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The GTPase TcRjl of the human pathogen *Trypanosoma cruzi* is involved in the cell growth and differentiation

Guilherme Rodrigo Reis Monteiro dos Santos [a], José Luciano Nepomuceno-Silva [b], Luiz Dione Barbosa de Melo [c], José Roberto Meyer-Fernandes [d], Didier Salmon [e], Ricardo Luiz Azevedo-Pereira [f], Ulisses Gazos Lopes [a,*]

[a] *Laboratório de Parasitologia Molecular, Instituto de Biofísica Carlos Chagas Filho, CCS, UFRJ, Rio de Janeiro, Brazil*
[b] *Laboratório Integrado de Bioquímica Hatisaburo Masuda, NUPEM/UFRJ, Pólo Barreto, Universidade Federal do Rio de Janeiro – Campus Macaé, Macaé, Brazil*
[c] *Laboratório de Genética Molecular, Departamento de Biotecnologia, Instituto Federal de Educação, Ciência e Tecnologia do Rio de Janeiro, IFRJ, Campus Maracanã, Rio de Janeiro, Brazil*
[d] *Laboratório de Bioquímica Celular, Instituto de Bioquímica Médica, CCS, UFRJ, Rio de Janeiro, Brazil*
[e] *Instituto de Bioquímica Médica, CCS, UFRJ, Rio de Janeiro, Brazil*
[f] *Instituto de Biofísica Carlos Chagas Filho, Instituto Nacional de Ciência e Tecnologia de Biologia Estrutural e Bioimagem – INBEB, Universidade Federal do Rio de Janeiro, Brazil*

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ABSTRACT

The protozoan parasite *Trypanosoma cruzi*, the etiological agent of Chagas Disease, undergoes through a complex life cycle where rounds of cell division and differentiation occur initially in the gut of triatominae vectors and, after transmission, inside of infected cells in vertebrate hosts. Members of the Ras superfamily of GTPases are molecular switches which play pivotal regulatory functions in cell growth and differentiation. We have previously described a novel GTPase in *T. cruzi*, TcRjl, which belongs to the RJL family of Ras-related GTP binding proteins. Here we show that most of TcRjl protein is found bound to GTP nucleotides and may be locked in this stage. In addition, we show that TcRjl is located close to the kinetoplast, in a region corresponding possibly to flagellar pocket of the parasite and the expression of a dominant-negative *TcRjl* construct (*TcRjl*S37N) displays a significative growth phenotype in reduced serum medium. Remarkably, overexpression of TcRjl inhibits differentiation of epimastigotes to trypomastigote forms and promotes the accumulation of intermediate differentiation stages. Our data suggest that TcRjl might play a role in the control of the parasite growth and differentiation.

1. Introduction

The Ras superfamily comprises a large group of monomeric GTP-binding proteins that perform essential roles in the dynamic regulation of many key events in eukaryotic cellular physiology, ranging from vesicle trafficking to cytoskeleton remodeling and mitogenic signaling [1,2]. Although substantially diverse on the activation mechanisms, the regulatory activity of this large group of GTPases is based on a simple cycle in which they achieve an active (effectors interacting) state when bound to GTP nucleotides and are kept on an inactive state after hydrolyzing this GTP to GDP [1]. Members of the Ras superfamily are further classified into at least seven families: Ras, Rho, Rab, Ran, Arf, RGK and RJL. The RJL family was first described in 2004 as a separate group of *Ras*-related GTPase genes whose cellular function is still unclear [3]. In contrast with other *Ras*-related families, which usually exhibit diverse and complex genomic structure, RJL family commonly presents only one representative ortholog per genome. Despite of such low intra-genomic diversity, RJL members can be clearly classified in two distinct subfamilies: Rjl and Rbj. *Rjls* genes are present in many protist lineages, encoding a core GTPase domain, without predictable membrane anchoring signal, while *Rbj* gene products are found in the choanoflagellata/metazoan lineage as chimaeras of a conventional Rjl protein fused to a DNAJ co-chaperone domain [3,4]. In striking contrast with others Ras-related GTPases, all known RJL proteins have in common an altered PM3 motif, theoretically rendering theses proteins unable to efficiently hydrolyze bound GTP [3].

