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Short communication

Expression of *GP82* and *GP90* surface glycoprotein genes of *Trypanosoma cruzi* during *in vivo* metacyclogenesis in the insect vector *Rhodnius prolixus*

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Abstract

Trypanosoma cruzi, the parasite causing Chagas' disease, relies on triatomines for its transmission. *T. cruzi* metacyclic trypomastigotes express GP82 and GP90, which are developmentally regulated surface proteins that have been implicated in host cell invasion. We used quantitative RT-PCR to quantify *GP90* and *GP82* mRNA levels expressed by *T. cruzi* in the digestive tract of experimentally infected *Rhodnius prolixus* at different times post infection. Translation of these transcripts was assessed by immunofluorescence using specific monoclonal antibodies against GP90 and GP82. We found that although GP82 and GP90 proteins were not detected in epimastigote cells by immunofluorescence, transcripts were present at lower levels. Increased levels of GP90 and GP82 transcripts and the appearance of these proteins on the parasite surface were accompanied by morphological differentiation from epimastigotes into metacyclic forms. Our data suggest that during *in vivo* metacyclogenesis there is a coordinated mechanism that links stabilization of *GP90* and *GP82* mRNAs with their translation.

Keywords: *Trypanosoma cruzi*; *Rhodnius prolixus*; Stage-specific surface glycoproteins; *In vivo* metacyclogenesis; Steady-state mRNA levels; Quantitative RT-PCR; Monoclonal antibodies

Trypanosoma cruzi, the etiological agent of Chagas' disease (American trypanosomiasis), is mainly transmitted by blood-sucking bugs of the subfamily Triatominae (Hemiptera: Reduviidae). In the insect vector, the parasites differentiate from epimastigotes to metacyclic trypomastigotes, which are the infective form for mammalian hosts. The entire process of differentiation, known as metacyclogenesis, occurs in the digestive tract of the triatomine vector (Garcia and Azambuja, 1991; Garcia et al., 1984; Kollien and Schaub, 2000). When a triatomine takes a blood meal from an animal carrying *T. cruzi* trypomastigotes, the parasites reach the insect's midgut and differentiate into epimastigotes, which proliferate by asexual reproduction. On reaching the rectum, these forms differentiate into the infective metacyclic trypomastigotes. During feeding on a new mammalian host, the insect defecates on the host's skin and the metacyclic trypomastigotes released in the feces can initiate infection.

Metacyclic trypomastigotes express two major stage-specific surface glycoproteins called GP82 and GP90, which have been implicated in the invasion of mammalian host cells (Teixeira and Yoshida, 1986; Yoshida, 2006). GP82 is a cell adhesion molecule that plays a key role in host cell invasion (Ramirez et al., 1993) by inducing Ca^{2+} response in target cells and in the parasite (Ruiz et al., 1998), an event that is required for parasite internalization (Moreno et al., 1994; Tardieux et al., 1994; Dorta et al., 1995). GP90 binds to mammalian cells in a receptor-mediated manner without triggering a Ca^{2+} signal

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(Ruiz et al., 1998) and functions as a down-regulator of the target-cell invasion (Málaga and Yoshida, 2001). Its expression is thus inversely correlated with the parasite's infectivity (Ruiz et al., 1998; Cortez et al., 2006). Previous *in vitro* studies showed that *GP82* and *GP90* genes are preferentially transcribed and expressed in the metacyclic trypomastigote stage (Teixeira and Yoshida, 1986; Araya et al., 1994; Carmo et al., 1999, 2002). What is not known is the transcription pattern of GP82 and GP90 genes, as well as the expression of the corresponding proteins, during metacyclogenesis in triatomines infected with *T. cruzi*.

The purpose of this work was to analyze the expression and distribution of *GP82* and *GP90* mRNAs in *T. cruzi* flagellates in the digestive tract of triatomines. Real time quantitative PCR (RTQ-PCR) using SYBR-Green I chemistry and the relative quantitation method were used to determine *GP82* and *GP90* mRNA levels. Expression of GP82 and GP90 proteins was also analyzed by immunofluorescence microscopy, using specific monoclonal antibodies (MoAbs) to these proteins (Teixeira and Yoshida, 1986).

