Impairment of cell division of *Trypanosoma cruzi* epimastigotes

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The mechanisms that facilitate the adaptation of Trypanosoma cruzi *to two distinct hosts, insect and vertebrate, are poorly understood, in part due to the limited ability to perform gene disruption studies by homologous recombination. This report describes a developmentally-defective phenotype that resulted from integration of a drug marker adjacent to the GAPDH gene in* T. cruzi.

Key words: *Trypanosoma cruzi* - life cycle - differentiation

Trypanosoma cruzi is a protozoan parasite of the ancient branch of eukaryotes (Kingdom Eukaryota, Order Kinetoplastida) (Stevens et al. 1999) and is endemic in South and Central America, and Mexico. *T. cruzi* is transmitted to vertebrates, including humans, predominantly by insect vector (subfamily *Triatoma*, family Reduviidae) and also occurs via blood transfusion, organ transplantation, and congenital routes. In humans, 30% of chronically infected individuals are estimated to develop Chagas disease, a distinct form of cardiomyopathy (Miles 2003). Despite the description of the association between *T. cruzi* transmission via the triatomine insect vector and heart disease in 1909 and decades of research illuminating the extraordinary mechanism of host cell infection (Hall 1993, Burleigh & Andrews 1998), no vaccine is available and only two anti-parasitic drugs have been licensed for treatment. However, these drugs are effective mainly at the acute stage of infection and are highly toxic (Barrett et al. 2003). The mechanisms that facilitate the adaptation of *T. cruzi* to two distinct hosts, insect and vertebrate, are poorly understood, in part due to the limited ability to perform gene disruption studies by homologous recombination. This report describes a developmentally-defective phenotype that resulted from integration of a drug marker adjacent to the *GAPDH* gene in *T. cruzi*. This study was initiated to evaluate the role of surface expressed glycosylphophatidylinositol (GPI)-anchored proteins in the complex life cycle of *T. cruzi* and utilized a homologous recombination-mediated approach to targeted disruption of the *TcGPI8* gene in the parasite, described in Zacks and Garg (2006) (Fig. 1). Electroporation with a *neomycin-resistance* (*neo*^{r})-based construct targeting *TcGPI8* and subsequent drug selection of transfectants resulted in genomic integration of the *neo*^{r} cassette, as confirmed by Southern blot analysis (Fig. 1) and PCR amplification of the 1.3 kilobase *GAPDH-IR-neo*^{r} fragment from gDNA of the transfectants (data not shown). However, *GAPDHIR-neo*^{r} did not integrate into the *TcGPI8* gene and the 3' and 5' ends of *TcGPI8* were absent (data not shown). Genome walking and PCR-cloning identified the site of insertion of the *GAPDHIR/neo*^{r} cassette (Fig. 2A-B) as adjacent to