At the present, the function of RJL genes on cell physiology remains unsolved, albeit some evidences derived from analysis of gene expression patterns point to a function of Rbj in the

* Corresponding author. Address: Laboratório de Parasitologia Molecular, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Cidade Universitária, Rio de Janeiro 21949-900, Brazil. Fax: +55 5521 2290 8193.
E-mail addresses: rodrigoreis@biof.ufrj.br (G.R.R.M. dos Santos), nepomuc@yahoo.com (J.L. Nepomuceno-Silva), luiz.dione.melo@gmail. com (L.D.B. de Melo), meyer@bioqmed.ufrj.br (J.R. Meyer-Fernandes), salmon@bioqmed.ufrj.br (D. Salmon), razevedo@biof.ufrj.br (R.L. Azevedo-Pereira), lopesu@biof.ufrj.br (U.G. Lopes).

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development of animal nervous system [3,5] and a comprehensive phyletic profile analysis of RJL suggests a flagellum-associated role [4].

Although functional studies of Ras-related GTPases are classically carried out in yeast and animal models, a growing body of knowledge has been accumulated regarding the function of Ras-related GTP-binding proteins in unicellular eukaryotes, with special emphasis on trypanosomatid parasites [6–8]. In this context, besides being regarded as valuable tools for studying parasite biology, Ras-related proteins have also been considered as potential targets for therapeutic intervention [6,9].

Rjls have been first described in the flagellate parasite *Trypanosoma cruzi*, the etiological agent of Chagas disease, which infects about eight million individuals in Latin America [10]. *TcRjl*, the *T. cruzi* RJL ortholog, is a simple copy gene which is normally transcribed and suffers regular trans-splicing in the three main life cycle stages of the parasite [11]. Here we report additional studies on the TcRjl protein of *T. cruzi*, providing the first experimental evidences for the function of RJL genes.

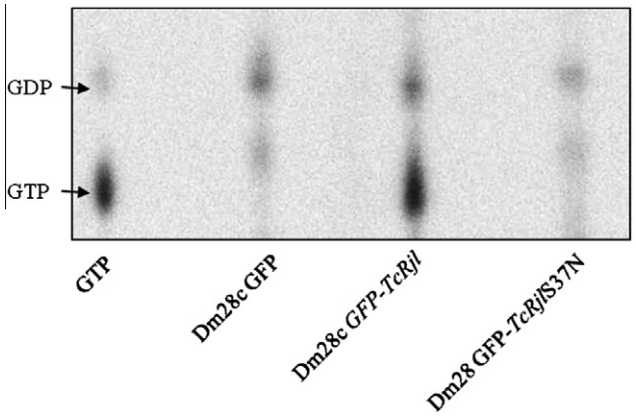

Fig. 1. TcRjl is predominantly bound to GTP nucleotides *in vivo*. *T. cruzi* epimastigotes overexpressing wild type and S37NTcRjl proteins were incubated in TAU-3AAG-H medium with ^{32}P orthophosphate. Cells were lysed and submitted to immunoprecipitation with anti-GFP IgG. Bound radioactive nucleotides were eluted and resolved by Thin-layer chromatography (TLC). Chromatograms of [γ-^{32}P] GTP and [γ-^{32}P] GDP were used as standards. Thin-layer chromatography and autoradiography was as indicated under methods.

2. Methods

2.1. Parasites

T. cruzi epimastigotes, clone Dm28c, were maintained at 28 °C in LIT medium (Liver Infusion Tryptose) [12] supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco) and 0.025 μg/mL hemin (Sigma–Aldrich). Transfected parasites were maintained in LIT medium supplemented with 300 or 600 μg/mL of G418 (Sigma–Aldrich).

