	
GALK-1	7.5–9.5	0.108 ± 0.15	0.36 ± 0.09	*	0.424 ± 0.07	22.9	212.04
GALK-2	ND	0.091 ± 0.11	0.1 ± 0.03	0.414 ± 0.02	ND	ND	ND

second day) than cultures growing in the presence of a high glucose concentration. The excretion of ammonium increased gradually until day four, when it reached the maximum value (32 mM and 12 mM for galactose and low glucose-based media, respectively). In each case this concentration was constant until the end of the stationary phase. By contrast, the culture in high glucose-based medium started to excrete ammonium around day four, just when the glucose was exhausted from the medium, increasing concomitantly with the age of the culture. Parasites growing in galactose-based medium excreted the highest concentration of ammonium (37 mM) compared to the two other conditions tested. Moreover, when ammonium excretion was normalized (ammonium concentration/number of parasites), cultures with galactose (20 mM) and their glucose depleted (1.5 mM), produced respectively 2.4 and 2.7 times more ammonium than cultures with 20 mM glucose.

4. Discussion

In this paper we described the cloning and expression in *E. coli* of two predicted *GALK* genes identified in the *T. cruzi* genome. The recombinant *Tc*GALK-1 and *Tc*GALK-2 proteins were biochemically characterized confirming them as galactokinases. Their sequences contain all residues known to be involved in the substrates binding sites and the characteristic motif of the GHMP superfamily. These proteins are localized in glycosomes, routed by two different canonical PTS-1 motifs, SNL (*Tc*GALK-1) and GKL (*Tc*GALK-2). In the epimastigote form both enzymes are active, as demonstrated in the experiment shown in Supplementary Figure S5, where they were separated by ion exchange and the enzymatic activity was detected. Additionally, both *Tc*GALKs appeared to be monomeric (Fig. 3), as other galactokinases studied previously [19,23,39]. These results demonstrated that *T. cruzi* is unique among the members of the trypanosomatid family in having two active galactokinases.

The recombinant enzymes *Tc*GALK-1 and *Tc*GALK-2 have a high specificity for the galactose like its homologue in yeast [20]. They showed kinetic characteristics similar to other members of the galactokinase family [38], however with no ability to catalyze the phosphorylation of glucose, N-acetyl-D-glucosamine or glucosamine. In this latter feature they differ from other galactokinases, which act on a broader range of substrates and can additionally use 2-deoxy-D-galactose, fucose, galactosamine and galacturonic acid [22,42]. The ability of galactokinases to phosphorylate other sugars has been exploited in the potential application for providing enzymatic routes to prepare unique sugar phosphates. Indeed, there is an increasing effort directed towards altering glycosylation patterns to produce new and potentially important therapeutics [43].

The presence of two isoenzymes in the same subcellular compartment may seem as redundancy in function if both would exhibit the same kinetic behavior. Indeed, the kinetic properties of galactokinases differ mainly in the K_m for ATP and in the special condition of *Tc*GALK-2 of being inhibited by substrate (ATP) with a K_i of 414 μM. Notably, all kinetic parameters are in the same order of magnitude as those of other kinases inside glycosomes [41,44]. Moreover, a similar situation has been found in glycosomal PGK isoenzymes [44]. It has been suggested that PGKC (inhibited by substrate and with a lower K_m) could function when the ratio of ATP/ADP is low, for example during gluconeogenesis. In contrast, PGKA (not inhibited by substrate and with a