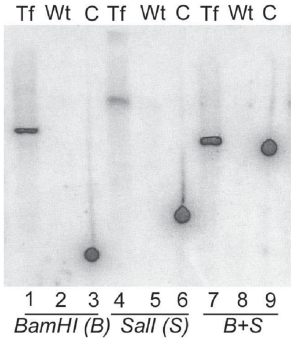

Fig. 1: genetic analysis of stable transfectants. Constructs for disruption of *TcGPI8* were designed such that drug resistance genes, *neo*^{r} or *ble*^{r}, were flanked by 400-600 base pairs of the 5' and 3' ends of the *TcGPI8* gene. The *GAPDH-intergenic region* (*GAPDH-IR*) sequence, present upstream of the *neo*^{r} or *ble*^{r} genes, provides the necessary splice acceptor site for mRNA processing (Nozaki & Cross 1995). *Trypanosoma cruzi* SylvioX10/4 strain (American Type Culture Collection, Manassas, VA) epimastigotes were electroporated with the linearized disruption cassette (10 µg) and incubated in selective drug (G418, 60 µg/ml) (Kelly et al. 1992). Following positive selection, as evidenced by the death of mock-transfected parasites cultured at the same drug concentration, the drug pressure for selection of transfectants was increased to 200-400 µg/ml; after greater than one month, chromosomal DNA (gDNA) was extracted from these stable transfectants (Medina-Acosta & Cross 1993). For Southern blot analysis, gDNA isolated from stable transfectants or from wild type, untransfected parasites was digested with restriction enzymes (*BamHI* and/or *SalI)* and resolved via agarose gel electrophoresis. Following DNA transfer to nylon membrane, hybridization was performed using a ^{32}P-labeled *neo*^{r} probe (random primer labeling) at 68^{o}C. After 6 days exposure, the image was scanned by phosphorimager. gDNA from *stable* transfectants hybridized with the *neo*^{r} probe (lanes 1, 4, and 7) indicating that *neo*^{r} is present in the transfectant genome. As expected, no hybridization with the *neo*^{r} probe was detected for wild type, untransfected gDNA (lanes 2, 5, and 8). Bands were detected at the predicted size for plasmid (*neo*^{r}*-TcGPI8*) DNA (positive control, lanes 3, 6, and 9).

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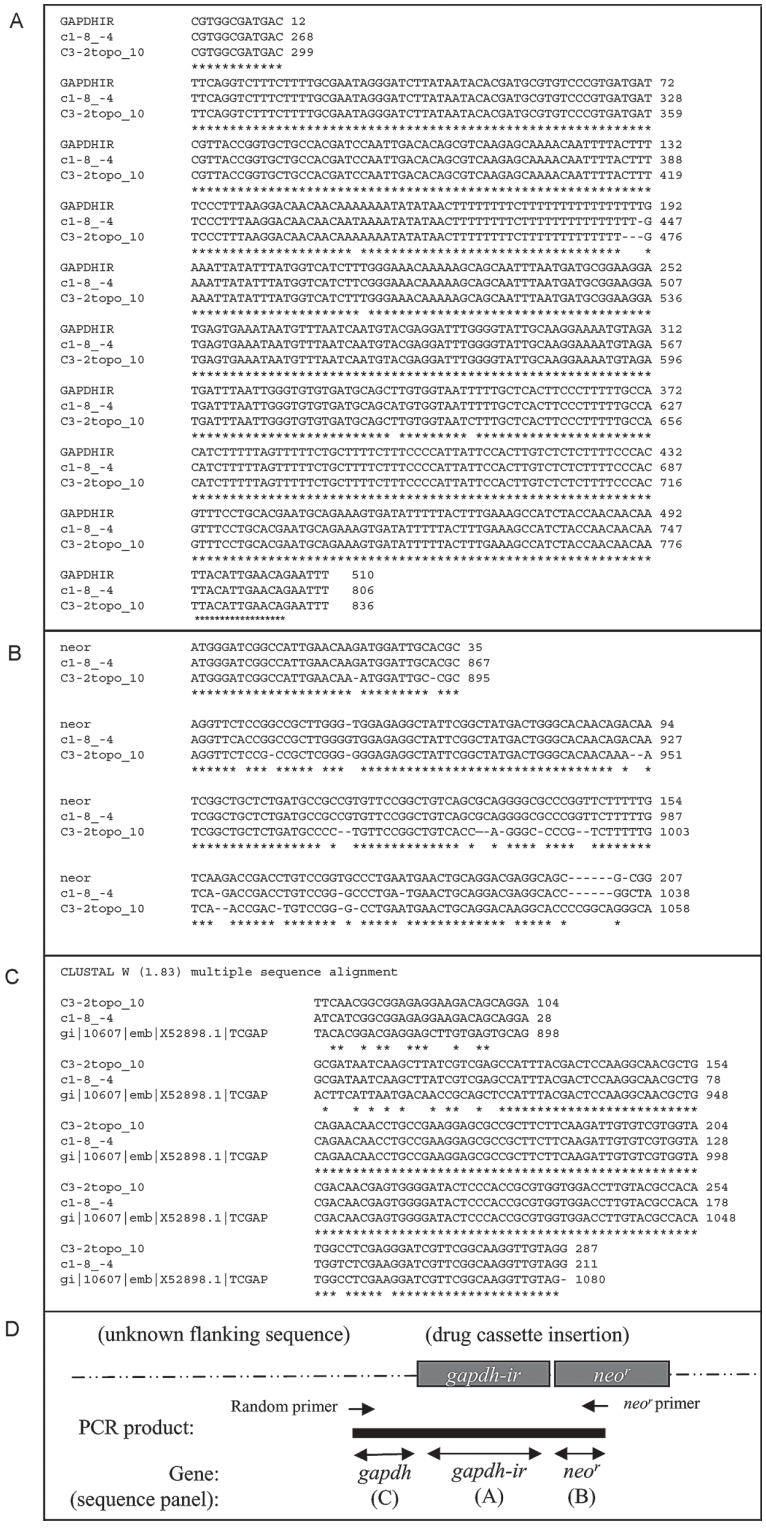