2.2. GFP and GFP-TcRjl constructions

T. cruzi lineages used in this work were obtained by transfection with pTEX based vectors [13]. Two derivatives of the pTEX plasmid were produced in order to allow either N or C terminal fusions of *TcRjl orfs* with the green fluorescence protein (GFP). GFP coding region was first amplified by PCR with *Pfx50*™ DNA polymerase (Invitrogen), using pEGFP-1 vector (Clontech) as template and two alternative pairs of primers: GFP-Forward-*Eco*RI (5′-GAATTC ATGGTGAGCAAGGGCGA-3′) and GFP-Reverse-*Sal*I (5′-GTCGACTT ACTTGTACAGCTCGTC-3′) or GFP-Forward-*Spe*I (5′-ACTAGTATGGTG AGCAAGGGCGA-3′) and GFP-ReverseΔstop-codon-*Sal*I (5′-GT CGA CTGCCTTGTACAGCTCGTCCA-3′). Amplicons were first cloned in the pGEM-T Easy vector (Promega) and then subcloned into *Eco*RI and *Sal*I or *Spe*I and *Sal*I sites of the pTEX vector, generating pTEX-GFPc for expression of C-terminal GFP fusion proteins or pTEX-GFPn for N-terminal GFP fusion proteins, respectively. The coding sequence of *TcRjl* was amplified by PCR using genomic DNA of clone Dm28c as template and primers TcRjl-Forward (5′-GTCGACATGAGTGCGAAGACCAG-3′) and TcRjl-Reverse (5′-GTC GACTTACATGAATGCTGCCAC-3′), both with artificial *Sal*I restriction sites. The PCR fragment of *TcRjl* was first cloned in pGEM-T Easy vector (Promega), then digested with *Sal*I and finally subcloned into *Sal*I digested pTEX-GFPn, creating the pTEX-GFP-*TcRjl* plasmid. Correct sequence and orientation were confirmed by automated DNA sequencing.

2.3. Mutagenesis and GFP-TcRjlS37N construction

Site directed mutagenesis was achieved by means of PCR-Directed Linker Scanning Mutagenesis method [14]. Two internal primers were designed (RJLS37N-Foward 5′-GGTGTTGGGAA-GAATTGTGTATCAAGCG-3′ and RJLS37N-Reverse 5′-CGCTTGAT AACACAATTCTTCCCAACACC-3′) to use together with external flanking primers (TcRjl-Forward and TcRjl-Reverse) amplify two overlapping fragments of *TcRjl* using genomic DNA of clone Dm28c as template. Resulting products were then combined in one PCR reaction with flanking primers TcRjl-Forward and TcRjl-Reverse. The PCR product originated from amplified genomic DNA was cloned in the pGEM-T Easy vector and sequenced. *TcRjl*S37N fragment was further subcloned into the *Sal*I restriction site of pTEX-GFPn vector, rendering the pTEX-GFP-*TcRjl*S37N plasmid.

2.4. Transfection assays and selection of lineages

Epimastigotes (10^8 cells) in log phase of growth were transfected with 100 μg of supercoiled DNA constructions in 400 μL of electroporation buffer (272 mM sucrose, 7 mM sodium phosphate, pH 7.2) using a Gene Pulser II Electroporation System (BioRad), with two pulses on the conditions of 50 μF, 1.5 kV, and 200 Ω. Transfectants were selected with G418. The following lineages were thus obtained: Dm28c pTEX-GFPc, Dm28c pTEX-GFP-*TcRjl* and Dm28c pTEX-GFP-*TcRjl*S37N.

2.5. Immunofluorescence

Wild-type Dm28c epimastigotes (2×10^6 cells) were washed twice with PBS and fixed for 30 min at room temperature in 4% paraformaldehyde. Parasites were washed twice again with PBS and adhered onto glass coverslips pre-coated with 0.1% poly-L-lysine in PBS for 30 min. Parasites were then incubated in blocking solution with 5% bovine albumin in PBS for 1 h at room temperature. Coverslips were washed twice with PBS and incubated overnight at 4 °C with anti-*TcRjl* (1:500) in PBS with 0.3% Triton X-100, followed by washing in PBS 0.3% Triton X-100 and incubated for 1 h with Alexa Fluor 488-conjugated goat anti-rabbit (1:500; Invitrogen). Nuclei were stained with SYTOX Green (1:500; Molecular Probes) for 20 min. Coverslips were mounted in Vecta Shield (Vector Laboratories, Inc., Burlingame, CA) and images were acquired using a confocal microscope (LSM 510 metal, Zeiss, Germany).