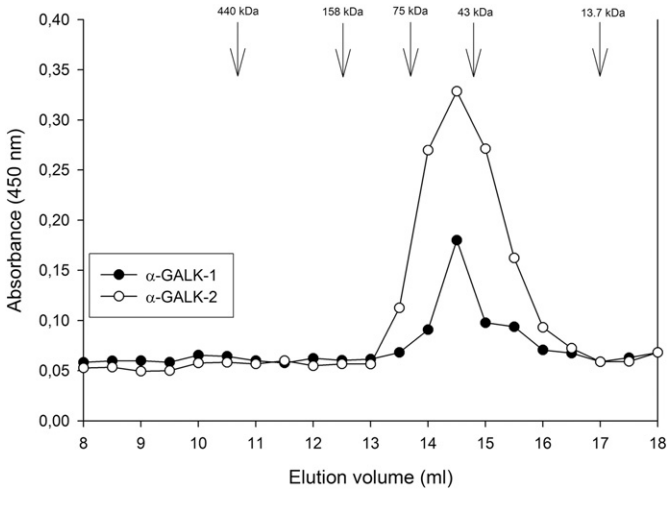


Fig. 3. Elution profile obtained by gel filtration chromatography of the natural *Tc*GALKs. The native molecular mass was determined by gel-filtration chromatography on a Superdex 200 10/300 GL column (Tricorn™-Amersham Biosciences) calibrated with molecular markers (Amersham Biosciences). For natural enzymes a glycosome-enriched fraction (GEF, 4.75 mg/ml), was applied onto the column. Eluted fractions of the GEF were collected and immune-probed using anti-*Tc*GALK-1 and anti-*Tc*GALK-2 by ELISA at 450 nm. The elution positions of the molecular weight markers used are indicated by arrows:ferritin (440 kDa), aldolase (158 kDa) conalbumin (75 kDa), ovalbumin (43 kDa), and ribonuclease A (13.7 kDa).

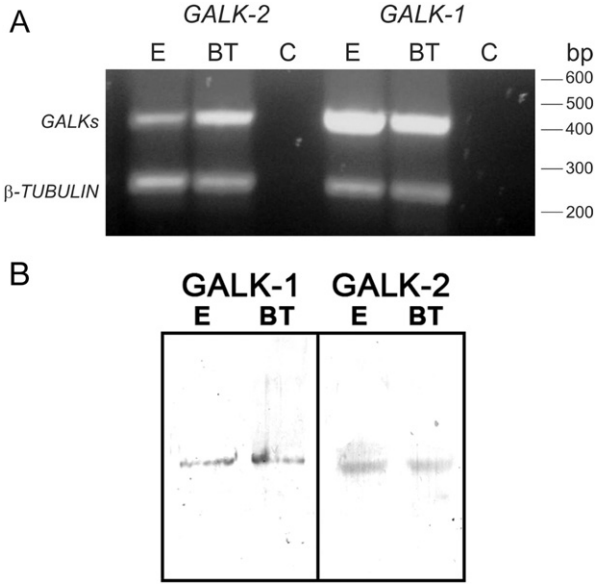


Fig. 4. Expression of galactokinases in *T. cruzi*. Panel A. Amplification by RT-PCR of the *TcGALKs* and β-*TUB* (tubulin) genes with total RNA from epimastigotes (E) and bloodstream trypomastigotes (BT), using a control (C) without reverse transcriptase. Panel B. Immunodetection of the *Tc*GALK-1 and *Tc*GALK-2 proteins in homogenates of epimastigotes (E) and bloodstream trypomastigotes (BT) using the antisera anti-*Tc*GALK-1 and 2.

higher K_m) could operate in the opposite condition by favoring glycolytic flux, instead gluconeogenesis [44]. Therefore, the difference in kinetic parameters between *Tc*GALKs suggests diverse roles in the glycosome,

