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Trypanosoma rangeli expresses a β-galactofuranosyl transferase

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ABSTRACT

Glycoconjugates play essential roles in cell recognition, infectivity and survival of protozoan parasites within their insect vectors and mammalian hosts. β-galactofuranose is a component of several glycoconjugates in many organisms, including a variety of trypanosomatids, but is absent in mammalian and African trypanosomes. Herein, we describe the presence of a β(1–3) galactofuranosyl transferase (GALFT), an important enzyme of the galactofuranose biosynthetic pathway, in *Trypanosoma rangeli*. The *T. rangeli GALFT* gene (*TrGALFT*) has an ORF of 1.2 Kb and is organized in two copies in the *T. rangeli* genome. Antibodies raised against an internal fragment of the transferase demonstrated a 45 kDa protein coded by *TrGALFT* was localized in the whole cytoplasm, mainly in the Golgi apparatus and equally expressed in epimastigotes and trypomastigotes from *T. rangeli*. Despite the high sequence similarity with *Trypanosoma cruzi* and *Leishmania* spp. orthologous *Tr*GALFT showed a substitution of the metal-binding DXD motif, conserved amongst glycosyltransferases, for a DXE functionally analogous motif. Moreover, a reduced number of *GALFT* genes were present in *T. rangeli* when compared with other pathogenic kinetoplastid species.

1. Introduction

The carbohydrates present in glycoconjugates of the cell surface of trypanosomatids are essential to a variety of cell events, including infectivity and/or virulence in insect vectors and mammalian hosts. Among these, galactofuranose, a glycoside present in most pathogenic trypanosomatids, fungi and bacteria but absence in mammals, represents an interesting target for therapeutic approaches of several diseases related to these organisms (de Lederkremer and Colli, 1995; Oppenheimer et al., 2011; Pedersen and Turco, 2003; Shibata and Okawa, 2011). In *Trypanosoma cruzi*, galactofuranose is found in the lipopeptidophosphoglycan (LPPG) coat (de Lederkremer et al., 1980, 1985; Golgher et al., 1993), in mucins and other proteins, including the 80–90 kDa glycoproteins and glycopeptides involved in host cell adhesion and parasite internalization (De Arruda et al., 1989; Haynes et al., 1996; Serrano et al., 1995). Galactofuranose is also present in *Leishmania* spp. glycoconjugates related to parasite structure, such as lipophosphoglycans (LPG) (McConville et al., 1990; Previato et al., 1997; Turco and Descoteaux, 1992).

The metabolic pathways involved in the removal or attachment of galactofuranose from trypanosomatids glycoconjugates have been reported, including a *T. cruzi* exo β-D-galactofuranosidase (Miletti et al., 2003). Genes encoding β(1–3) galactofuranosyl transferase (*GALFT*) enzymes have been described for *T. cruzi* (El-Sayed et al., 2005) and *Leishmania* spp. (Ivens et al., 2005; Spãth et al., 2000; Zhang et al., 2004), but expression and protein characterization was achieved only for *Leishmania* sp. On the other hand, the absence of *GALFT* in African trypanosomes remains an intriguing question (Berriman et al., 2005).

Considering the importance of galactofuranose glycoconjugates for mammalian pathogenic trypanosomatids, in this work, we described for the first time the presence of GALFT in the protozoan parasite *Trypanosoma rangeli*. This parasite infects a variety of mammalian species, including humans in Central and South America, and probably has an impact on the misdiagnosis of Chagas disease (Grisard et al., 1999a, 2010).

After colonization of the triatomine vector gut, the parasites reach the hemocoel, where the epimastigotes multiplicate in the hemolymph, invade the salivary glands and transform into metacyclic trypomastigotes (D'Alessandro, 1976; Grisard et al., 1999b; Guhl and Vallejo, 2003). Transmission to the mammalian host occurs by the bite of infected triatomines, in particular those from the genus *Rhodnius*. Although *T. rangeli* is considered pathogenic to the invertebrate host, this parasite is completely harmless to the mammalian host, where its biology, including intracellular multiplication ability, remains controversial.

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While several aspects of the *T. rangeli* life cycle within triatomine bugs are well known (Grisard et al., 1999a, 2010; Guhl and Vallejo, 2003), the parasite development in the mammalian host remains unknown. This lack of information emphasizes the need for further studies into the parasite–host cell interaction, where galactofuranose glycoconjugates might play an important role. As several glycoconjugates have important roles in cell–parasite interactions in pathogenic protozoa, the expression of beta galactofuranosyl transferase, a key enzyme in the galactofuranose pathway, was investigated in the non-pathogenic parasite *T. rangeli*.

2. Materials and methods

2.1. Parasites and culture conditions

Epimastigotes of *T. rangeli* Choachí strain and *T. cruzi* Y strain were maintained in liver infusion tryptose (LIT) medium supplemented with 10% FCS at 27 °C after cyclic passages in mice-triatomine-mice. Differentiation of *T. rangeli* epimastigotes to trypomastigotes was accomplished *in vitro* under chemically defined conditions, as described previously (Koerich et al., 2002).

T. cruzi trypomastigotes were obtained from the supernatant of Vero cells (ATCC-CCL81) cultures infected with bloodstream trypomastigote forms and maintained in Dulbecco's modified Eagle's Medium – DMEM (Sigma–Aldrich), pH 7.4, supplemented with 5% FCS (Carvalho and De Souza, 1983; Eger-Mangrich et al., 2001).

2.2. T. rangeli DNA and RNA isolation

Total DNA was purified using 2×10^8 *T. rangeli* epimastigotes by standard phenol–chloroform procedures. Total RNA was obtained using the Trizol® reagent (Invitrogen). Messenger RNA (mRNA) was purified using the μMACs mRNA Isolation (Miltenyi Biotec). Assessment of purity and quality was performed spectrophotometrically (260/280 nm) and by ethidium bromide-stained agarose gel electrophoresis.