2.6. Metacyclogenesis

In vitro metacyclogenesis was carried out as described by Contreras et al. [15]. Epimastigotes grown in LIT medium were harvested by centrifugation at 1000*g* for 10 min, washed three times in PBS and suspended in TAU medium (190 mM NaCl, 8 mM

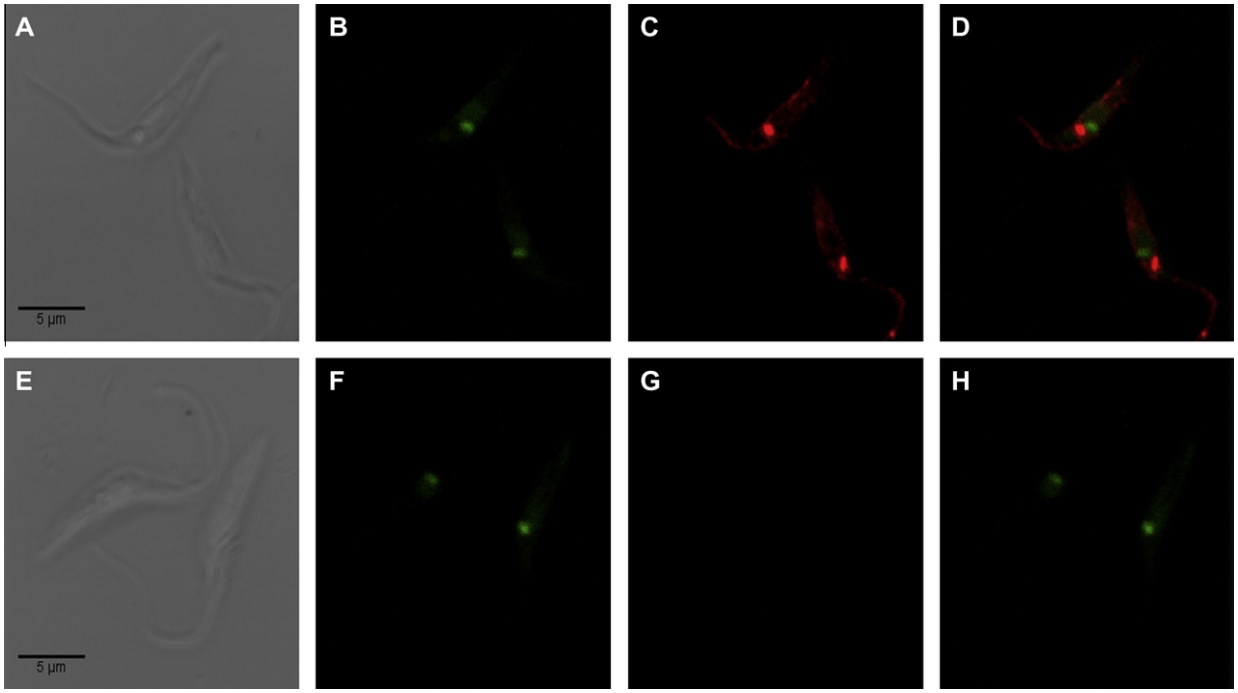

Fig. 2. Immunolocalization of TcRjl in wild-type Dm28c epimastigotes. (A–D) Incubation with anti-TcRjl; (E and F) incubation with preimmune serum; (A and E) phase contrast; (B and F) SYTOX Green to kinetoplast and nuclear labeling; (C) immunofluorescence using rabbit anti-TcRjl serum; (G) immunofluorescence with preimmune rabbit antiserum; (D) merging C and B images; (H) merging G and F images.

sodium phosphate buffer, pH 6.0, 17 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 0.35% $NaHCO_3$) to reach a density of 5×10^8 cells/mL. After 2 h of incubation at 28 °C, parasites were transferred to culture flasks containing TAU-3AAG (TAU supplemented with 10 mM glucose, 2 mM L-aspartate, 50 mM L-glutamate, and 10 mM L-proline) to a final density of 5×10^6 cells/mL. Metacyclogenesis assay was carried out in triplicates and followed for 72 h. The evolutive cell forms were determined by optical microscopy in Giemsa stained slides. The results were statistically validated with the *t*-test, using the Prism 5 software (Graph Pad).

2.7. Growth curve

Epimastigotes in exponential growth were harvested, washed twice in PBS, counted in Neubauer chamber and distributed in triplicate (5×10^5 cells/mL) in LIT medium supplemented with 10% or 4% of FCS. Growth was accompanied daily by counting parasites in Neubauer chamber during 10 days.