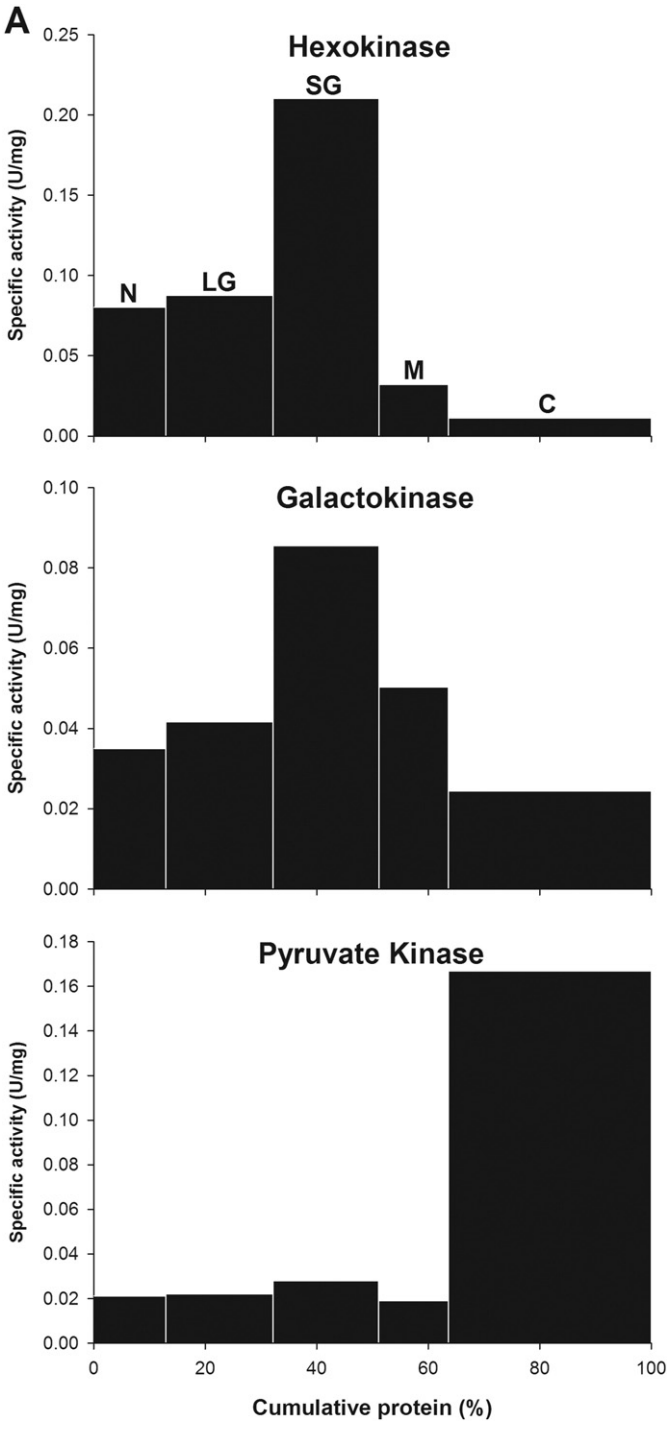


Fig. 5. Subcellular localization of galactokinase in *T. cruzi* epimastigotes. A homogenate of *T. cruzi* epimastigotes was prepared by grinding washed cells with silicon carbide (200 mesh), and fractionated by both, differential and isopycnic centrifugation. Fractions obtained were analyzed by enzymatic assay and immunodetection. Panel A. Distribution profile of GALK activity and marker enzymes (hexokinase for glycosomes and pyruvate kinase for cytosol) obtained by differential centrifugation of a homogenate of *T. cruzi* epimastigotes. Panel B. Western blot analysis. Proteins (60 μg/lane) of subcellular fractions were separated by SDS-PAGE, blotted and probed with the antisera anti-*Tc*GALK-1 and anti-*Tc*HK. Abbreviations in the different panels indicate the fractions: N Nuclear, LG Large Granular, SG Small Granular, M Microsomal, C Cytosol, PG Purified Glycosomes. Panel C. Western blot analysis of the fractions collected from the isopycnic gradient centrifugation using anti-*Tc*GALK-2 and anti-*Tc*HK.

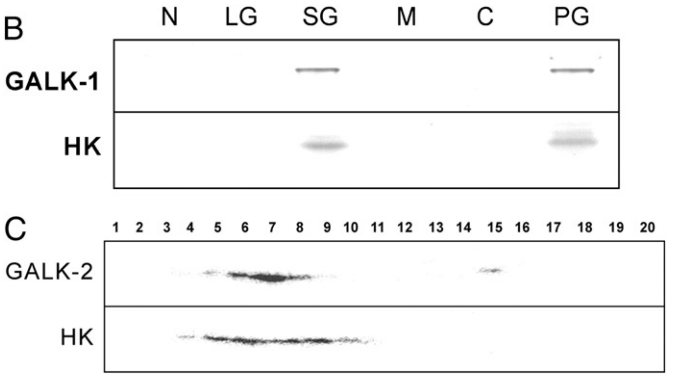


Fig. 5 (*continued*).

with the isoenzymes operating differentially in distinct metabolic situations. Specifically, *Tc*GALK-2 could operate when the ratio of ATP/ADP is low; for instance, in metacyclic trypomastigotes when they reach the rectal vault of triatomines. In that location, there is no glucose availability, however the parasites maintain their cellular surface covered with glycoconjugates rich in terminal β-galactopyranosyl residues, with attached the sialic acid transferred from host cells.These glycoconjugates are involved in the parasite's survival in the mammalian host [45,46]. In contrast, *Tc*GALK-1 could operate in a condition with a high ATP/ADP ratio, where *Tc*GALK-2 is inhibited by its nucleotide substrate and *Tc*GALK-1 is active. It provides the parasite with the ability to phosphorylate galactose no matter phosphate potential inside the glycosomes.