T. cruzi G strain (Yoshida, 1983) was used throughout this study. Parasites were maintained alternately in mice and liver infusion tryptose (LIT) medium containing 5% fetal calf serum at 28 °C. NMRI mice were infected by intraperitoneal injection of 10^6 metacyclic trypomastigotes. Fourth instar nymphs of *R. prolixus* were fed on a mouse infected with *T. cruzi* (G strain) and maintained without further feeding until dissection. Starting on the 5th day after the blood meal, urine was observed daily for microscopic detection of trypanosomes, and positive insects (one per experiment) were used for immunofluorescence analysis and RNA isolation. Once the infection was confirmed, the nymphs were dissected out and the rectum contents were removed with 20 μL of phosphate buffered saline (PBS) and transferred to Eppendorf tubes. Aliquots were used to estimate the number of parasites, to isolate total RNA, and to make slides for immunofluorescence and Giemsa staining.

Total RNA was extracted from $\sim 2.5 \times 10^3$ parasites (volume 10–15 μL) with Trizol (Invitrogen, Carlsbad, CA, USA) and treated with RNAse-free DNase (Invitrogen). First-strand cDNA was synthesized using the ThermoScript Preamplification System according to the manufacturer's instructions (Invitrogen). QRT-PCR reactions were performed using 1.0 μL of the cDNA reactions and 200 nM of primers in a final volume of 20 μL of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). *GP82* cDNA was measured with the F82qRT 5′-TGC CTC CTT CTC CGC TTC T-3′ and R82qRT 5′-CGC TGG CCG AAT TGG A-3′ oligonucleotides; and *GP90* cDNA was measured with the ABI9F 5′-TCA TGC GGT CGA TCT ATT TTT G-3′ and ABI9R 5′-AAT GCT TCC CTC GTA GTC TCT TGA-3′ oligonucleotides. *GADPH* cDNA (used as internal control) was measured with the ABIGF 5′-AGCGCGCGTCTAAGACTTACA-3′ and ABIGR 5′-TGGAGCTGCGGTTGTCATT-3′ oligonucleotides. The reactions were carried out with the ABI Prism 7000 (Applied Biosystems) and analyzed with ABI Prism 7000 SDS version 2.0 software using the standard protocol. The primers were designed to achieve maximum polymerase efficiency. Each amplicon was about 69 bp in length. For each sample, we determined the threshold cycle (Ct), which was normalized using the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) housekeeping gene. The comparative mRNA levels were determined after normalization with *GADPH* amplicons. Reverse transcription polymerase chain reaction (RT-PCR) was employed to clone and sequence the GP82 transcripts using the following primers; GP82F1 5′-TTG GAC TCC TGT CCA ATT CG-3′ and GP82R 5′-TTA TCT TCC TCG TCT TCG CC-3′ (amplicon length = 500 bp); and GP82F2 5′-GGC CGC ACA CTT TTG TAA TT-3′ and GP82Rint 5′-GTA GAA GTC CGC GAT TTC AGC-3' (amplicon length = 335 bp). For comparison purposes, we also included in this study the α-tubulin gene, which is expressed in all developmental forms of the parasite (da Silva et al., 2006). The α-tubulin cDNA was measured with the ABITF 5′-CAC TGC TTG AAC ACA CCG ATG T-3′ and ABITR 5′-CGA GGT TAC GAC GAG TTA AAT CAT AG-3′ oligonucleotides.

Fourth instar nymphs of *R. prolixus* with established *T. cruzi* infections were dissected after different periods of starvation to determine the population density (*n*) and the percentage of different developmental stages of the parasite in the rectal portion of the digestive tract (Table 1). Epimastigotes and spheromastigotes were detected in the rectal ampulla after the 15th day, while metacyclic trypomastigotes were detected from the 25th day (5%) to the 40th day (62%). *GP90* and *GP82* gene transcription patterns were similar, and transcripts of both genes were detected after the 15th day, with their maximum levels being

Table 1
Population density (*n*) and percentage of *T. cruzi* epimastigotes and metacyclic trypomastigotes collected from the rectal ampulla of *Rhodnius prolixus*