2.8. GTP bounding and hydrolysis

This experiment was performed according to Tatsis et al. [16]. *T. cruzi* epimastigotes (5×10^7 cells) were incubated in 3 mL of phosphate-free TAU3AAG-H medium (equilibrated with HEPES, pH 6) for 8 h and then for 16 additional hours in the same medium containing 0.5 mCi of [^{32}P] orthophosphate/point at 28 °C. Cells were harvested by centrifugation at 1000*g* for 10 min, washed three times in PBS and disrupted in lysis buffer (100 mM NaCl, 50 mM Tris–HCl pH 8, 0.25% NP-40, 5 mM $MgCl_2$, 30 μM GTP and GDP, supplemented with cocktail of protease inhibitors (Thermo Life)). GFP-TcRjl proteins were immunoprecipitated with anti-GFP (Abcam). Once proteins were washed in lysis buffer and bound nucleotides were eluted by heating the recovered antibody–antigen complex to 68 °C in elution buffer (20 mM HEPES (pH 7.4), 100 mM NaCl, 5 mM EDTA, 5 mM GTP, and 5 mM GDP). An aliquot (2000 CPM) of the eluted material (with radioactive nucleotides) was then spotted onto a polyethyleneimine–cellulose plate, resolved by ascending thin-layer chromatography (TLC) using 0.75 M KH_2PO_4 (pH 3.4) as solvent and visualized by autoradiography. The radioactive spots were detected by autoradiography (Kodak XK-1 film exposed for 24 h at room temperature.

3. Results and discussion

3.1. TcRJL is mainly found in the GTP-bound state

Although the comprehensive phylogenetic analysis of several sequenced genomes have confirmed that RJL is an independent lineage of the Ras superfamily of GTPases present in a wide diversity of organisms [4], the cell function and the biochemical features of this GTPase family remain unclear.

Predicted aminoacid sequences of all known RJL proteins reveals an alteration in the canonical glutamine residue of PM3 motif (Q61 for Ras) which has an important role in coordinating a water molecule that is required for GTP hydrolysis [17]. TcRjl predicted protein, for instance, has an arginine residue occupying this position [3]. It is well known that some *Ras*-derived oncogenes present mutations at this position that renders their products constitutively activated [18]. Also, artificial site-directed mutagenesis procedures designed to produce dominant positive GTP-binding proteins usually target this same position. There are evidences in literature that other Ras-related proteins with similar modifications in this position generate natural GTP locked proteins [19–21], thus our hypothesis was that RJL proteins may be ubiquitously locked in the GTP bound state [3]. In order to investigate the GTP/GDP bounding state of *TcRjl*, we performed the immunoprecipitation of TcRjl proteins followed by TLC analysis of bound nucleotides. Proteins were immunoprecipitated using anti-GFP antibodies from stable *T. cruzi* lineages expressing GFP-fusions of wild type TcRjl or dominant negative mutant TcRjlS37N (Fig. 1). Serine residue alteration at position 17 was reported to induce dominant negative phenotype in many Ras-related proteins [22], thus we have construct the mutant at position 37 of TcRJL, that corresponds to position 17 in many Ras-related proteins to assess the effects of such change in TcRjl protein.