Trypanosomatids depend completely on the carbon sources present in their hosts for their energy metabolism. *T. cruzi* has a complex life cycle, involving a hematophagous insect vector (triatomine bug) and a mammalian host, where the parasite undergoes different morphological and metabolic, adaptive changes. The epimastigotes present in the triatomine gets its free energy using L-proline, glutamine and histidine, the constituents found in a large proportion in the hemolymph of the insect vectors [47]. Nevertheless, when epimastigotes are cultured in axenic medium, they use preferentially glucose [48]. In mammals, the trypomastigote form uses glucose, an abundant sugar in the bloodstream of these hosts, and exhibits a glycolytic metabolism [48]. The amastigotes reside in the cytosol of the mammalian host cells with sugar phosphate availability, but for its energy generation depends mostly on fatty acid catabolism [49]. Indeed, the presence in the parasite genome of hexose phosphate transporters, not present in *T. brucei* and *L. major*, as well as a diversity of sugar kinases, also present in *L. major* but not in *T. brucei*, may be linked to the possible carbohydrate availability for the amastigotes in the cytosol of host cells [12,50]. In that respect, galactose is another hexose present in the bloodstream and in the cytosol of mammalian host cells, which can be used for the UDP-Gal synthesis and also as a free energy source. This sugar is taken up, by the mammalian host, usually in the form of disaccharide lactose, which is split by the digestive enzyme lactase into its hexose moieties, glucose and galactose. This latter hexose uses the same transport mechanism as glucose and its concentration in the systemic blood is around 1 mM [51].

In many organisms, galactose is metabolized by the Leloir pathway to produce UDP-galactose, the substrate of the glycosylation route, or to produce glucose 6-phosphate, an intermediate of the glycolytic and pentose phosphate pathways, that can be used to generate ATP and NADPH [17]. In higher plants, enzymes of this Leloir pathway are required for the synthesis of galactose to be incorporated into the cell wall [20]. In the trypanosomatids *T. cruzi* and *T. brucei*, but not in *L. major*, the biosynthesis of UDP-galactose has been proposed to occur only via epimerization of the abundant nucleotide sugar UDP-glucose by the UDP-Glc 4-epimerase [2], since these two parasites are unable to take up galactose from the environment by the *Tcr*HT1, *Tb*HT1 and *Tb*HT2 transporters [7,8]. Furthermore, the gene which encodes the