Day	Parasites/ml	*n*	Epimastigotes (%)	Metacyclic trypomastigotes (%)
15th	4×10^5	8,000	100	0
20th	3×10^5	6,000	100	0
25th	1.2×10^6	24,000	96	4
30th	4.5×10^5	9,000	95	5
35th	2×10^5	4,000	88	12
40th	1.6×10^6	32,000	38	62

Fourth instar nymphs were fed on a mouse infected with *T. cruzi* and maintained without further feeding until dissection. Starting on the 5th day after the blood meal, urine was observed daily for microscopic detection of trypanosomes. The number of parasites is expressed as the number of parasites/field and was determined by direct counting using a light microscope with a 40× lens and a Neubauer chamber. The percentage of metacyclic trypomastigotes was also determined by differential counting in Giemsa-stained smears.

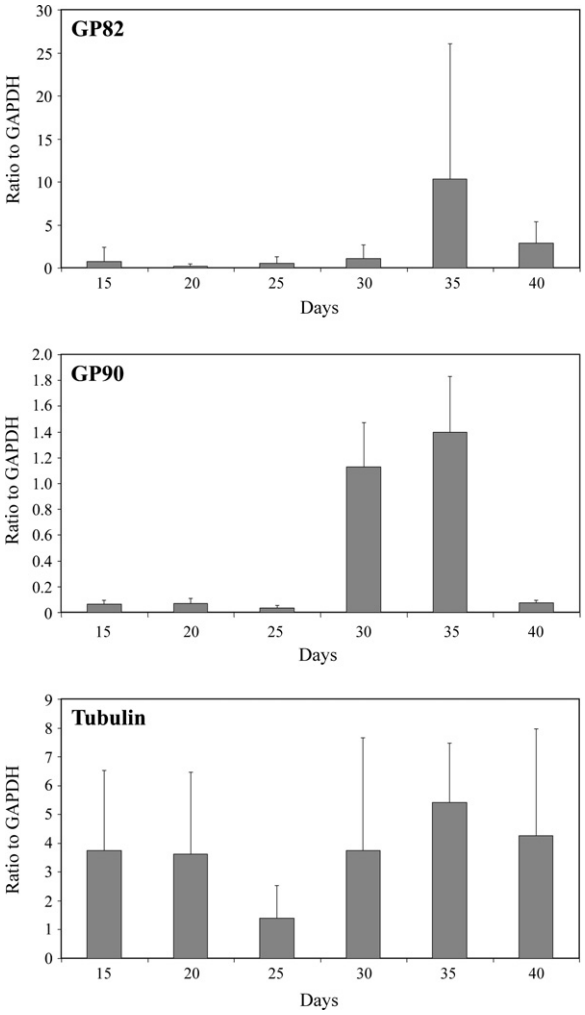

Fig. 1. Relative amount of *GP82* and *GP90* mRNAs during *T. cruzi* metacyclogenesis in infected *R. prolixus*. Transcript levels were determined by real-time quantitative PCR (RTQ-PCR) using SYBR-Green I chemistry. RTQ-PCR was performed on RNA samples from trypanosomes collected in the rectal ampulla of *R. prolixus* on various days post infection. The comparative mRNA levels were determined after normalization with *GADPH* amplicons. Standard deviations are derived from four replicas.

reached on the 35th day (Fig. 1 and Table 2). The levels of *GP90* and *GP82* transcripts from parasites collected on the 35th day were 21.85 and 12.38 times higher, respectively, than those collected on the 15th day, when epimastigotes were the predominant form present in the digestive tract (Table 2). The increase in *GP82* and *GP90* mRNAs levels coincided with the enrichment of metacyclic forms from the 15th to the 35th day after infection, as observed by Giemsa staining of the infected bug's intestinal content (Table 1 and Fig. 2).

Table 2
Comparison of *GP90*, *GP82* and tubulin-gene transcription rates during *T. cruzi* metacyclogenesis in naturally infected *R. prolixus*

	Days after infection with *T. cruzi*					
	15th	20th	25th	30th	35th	40th
mRNA ratio						
GP82	1.00	0.31	0.81	2.62	12.38	2.73
GP90	1.00	1.10	0.55	17.62	21.85	1.20
Tubulin	1.00	0.97	0.37	1.00	1.44	1.13

The mRNA levels of a given gene from parasites collected on the 15th day were compared with those from parasites collected during metacyclogenesis. The results are expressed as a ratio between the normalized transcription values obtained each day and the value obtained on the 15th day. All measurements were normalized relative to the corresponding *GADPH* level before comparison.