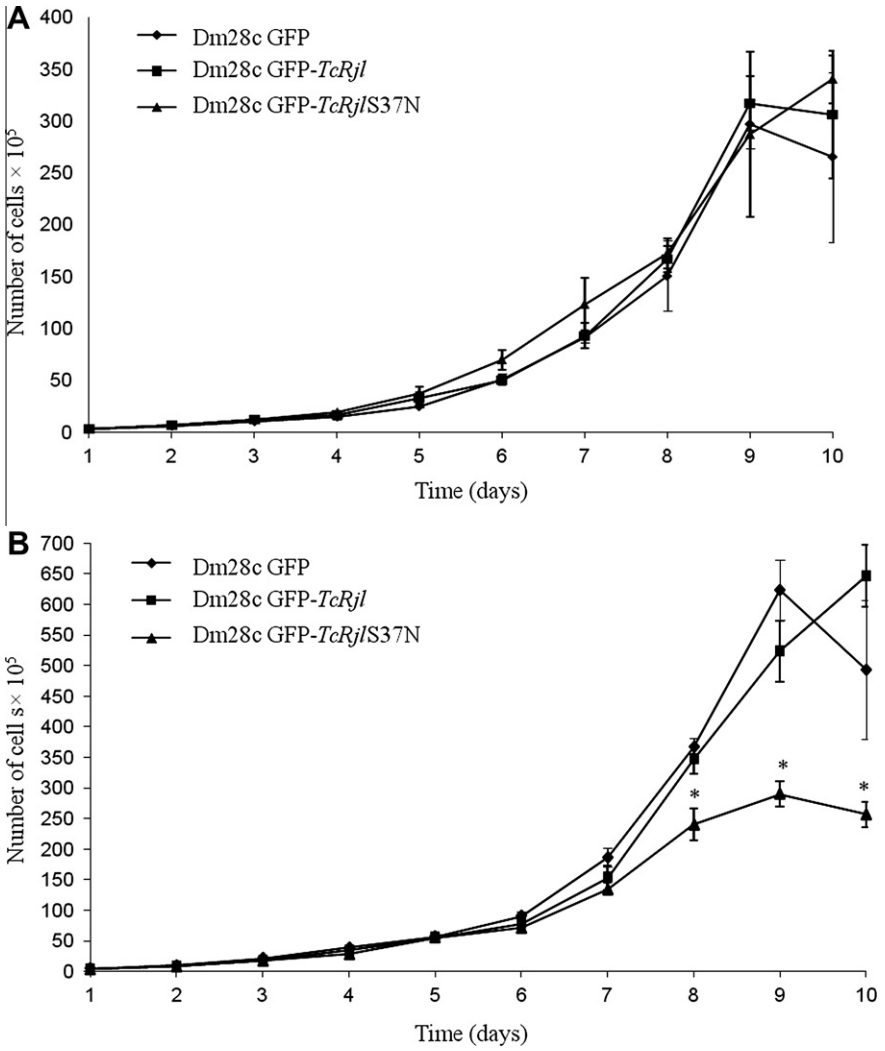

Fig. 3. *TcRjl* is involved in the control of *T. cruzi* growth. (A and B) Growth curves in different concentrations of FCS. Plotted values are means of experiments conducted in triplicate and the standard deviation for each point is indicated in bars. Each experiment was independently repeated three times. In usual condition of *in vitro* growth (10% of FCS) no effect is observed (A), but when the concentration of FCS drops to 4%, a significant decrease is observed only in the *TcRjl*S37N expressing lineage (B) (*$p < 0.5$).

Our analysis revealed that GFP-TcRjl protein is mainly found bound to GTP, while GFP-TcRjlS37N is enriched in the GDP-bound form. Predominance of a GTP bound TcRjl may be due to an intrinsic inability of this protein to hydrolyze bound GTP. Nevertheless we cannot discard the possibility that high TcRjl GTP loading might be caused by intense signaling activation during epimastigote *in vitro* proliferation. These data corroborate our prediction that TcRjl may be naturally locked in the GTP-bound stage as well as reveals the feasibility of using TcRjlS37N as a dominant negative to evaluate the effects of loss of TcRjl function.

3.2. Immunolocalization of TcRjl

Recently Elias and Archibald suggested, based on comprehensive sequence inference, that RJL proteins are somehow related to the flagellar apparatus of a wide diversity of organisms [4], although no ground experimental evidence have been shown. As *T. cruzi* exhibits a high polarized cell organization of organelles [23,24], cellular localization of TcRjl protein may corroborate the putative relationship between these proteins and the flagellar apparatus. We developed a TcRjl rabbit antiserum raised against conserved C-terminal TcRjl peptide which was used in immunolocalization studies (Fig. 2) on Dm28c epimastigotes. Images revealed a conspicuous signal close to the kinetoplast structure and the flagellar pocket of the parasite. Minor signal was evident in the flagellum projection and adjacent along cell body to the cell membrane. Further studies are in progress to disclose the association between function and localization of TcRjl.