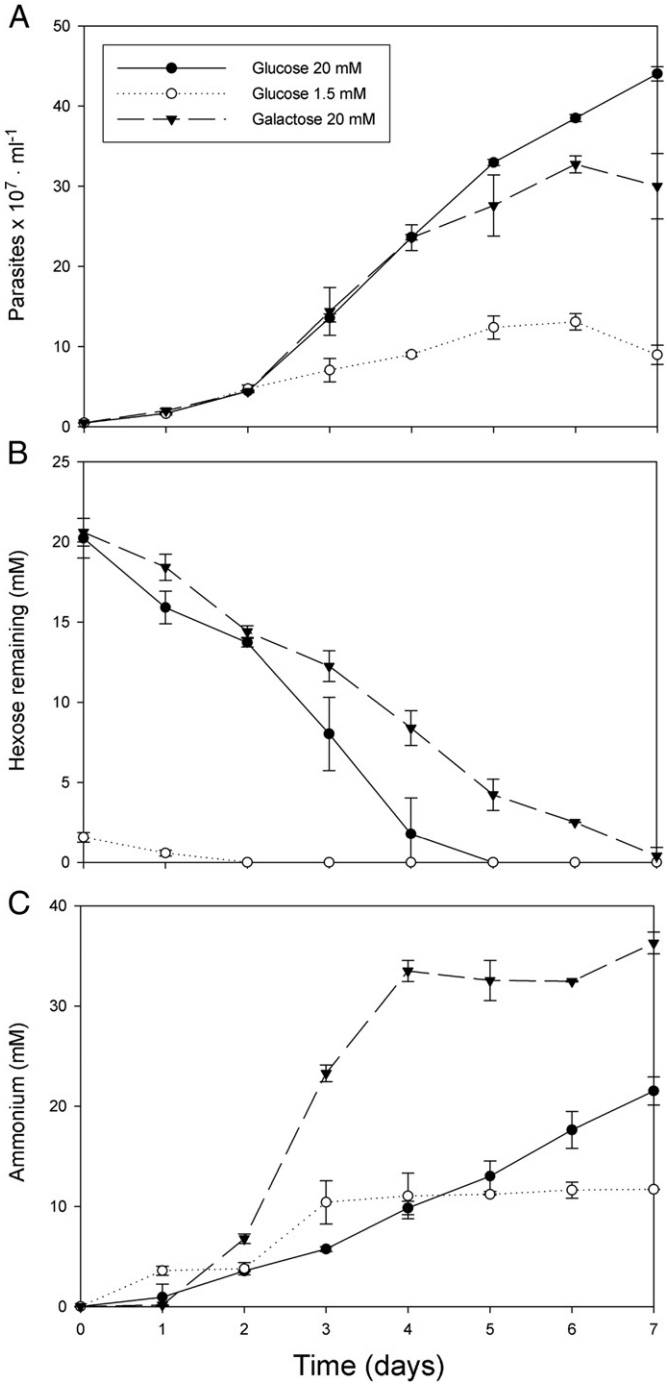


Fig. 6. Growth of epimastigotes in LIT medium, partially depleted in glucose (1.5 mM of glucose), and then supplemented with glucose or galactose (20 mM in each case). All cultures of epimastigotes (100 ml) were started with 5×10^6 cells/ml and incubated at 28 °C. Growth was then followed by counting numbers of parasites in a Neubauer chamber; samples were collected every 24 h until the culture reached the stationary growth phase. Supernatants were used for determination of glucose, galactose and ammonium concentrations in the culture medium. Panel A. Growth curve of epimastigotes. Panel B. Levels of hexose. Panel C. Ammonium concentration.

Leloir pathway enzyme, galactose 1-phosphate uridyltransferase, is absent in all trypanosomatids [10,11,12]. Nevertheless, recently in *T. cruzi* and *L. major*, but not in *T. brucei*, have been detected and characterized the UDP-sugar pyrophosphorylase, an enzyme so far found in plants that utilize diverse sugar 1-phosphate and UTP to form UDP-sugars [3,15]. This finding together with the presence of two active galactokinases in *T. cruzi*, may explain the existence of an alternative pathway for the synthesis of UDP-galactose, which could function independently of the UDP-Glc biosynthesis. This salvage route uses free galactose that can be generated from the degradation of glycoproteins or glycolipids, which are recycled into the cell by specific transporters [3]. Then, the enzyme galactokinase could catalyze the activation of galactose and produce galactose 1-phosphate, that together with UTP could be utilized by UDP-sugar pyrophosphorylase to generate UDP-galactose.

As mentioned above, the pathway that allows the formation of UDP-Gal could be linked to the generation of glucose 6-phosphate, since all enzymes needed for this purpose are located in the same organelle, the glycosome [4]. In that way UDP-Gal could be epimerized by the enzyme UDP-Glc 4-epimerase to UDP-Glc that is then transformed into α-D-Glc-1-P and UTP by the enzyme USP. Subsequently, PGM could catalyze the interconversion of α-D-Glc-1-P into α-D-Glc-6-P, and this latter metabolite may be able to enter into the glycolytic pathway in order to generate ATP and feed the Pentose Phosphate Pathway too [17]. However, glucose and galactose in the glycosome may have different oxidation routes, since the parasites growing with galactose produce higher ammonium concentrations than those with glucose. The transport of this hexose in epimastigotes of *T. cruzi*, a strain from Brazil, was studied by Warren and co-workers [52]. They reported that galactose supported the respiration of the parasites and proposed the existence of an active transporter functioning at low concentrations of this sugar and a facilitated diffusion carrier operative at high concentrations [52]. However, Tetaud et al. [8] using ultra-pure D-galactose as a probe, found that this sugar is not a substrate of the *T. cruzi* HT1 transporter (*Tcr*HT1). Furthermore, Tetaud et al. [53] studied the uptake of several hexoses in epimastigotes through inhibition of glucose transport, among them, galactose; and they found it does not inhibit the glucose uptake. In contrast, we observed that *T. cruzi* epimastigotes of the EP strain are able to grow in LIT medium supplemented with galactose, but it is still unknown how this sugar is transported into the parasite. This discrepancy could be either due to that the specific transporter of galactose should be different from the *Tcr*HT1, or it may be due to strain-specific differences. On the other hand, in the yeast *S. cerevisiae*, this sugar is internalized by two transporters, one with a high affinity and specificity, Gal2p, expressed in the presence of galactose and repressed in the presence of glucose. The other transporter is less selective with regard to the nature of the hexose and is a member of the HXT family [54]. We cannot exclude that in *T. cruzi* galactose could enter by a transporter similar to Gal2p. In the genome of this parasite there are genes encoding sequences homologous to proteins of the MFS superfamily, although the specific functions of these genes products are still unknown.