An unexpected finding was a sudden significant decline in the levels of transcripts for *GP90* and *GP82* in parasites obtained on the 40th day, when metacyclic trypomastigotes were the predominant form (62%). By the 40th day, transcript levels had declined significantly compared with those observed on the 35th day. However, it is interesting to note that *GP90* and *GP82* levels on the 40th day remained higher than those in epimastigotes collected on the 15th day (2.73 and 1.20 times higher for *GP90* and *GP82*, respectively) (see Table 2). The declining *GP90* and *GP82* transcript levels on the 40th day could not be due to the presence of a putative inhibitor in the RNA samples, as the levels of tubulin transcripts were very similar on the 35th and 40th days (1.44 and 1.13, respectively) (Fig. 1).

Expression of surface proteins was analyzed with MoAbs 3F6 and 1G7, which are specific for GP82 and GP90, respectively (Teixeira and Yoshida, 1986). We also used the MoAb 10D8 (Teixeira and Yoshida, 1986), which reacts specifically with mucins present in epimastigotes and metacyclic trypomastigotes (Fig. 2). Although *GP82* and *GP90* transcripts could be detected in epimastigotes, no fluorescence signals were detected in this form (Fig. 2). The most likely explanation for this finding is that *GP82* and *GP90* transcripts are not translated. Another possibility is that these proteins are rapidly degraded in epimastigotes. However, it is unlikely that the proteins were destroyed by proteases or masked or modified by insect factors, as they were easily detected in the metacyclic forms. On the 25th day (Fig. 2), positive reactions with MoAbs 3F6 and 1G7 coincided with the appearance of metacyclic forms.

To determine the repertoire of *GP82* transcripts in trypanosomes on the 40th day of infection, we designed different combinations of oligonucleotide primers for specific RT-PCR amplification. The specific forward and reverse primers, whose design was based upon the nucleotide sequences of the *GP82* gene (GenBank accession no.L14824.1, Araya et al., 1994), were used to amplify the 500-bp and 300-bp fragments encoding the carboxy-terminal domain of GP82. Amplicons were separated by agarose gel electrophoresis, purified and cloned into a plasmid vector. Analysis of 40 clones showed that they share a high degree of sequence similarity (only 5 clones displayed one or two nucleotide substitutions) (data not shown). The differences found between them were consistent with point mutations that result in synonymous codons (2 clones) or encode amino acids with the same polarity (3 clones), suggesting that *GP82* genes are less vulnerable to mutations at the protein level. This finding is in agreement with our previous study on the robustness of *T. cruzi* surface protein genes in *in vitro* cultured trypanosomes (Azuaje et al., 2007). *GP82* genes display low volatility, which confers a "robust" genetic pattern on them (Azuaje et al., 2007). Thus, as they are more robust (less volatile), these genes tend to encode the same amino acid (or similar amino acids) when affected by mutations. Of note in this regard is the high conservation (97.9%) of GP82 amino acid sequences in two *T. cruzi*

(A)

Reactivity with monoclonal antibody

	15th	20th	25th	30th	35th	40th
1G7 (GP90)	-	-	-	+	+	+
3F6 (GP82)	-	-	+	+	+	+
10D8(GP35/50)	+	+	+	+	+	+

(B)