3.3. TcRjl is involved T. cruzi cell growth and differentiation

Epimastigotes of *T. cruzi* proliferate in the midgut of the invertebrate triatominae vector and they differentiate into infective metacyclic trypomastigotes, which are mainly present in the rectum of the infected bug [23,25]. Cell growth and metacyclogenesis are easily achieved *in vitro* and have been consistently used to investigate key features of cell cycle regulation in this parasite [15,26]. Therefore, we investigated whether the overexpression of *TcRjl* or dominant negative *TcRjl*S37N could affect epimastigote growth capacity (Fig. 3). When LIT medium supplied with 10% of fetal calf serum was used to support the growth, no significant differences were observed in the growth curve (Fig. 3A). However, when the content of fetal calf serum in the medium dropped to 4%, a significant decrease of cell growth was observed in the *TcRjlS37N* lineage (Fig. 3B). Interestingly, the expression of GFP-TcRjlS37N protein was consistently low when compared to either GFP or GFP-TcRjl, suggesting a limiting growth effect of the mutant *TcRjl* gene, which, by unknown mechanisms, may affect, the cell cycle, data not shown.

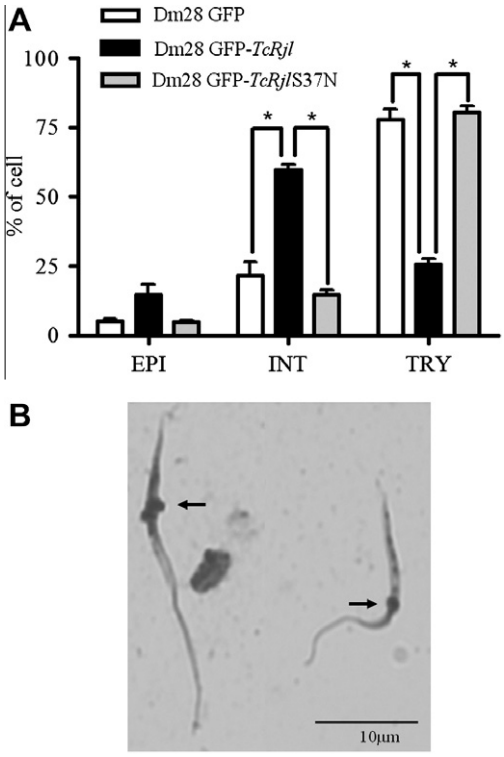

Fig. 4. Effects of *TcRjl* overexpression in metacyclogenesis. (A) Percentage of epimastigotes, intermediated forms and trypomastigotes after 72 h of incubation in TAU-3AAG medium. Values on columns are means of experiments conducted in triplicate and standard deviation is indicated in bars. The experiment was independently repeated three times (*$p < 0.05$); (B) Giemsa stained smears showing morphology of intermediate forms, which are predominant in Dm28c GFP-*TcRjl* lineage.

A remarkable phenotype was observed in the metacyclogenesis assays. Epimastigotes submitted to TAU differentiating medium are known to suffer dramatic morphological modifications, involving the migration of the kinetoplast towards the posterior end of the cell accompanied by the flagellar pocket [26]. Intermediate forms are observed throughout the process, which culminates with the formation of trypomastigotes, which are highly infective and unable to divide [23,26]. We observed that TcRjl overexpression impaired metacyclogenesis, leading to the accumulation of intermediary forms (Fig. 4A and B). Expression TcRjlS37N did not affect cell differentiation and originated significant number of trypomastigotes. It is conceivable that TcRjl function is required for cell growth, as observed in Fig. 3, while its overexpression blocks the differentiation process. The role of Ras-related GTPase in the *T. cruzi* metacyclogenesis was investigated by our group with the Rho-homolog TcRho1 [27]. However, the effects observed on differentiation were strikingly different between these two GTP binding proteins. *T. cruzi* cells expressing a mutant TcRho1 lacking the CAAX-terminal motif died synchronously during metacyclogenesis. In the case of TcRjl, there is a clear effect in the completion of differentiation that might be related to the interruption of the cytoskeleton modification needed for the process.

In conclusion, we hereby provided data indicating that, in *T. cruzi* parasites, RJL proteins are involved in cell growth and differentiation. Further studies are in progress to pinpoint the signaling events associated to this family of Ras-related GTP binding proteins.

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