Fig. 2: identification of the site of integration of *GAPDHIR-neo*^{r} cassette in *Trypanosoma cruzi* genome. Genome walking via an arbitrarily primed PCR approach was used to determine the site of integration (Parker et al. 1991, Liu & Whittier 1995). Briefly, random, degenerate primers (Liu & Whittier 1995) were used in pairs with *neo*^{r}-specific primers (nested primers R1, 5'-TTTCGCTTGGTGGTCGAATGGGCAGGTA-3'; R2, 5'-GCACAGCTGCGCAAGGAACGCCC-3': R3, 5'-GCCGCGCTGCCTCG-3') to amplify fragments from gDNA of stable *neo*^{r}*-TcGPI8* transfectants containing the unknown sequence flanking *GAPDHIR-neo*^{r} using modified cycling parameters (Liu & Whittier 1995). PCR fragments were cloned and sequenced (UTMB Protein Core Facility). Three informative clones were obtained, e.g., clones in which the DNA sequence matched to the expected *GAPDHIR*, and *neo*^{r} portions and in which additional flanking sequence was present. *T. cruzi* blast search was performed using these flanking sequences. The sequence of the third clone was identical to c1-8_-4. Sequence alignments of TAIL-PCR clones with A) *GAPDHIR*, B) *neo*^{r}, and C) GAPDH are shown. The *GAPDHIR* and *neo*^{r} portions of the disruption cassette were confirmed and *T. cruzi* blast search identified the sequence flanking the 5' end of the *GAPDHIR-neo*^{r} insertion as matching to the 3' end of the *T. cruzi GAPDH* gene (accession #X52898) (Kendall et al. 1990).

the 3' end of *GAPDH* (Fig. 2C) (Kendall et al. 1990). The orientation of the insertion is shown in Fig. 2D.

The morphology of epimastigote-stage transfectants was documented via confocal microscopy (Fig. 3). Stable *neo*^{r}*-TcGPI8* transfectants (Fig. 3A) showed unusual morphologies, first with the appearance of thin extended forms that appeared to be reduced in motility. Highly motile parasites with two or three flagella were present with frequent observation (> 2 per microscopy field) of two parasites with fused membranes, either in apical or longitudinal style. The nucleic acid staining pattern observed in doublet parasites incubated with the fluorescent Syto11 dye indicated that duplication of kinetoplast and nuclei was not impaired. However, in many cases, cellular membranes appeared to be fused. The possibility that these represent intermediate morphological forms in normal *T. cruzi* division cannot be excluded. However, such parasites were not found in either routine (e.g. daily) light microscope examination of non-transformed cultures during consideration over an extensive period of cultivation with selective drug (e.g., G418 for minimum of 2-3 months) or without selective drug while maintained in parallel cultures using liver infusion tryptose medium. Nor, as presented here, were such forms documented during confocal microscopy. This observation is consistent for this study as well as for cultures of pTEX transformed cultures that were continuously cultured for > 2 years in the presence of selective drug (e.g., G418, as reported here), under daily to weekly monitoring. At the time this phenotype became pronounced, growth of the transformant population declined and could not be further maintained, most likely as a result of their inability to complete their replication cycle. Thus, at the epimastigote stage, a striking defect in cell division occurred in transfectants.