2.3. Cloning and sequencing of TrGALFT gene

Using *T. rangeli* transcriptome data (Grisard et al., 2010), two primers (GalF 5′-GAG CTT GAG AAG ATT TAT GGG TGG-3′/GalR 5′-GTT CTC GTC AAA ATA TCC CAC CG-3′) were designed to obtain the entire 1.2 Kb of the *T. rangeli* β-galactofuranosyl transferase gene sequence by RT-PCR using SuperScript II® reverse transcriptase (RT) (Invitrogen). Amplification of the 5′ and 3′ ends of *TrGALFT* cDNA was performed using the specific primers and a primer directed to the spliced leader sequence (5′-CCC GAA TTC TGT ACT ATA TTG GT-3′) or an oligo(dT)35 primer (Invitrogen).

All amplification products were cloned in pGEM-T-Easy vector (Promega) and sequenced on both strands in a Megabace 1000® DNA Analysis System using the DYEnamic ET terminators kit (GE Healthcare) according to the manufacturer's conditions.

2.4. Sequence assembling and analysis

All high quality DNA sequences (Phred $\geqslant$20) were assembled and analyzed using the Phred/Phrap/Consed package (Ewing and Green, 1998) and then compared with public databases using the BLAST algorithm (Altschul et al., 1997). Analysis of deduced protein sequences was carried out using the Proteomic Tools provided by the ExPASy (http://www.expasy.org). Amino acid sequences of other kinetoplastid species GALFT's were retrieved from the TriTrypDB (Aslett et al., 2010) for comparative and phylogenetic analysis (Supplementary material). Multiple alignments of amino acid sequences were performed using CLUSTAL W package (Thompson et al., 1994), leading to construction of phylogenetic trees using neighbor joining method and bootstrap analysis by the MEGA 4.1 software (Tamura et al., 2007).

2.5. Southern blot

T. rangeli genomic DNA (15 μg/lane) was digested with *Taq*I, *Eco*RI and *Pst*I restriction endonucleases, separated by electrophoresis, and transferred onto nylon membranes (Sigma–Aldrich). A peroxidase-labeled probe was prepared using a 519 bp PCR fragment (nucleotides 304–807) and the ECL Direct Nucleic Acid Labeling and Detection System (GE Healthcare). The membrane was hybridized and washed using a standard protocol and developed using the ECL kit according to the manufacturer's directions.

2.6. Heterologous expression and antibody production

For expression, a *TrGALFT* gene fragment (aa 96–269) (Fig. 1) was amplified by PCR using gene specific primers ExpGal-F (5′-CTC GAG GAG CTT GAG AAG ATT TAT GGG TGG-3′) and ExpGal-R (5′-GGA TCC GTT CTC GTC AAA ATA TCC CAC CGT-3′) modified with appropriate restriction sites (the underlined nucleotides correspond to *Xho*I and *Bam*HI restriction sites, respectively). The 519 bp amplicon pre-digested with *Xho*I and *Bam*HI, was cloned into a pET14b expression vector (Novagen) and used to transform *Escherichia coli* BL21 (DE3) competent cells.

The His6-tagged *Tr*GALFT recombinant protein was obtained by induction of the cells at OD600 of 0.6 with 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) at 27 °C for 3 h. Cells were then harvested by centrifugation (6000*g* for 15 min at 4 °C) and lysed using a denaturing buffer (8 M Urea; 10 mM Tris; 100 mM NaH_2PO_4, pH 8.0). The samples were then incubated at 65 °C to dissolve inclusion bodies and centrifuged (10,000*g* for 30 min at 4 °C). The supernatants were submitted to purification using Ni–NTA affinity chromatography columns (Qiagen) and the purity of the obtained protein was assessed by 12% SDS–PAGE stained with Coomassie brilliant blue R-250. Protein concentrations were determined by the Bradford method using bovine serum albumin as standard.

The purified 22 kDa recombinant *Tr*GALFT (r*Tr*GALFT) was subcutaneously inoculated in Balb/C mice (50 μg) using Freund's complete adjuvant (Sigma–Aldrich), with two consecutive inoculations at 10 days intervals using Alu-Gel (Serva). Mouse serum was collected 10 days after the third injection and tested for anti-*Tr*GALFT antibodies. The UFSC Ethics Committee for Animal Care approved all procedures involving experimental animals (Protocol 23080.025618/2009-81).

2.7. Western blot

*Tr*GALFT recombinant protein and total epimastigotes and trypomastigotes protein extracts (30 μg) from *T. rangeli* and *T. cruzi* were mixed with Laemmli sample buffer, boiled for 5 min and resolved on 12% SDS–PAGE gels. Protein separations were transferred onto nitrocellulose membranes (GE Healthcare) in appropriate buffer (25 mM Tris; 192 mM glycine; 20% v/v methanol, pH 8.3) in a TE 70 Semi-Dry Transfer Unit (GE Healthcare). Membranes were then blocked with 5% non-fat milk in PBS (pH 7.4) with 0.1% Tween-20 (PBS-T) for 1 h at room temperature. After blocking, membranes were incubated for 1 h with anti-*Tr*GALFT polyclonal serum (1:500), anti-His-Tag monoclonal antibody (1:10,000) or anti-α tubulin monoclonal antibody (1:10,000) used as loading control. After four washes in PBS-T, membranes were incubated for 1 h with anti-mouse IgG antibody conjugated with peroxidase (1:10,000) (Sigma–Aldrich). Membranes were developed using ECL reagent (GE Healthcare) and detection was achieved in radiographic films.