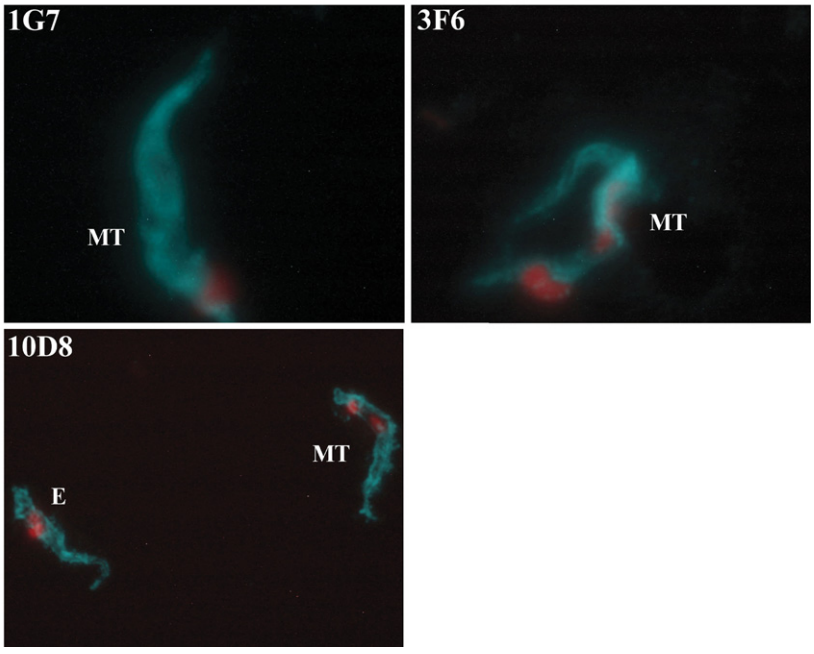

Fig. 2. (Panel A) Reactivity of monoclonal antibodies specific for GP90 (MoAb 1G7) and GP82 (MoAb 3F6) during *T. cruzi* metacyclogenesis in *R. prolixus*. Control experiments were performed with MoAb 10D8 (specific for *T. cruzi* mucins), which reacts with both epimastigote and metacyclic forms. (Panel B) Immunofluorescence staining of parasites found in the ampulla of *R. prolixus* on the 40th day of infection. Metacyclic trypomastigotes (MT) stained with either MoAb 3F6 or 1G7 (blue). Epimastigotes (E) and metacyclic trypomastigotes stained with MoAb 10D8 (blue). DAPI-staining of parasite nuclei and kinetoplasts appears in red.

strains (G and CL) (Yoshida, 2006) belonging to distant genetic groups.

We found that *GP90* and *GP82* transcript levels peaked on the 35th day and had dropped significantly by the 40th day, when metacyclic trypomastigotes accounted for 62% of the parasite population. Despite this sharp decline in transcript levels, GP90 and GP82 proteins were present in the metacyclic forms, as demonstrated by immunofluorescence with the 1G7 and 3F6 MoAbs. This result indicates that *GP90* and *GP82* transcripts had already been translated or were translated in the metacyclic form on the 40th day. Preliminary results obtained with *in vitro* cultured trypanosomes showed that *GP82* transcript half-lives were greater than 8 h in metacyclic trypomastigotes and less than 0.5 h in epimastigotes (Gentil LG, unpublished results). The reduction in *GP90* and *GP82* transcripts in metacyclic forms on the 40th day could be explained by a self controlling mechanism regulating *GP82* and *GP90* gene expression. As suggested to explain the regulation of *T. cruzi* tubulin genes (Urmenyi et al., 1992; da Silva et al., 2006), the decline in *GP90* and *GP82* transcripts may be the result of a destabilizing effect caused by the increase in the intracellular concentration of GP90 and GP82 proteins. Such an effect could lead to a reduction in the levels of *GP90* and *GP82* mRNAs without affecting the transcriptional rate of their genes.

Our data suggest that there is a coordinated mechanism during *in vivo* metacyclogenesis linking stabilization of *GP90* and *GP82* mRNAs and translation. Although *GP82* and *GP90* mRNAs have been detected in epimastigotes, their steady-state levels are lower than those in metacyclic forms and are not translated. Previous *in vitro* studies demonstrated that gene expression in *T. cruzi* is essentially regulated at the post-transcriptional level (Avila et al., 2001, 2003). Taken together, these results suggest that a post-transcriptional regulation mechanism governs *GP90* and *GP82* gene expression in the insect vector.

Finally, previous studies with *in vitro* cultured trypanosomes have shown that metacyclogenesis is triggered by nutritional stress (Contreras et al., 1985; Figueiredo et al., 2000). In our study the triatomines were fed once on an infected mammalian host and subsequently starved. The resulting nutritional stress

may have caused epimastigotes to differentiate into metacyclic forms and to proliferate rapidly. We suggest that programmed expression of GP82 and GP90 surface glycoproteins accompanies further rounds of differentiation, enabling the parasite to progress to the metacyclic infective form.

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