The effect of this transformation on the life cycle development of transfectants, e.g., differentiation of epimastigote to the infective metacyclic forms and infection of mammalian cells, was further evaluated. Transfectant epimastigotes and, as control, wild type epimastigotes were grown in liver infusion tryptose medium for ≥ 10 days without addition of media to achieve stationary-growth phase for the conversion of parasites to metacyclic trypomastigotes and were used to infect fibroblast monolayers at a parasite to cell ratio of 50:1. At 24 h after infection, medium containing free

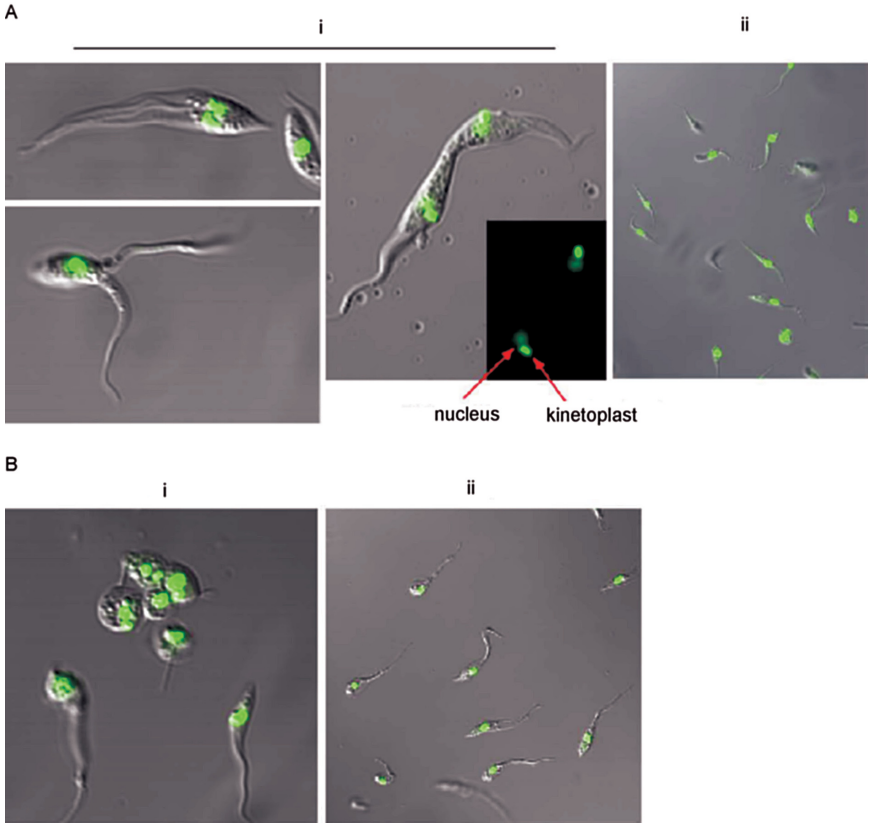

Fig. 3: morphological characteristics of stable transfectants. Stable *neo*^{r}*-TcGPI8* transfectants were generated and genetically characterized, as described in Figs 1 and 2. Parasites were harvested by centrifugation and washed in ice-cold PBS. Epimastigote morphology was documented via confocal microscopy of live parasites. To visualize kinetoplast and nuclear DNA, epimastigotes were incubated on ice for 10 min with Syto11 (Molecular Probes, 1:200 dilution), a cell-permeant nucleic acid binding fluorescent dye (excitation $\lambda_{Max.}$ 515 nm; emission λ_{Max}, 543 nm). Confocal images were captured on a Zeiss LSM 510 UV Meta Laser Scanning Confocal Microscope (UTMB Optical Imaging Core Facility) at a magnification of 63X. Signals were overlaid with Nomarski differential interference using Zeiss AxioVision Viewer software. *A:* stable *neo*^{r}*-TcGPI8* transfectants. i: transfectants; ii: wild type untransfected *T. cruzi*. Kinetoplast and nuclear DNA, which replicated in doublet parasites, are indicated by arrow; *B: neo*^{r}*-TcGPI8* transfectants following targeting with *ble*^{r}*-TcGPI8.* Stable *neo*^{r}*-TcGPI8* transfectants were electroporated with a second, *ble*^{r}-based disruption construct and selected in phleomycin, as described in the text. i: transfectants; ii: wild type untransfected *T. cruzi*.