5. Conclusions

Two different galactokinase genes are expressed in *Trypanosoma cruzi*, both enzymes are active, located in glycosomes, which suggests an alternative pathway to synthesize UDP-galactose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.parint.2016.06.008.

References

[1] Z. Brener, Biology of *Trypanosoma cruzi*, Annu. Rev. Microbiol. 27 (1973) 347–382.

[2] D.C. Turnock, M.A. Ferguson, Sugar nucleotide pools of *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania major*, Eukaryot. Cell. 6 (2007) 1450–1463.
[3] T. Yang, M. Bar-Peled, Identification of novel UDP-sugar pyrophosphorylase with broad substrate specificity in *Trypanosoma cruzi*, Biochem. J. 429 (2010) 533–543.
[4] F. Opperdoes, J.P. Szikora, In silico prediction of the glycosomal enzymes of *Leishmania major* and trypanosomes, Mol. Biochem. Parasitol. 147 (2006) 193–206.
[5] S. Kuettel, C.T. Majken, M.L. Wadum, S. Güther, K. Mariño, C. Riemer, M.A. Ferguson, The *de novo* and salvage pathways of GDP-mannose biosynthesis are both sufficient for the growth of bloodstream-form *Trypanosoma brucei*, Mol. Microbiol. 84 (2012) 340–351.
[6] K. Mariño, M.L. Güther, A.K. Wernimont, M. Amani, R. Hui, M.A. Ferguson, Identification, subcellular localization, biochemical properties, and high-resolution crystal structure of *Trypanosoma brucei* UDP-glucose pyrophosphorylase, Glycobiology 20 (2010) 1619–1630.
[7] J.R. Roper, M.A. Ferguson, Cloning and characterisation of the UDP-glucose 4′-epimerase of *Trypanosoma cruzi*, Mol. Biochem. Parasitol. 132 (2003) 47–53.
[8] E. Tetaud, M.P. Barrett, F. Bringaud, T. Baltz, Kinetoplastid glucose transporters, Biochem. J. 325 (1997) 569–580.
[9] J.I. MacRae, S.O. Obado, D.C. Turnock, J.R. Roper, M. Kierans, J.M. Kelly, M.A. Ferguson, The suppression of galactose metabolism in *Trypanosoma cruzi* epimastigotes causes changes in cell surface molecular architecture and cell morphology, Mol. Biochem. Parasitol. 147 (2006) 126–136.
[10] N.M. El-Sayed, P.J. Myler, D.C. Bartholomeu, et al., The genome sequence of *Trypanosoma cruzi*, etiologic agent of Chagas disease, Science 309 (2005) 409–415.
[11] A.C. Ivens, C.S. Peacock, E.A. Worthey, et al., The genome of the kinetoplastid parasite, *Leishmania major*, Science 309 (2005) 436–442.
[12] M. Berriman, E. Ghedin, C. Hertz-Fowler, et al., The genome of the African trypanosome *Trypanosoma brucei*, Science 309 (2005) 416–422.
[13] K.J. Isselbacher, A mammalian uridine diphosphate galactose pyrophosphorylase, J. Biol. Chem. 232 (1958) 429–444.
[14] A.C. Lamerz, S. Damerow, B. Kleczka, M. Wiese, G. van Zandbergen, J. Lamerz, A. Wenzel, F.F. Hsu, J. Turk, S.M. Beverly, F.H. Routier, Deletion of UDP-glucose pyrophosphorylase reveals a UDP-glucose independent UDP-galactose salvage pathway in *Leishmania major*, Glycobiology 20 (2010) 872–882.
[15] S. Damerow, A.C. Lamerz, T. Haselhort, J. Führing, P. Zarnovican, M. von Itztein, F.H. Routier, *Leishmania* UDP-sugar pyrophosphorylase: the missing link in galactose salvage? J. Biol. Chem. 285 (2010) 878–887.
[16] M.D. Urbaniak, D.C. Turnock, M.A. Ferguson, Galactose starvation in a bloodstream form *Trypanosoma brucei* UDP-glucose 4′-epimerase conditional null mutant, Eukaryot. Cell. 5 (2006) 1906–1913.