parasites was replaced and cells monitored for the appearance of trypomastigotes. Three independent in vitro infection experiments were performed in two different fibroblast cell lines (C2C12 and BHK21) using stationary-phase cultures of stable transfectants or of wild type untransfected *T. cruzi*. Transfectants did not convert to the typical morphology of the infective metacyclic form following ≥ 10 days of cultivation without addition of new growth medium. No trypomastigotes were seen when monitoring fibroblasts infected with the stationary phase cultures of these *neo^r-TcGPI8* transfectants during a 10-12 day incubation period. However, wild type, untransfected parasites were capable of infecting either fibroblast line, with appearance of trypomastigotes and amastigotes within 6 and 11 days, respectively. In summary, morphological transformation of the *neo^r-TcGPI8* transfectant epimastigotes into metacyclic trypomastigotes in culture conditions of "starvation" did not occur, unlike for wild type parasites. In conclusion, these stable *neo^r-TcGPI8* transfectants were not infective for mammalian cells in vitro.

In effort to obtain homozygous transfectants, the stable *neo^r-TcGPI8* transfectants were subsequently electroporated with a similar disruption construct containing the *phleomycin-resistance* (*ble^r)* gene in place of the *neo^r* gene. During drug selection (500 µg/ml ble), drastic changes from normal epimastigote morphology in axenic cultures were observed in these transfectants (Fig. 3B, panel i) but not in wild type parasites, which were cultured in parallel without selective drug (Fig. 3B, panel ii). Amastigote-like forms with short, retracted flagella were abundant (> 50% of the culture) among the transfected epimastigote population (Fig. 3B, panel i), whereas the morphology of the parallel wild-type untransfected culture was normal (Fig. 3B, panel ii). Uptake of propidium iodide in these forms indicated that parasite membranes were disrupted (data not shown). These parasites could not be maintained in culture, precluding isolation of gDNA for further genetic analysis.

In summary, this report documents an interesting and selective defect in which organelle duplication (kinetoplast and nuclei) does not appear to be blocked, in which the flagella replicate but in some cases, in which the plasma membranes do not appear to separate effectively. From these studies, it can be surmised that cell division of epimastigotes was impaired selectively. To date, few studies have been published on the mechanism of cell division of *T. cruzi* (Gomez et al. 1998, 2001, Bogitsh et al. 1999, Grellier et al. 1999a, b, Orr et al. 2000, Santori et al. 2002). The pattern of development, e.g., the time course of DNA replication, organelle duplication and cell division of *T. cruzi* cannot be assumed to occur as has been demonstrated in elegant studies of the related trypanosomatid, *T. brucei* (Hendriks et al. 2000, McKean 2003). In *T. cruzi*, targeted disruption of genes for functional analysis remains a challenge and inducible expression systems have not been possible (DaRocha et al. 2004). In addition to the deficit of tools for genetic manipulation of *T. cruzi*, we lack an understanding of the mechanisms regulating *T. cruzi* gene expression and governing the cellular processes that enable the parasite to transform morphologically. However, recently, the genome sequence of *T. cruzi* CL-Brener strain was completed (El-Sayed et al. 2005). It is anticipated that future developments in transformation, gene disruption and/or inducible expression systems, combined with a comparative genomics approach (Parsons et al. 2005) will enhance our understanding of the complex developmental cycle and morphological transformation required for *T. cruzi* survival and may identify specific drug targets. Further molecular-genetic studies will be required to evaluate the potential role of the flanking sequences of *GAPDH*, such as promoter functions, in the regulation of cell division and differentiation in *T. cruzi.*

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