[17] L.F. Leloir, The enzymatic transformation of uridine diphosphate glucose into a galactose derivative, Arch. Biochem. Biophys. 36 (1951) 186–190.
[18] P.A. Frey, The Leloir pathway: a mechanistic imperative for three enzymes to change the stereochemical configuration of a single carbon in galactose, FASEB J. 10 (1996) 461–470.
[19] J.R. Sherman, J. Adler, Galactokinase from *Escherichia coli*, J. Biol. Chem. 238 (1963) 873–878.
[20] M.R. Heinrich, The purification and properties of yeast galactokinase, J. Biol. Cell. 239 (1964) 50–53.
[21] F.J. Ballard, Kinetic studies with liver galactokinase, Biochem. J. 101 (1966) 70–75.
[22] P. Dey, Galactokinase of *Vicia faba* seeds, Eur. J. Biochem. 136 (1983) 155–159.
[23] H.M. Holden, J.B. Thoden, D.J. Timson, R.J. Reece, Galactokinase: structure, function and role in type II galactosemia, Cell. Mol. Life Sci. 61 (2004) 2471–2484.
[24] P. Bork, C. Sander, A. Valencia, Convergent evolution of similar enzymatic function on different protein folds: the hexokinase, ribokinase, and galactokinase families of sugar kinases, Protein Sci. 2 (1993) 31–40.
[25] E. Camargo, Growth and differentiation in *Trypanosoma cruzi*. I. Origin of metacyclic *Trypanosoma* in liquid media, Rev. Inst. Med. Trop. Sao Paulo 6 (1964) 93–100.
[26] M.S. Bertelli, Z. Brener, Infection of tissue culture cells with bloodstream trypomastigotes of *Trypanosoma cruzi*, J. Parasitol. 66 (1980) 992–997.
[27] E. Zuckerkandl, L. Pauling, Evolutionary divergence and convergence in proteins, in: V. Bryson, H.J. Vogel (Eds.), Evolving Genes and Proteins, New York, Academic Press 1965, pp. 97–166.
[28] K. Tamura, J. Dudley, M. Nei, S. Kumar, MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0, Mol. Biol. Evol. 24 (2007) 1596–1599.
[29] W. Studier, Protein production by auto-induction in high-density shaking cultures, Protein Expr. Purif. 41 (2005) 207–234.
[30] O. Lowry, N. Rosebrough, L. Farr, R. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[31] A.J. Cáceres, W. Quiñones, M. Gualdrón, A. Cordeiro, L. Avilán, P.A. Michels, J.L. Concepción, Molecular and biochemical characterization of novel glucokinases from *Trypanosoma cruzi* and *Leishmania* spp. Mol. Biochem. Parasitol. 156 (2007) 235–245.
[32] H.U. Bergmeyer, Methods of Enzymatic Analysis, Academic Press, New York, 1983.
[33] U.K. Laemmli, Cleavage of structural proteins during assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[34] J.L. Concepción, D. Gonzalez-Pacanowska, J.A. Urbina, 3-hydroxy-3-methyl-glutaryl-CoA Reductase in *Trypanosoma* (*Schizotrypanum*) *cruzi*: subcellular localization and kinetic properties, Arch. Biochem. Biophys. 352 (1998) 114–120.
[35] W. Quiñones, J.A. Urbina, M. Dubourdieu, J. Concepción, The glycosome membrane of *Trypanosoma cruzi* epimastigotes: protein and lipid composition, Exp. Parasitol. 106 (2004) 135–149.
[36] J.R. Wiśniewski, A. Zougman, N. Nagaraj, M. Mann, Universal sample preparation method for proteome analysis, Nat. Methods 6 (2009) 359–362.
[37] A.J. Cáceres, P.A. Michels, V. Hannaert, Genetic validation of aldolase and glyceraldehyde-3-phosphate dehydrogenase as drug targets in *Trypanosoma brucei*, Mol. Biochem. Parasitol. 169 (2010) 50–54.
[38] T. Yang, L. Bar-Peled, L. Gebhart, S.G. Lee, M. Bar-Peled, Identification of galactouronic acid-1-phosphate kinase, a new member of the GHMP kinase superfamily in plants, and comparison with galactose-1-phosphate kinase, J. Biol. Chem. 284 (2009) 21526–21535.
[39] J.B. Thoden, H. Holden, Molecular structure of galactokinase, J. Biol. Chem. 278 (2003) 33305–33311.
[40] K. Fung, C. Clayton, Recognition of a peroxisomal tripeptide entry signal by the glycosomes of *Trypanosoma brucei*, Mol. Biochem. Parasitol. 45 (1991) 261–264.
[41] A. Cáceres, R. Portillo, H. Acosta, D. Rosales, L. Avilán, L. Salazar, M. Dubourdieu, P.A.M. Michels, J. Concepción, Molecular and biochemical characterization of hexokinase from *Trypanosoma cruzi*, Mol. Biochem. Parasitol. 126 (2003) 251–262.
[42] L. Li, Y. Liu, W. Wang, J. Cheng, W. Zhao, P. Wang, A highly efficient galactokinase from *Bifidobacterium infantis* with broad substrate specificity, Carbohydr. Res. 355 (2012) 35–39.
[43] J. Yang, D. Hoffmeister, L. Liu, X. Fu, J.S. Thorson, Natural product glycorandomization, Bioorg. Med. Chem. 12 (2004) 1577–1584.
[44] X. Barros-Álvarez, A.J. Cáceres, P.A. Michels, J.L. Concepción, W. Quiñones, The phosphoglycerate kinase isoenzymes have distinct roles in the regulation of carbohydrate metabolism in *Trypanosoma cruzi*, Exp. Parasitol. 143 (2014) 39–47.
[45] V.L. Pereira-Chioccola, A. Acosta-Serrano, I. Correia de Almeida, M.A. Ferguson, T. Souto-Padron, M.M. Rodrigues, L.R. Travassos, S. Schenkman, Mucin-like molecules form a negatively charged coat that protects *Trypanosoma cruzi* trypomastigotes from killing by human anti-alpha-galactosyl antibodies, J. Cell Sci. 113 (2000) 1299–1307.
[46] L. Freire-de-Lima, L.M. Fonseca, T. Oeltmann, L. Mendonça-Previato, J.O. Previato, The trans-sialidase, the major *Trypanosoma cruzi* virulence factor: three decades of studies, Glycobiology 25 (2015) 1142–1149.
[47] J.A. Atwood 3rd, D.B. Weatherly, T.A. Minning, B. Bundy, C. Cavola, F.R. Opperdoes, R. Orlando, R.L. Tarleton, The *Trypanosoma cruzi* proteome, Science 309 (2005) 473–476.
[48] F. Bringaud, L. Rivière, V. Coustou, Energy metabolism of trypanosomatids: adaptation to available carbon sources, Mol. Biochem. Parasitol. 149 (2006) 1–9.
[49] J.J. Cannata, J.J. Cazzulo, The aerobic fermentation of glucose by *Trypanosoma cruzi*, Comp. Biochem. Physiol. B 79 (1984) 297–308.
[50] M.L. Ginger, P.G. McKean, R. Burchmore, K.M. Grant, Proteomic insights into parasite biology, Parasitology 139 (2012) 1101–1102.
[51] N.L. Keim, R.J. Levin, P. Havel, Carbohydrates, in: M.D. Shils, M. Shike (Eds.), Modern Nutrition in Health and Disease, 10th ed.Lippincott Williams α Wilkins 2006, p. 74.
[52] L.G. Warren, W.B. Kitzman, Metabolism of *Schizotrypanum cruzi* Chagas. II. Galactose utilization, J. Parasitol. 49 (1963) 808–813.
[53] E. Tetaud, S. Chabas, C. Giroud, M.P. Barrett, T. Baltz, Hexose uptake in *Trypanosoma cruzi*: structure–activity relationship between substrate and transporter, Biochem. J. 317 (1996) 353–359.
[54] D.J. Timson, Galactose metabolism in *Saccharomyces cerevisiae*, Dyn. Biochem. Process. Biotechnol. Mol. Biol. 1 (2007) 63–73.