Supplemental information – Diaz-Laxman

Experimental

Amplification of genomic DNA from *T.* cruzi. The CL Brenner strain of *T. cruzi* was obtained from the laboratory of Dr. Bianca Zingales (Instituto de Química, Universidade de Sao Paulo, Brasil) through Dr. José Luis Ramírez (Instituto de Biología Experimental, Universidad Central de Venezuela). Parasite genomic DNA was isolated from 10^{10} parasites washed twice with NaCl 0.85%, and lysed in 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.5% SDS, 1 mM EDTA, plus Proteinase K (10 μg/ml) with gentle agitation at 37 ºC for 14-16 h. DNA was extracted twice with one volume of phenol:chloroform (1:1 v/v) and once with one volume of a chloroform:isoamyl alcohol (24:1 v/v). Phases were separated by centrifugation at 10,000 x g for 30 min. DNA was precipitated with two volumes of absolute ethanol and fibers collected by spooling. The DNA was then air dried and resuspended in 10 mM Tris-HCl pH 8.0, 1 mM EDTA. Primers were synthesized based on two ESTs found in the database for sequences homologous to previously described PDEs, and used for PCR amplifications. Their sequence and designations are:
AQ44.mS (GGCATTGTGCGCCACGTGCTTCACG); AQ44.mAS (GGATGTCGGTGTTTGACAGCAGCGTC); AQ44.1S (GTACGAGGGGTCGATTGTGGCTGTTG), AQ44.1AS (GTCCGGTACCCAAAGCTGCTTGTCC); AA95.1S (GGGAAGCCTTTGTGCTGAACGGTG); AA95.1AS (CCAGTACGGCCACCAGCCAATGCTCGG).
Nucleotide sequences were assembled with the SEQUENCHER 3.0 program (Gene Codes Co. Ann Arbor, MI). The fragments obtained were cloned into the pCRII-TOPO vector, and sequenced using an Applied Biosystems PRISM Dye Terminator sequencing kit (Perkin-Elmer).

Screening of a *T. cruzi* genomic library. A probe designated Tc13, was labeled with ^{32}P using the Multiprime DNA labeling system (Amersham). This probe was obtained as an *Eco*RI digestion product of a 4.3 kbp nucleotide fragment, containing the sequences corresponding to the GAF B domain and most of the catalytic domain of TcrPDEB1 (Fig. 1B, amino acids 408 to 842). Probe Tc13 was used to screen a *T. cruzi* genomic library as follows: Filters were pre-treated for up to 4 h with 10 ml of hybridization solution (0.5 M Sodium Phosphate Buffer, pH 7.2; 7% SDS; 1 mM EDTA; 100 μg/ml Herring Sperm DNA). After that, the radioactive probe was added to the solution, and the filters were incubated with agitation at 65ºC overnight. Membranes were briefly washed twice with 40 mM Sodium Phosphate Buffer, pH 7.2 at room temperature, and once for 15 min at 65ºC. Signals were detected on Kodak Biomax MS films. Positive cosmids were identified and their DNA isolated (Rapid Plasmid Miniprep System, Qiagen), digested with *Pst*I and re-probed with Tc13. Positive bands were cloned and sequenced as described above.

Hybridization of a *T. cruzi* electrokariotype. In order to separate chromosomal bands of the parasites, probe Tc13 was used to hybridize *T. cruzi* genomic DNA separated by Pulse Field Gel Electrophoresis. Agarose embedded chromosomes were separated in 1% agarose gels. Electrophoresis was performed in TBE 0.5X at 14ºC, with an electrode angle of 120º, and three different switching parameters: ramping from 30 to 120 s pulses for 18h, from 120 to 200 s pulses for 16 h and from 200 to 240 s pulses for 8 h. Gels were stained with ethidium bromide and transferred to nylon membranes (Hybond) by capillary action. Hybridization conditions were of high stringency.

Generation of the complete ORFs. Cloning of the first ORF (TcrPDEB1) was carried out with primers Tc2cATG.S (ATGTTTACTCAACAACGCCTGCGTC) and Tc2cExp.AS (CTTGGCGTCAAGTGATCGCTGCC). Cloning of the second ORF, corresponding to TcrPDEB1 was carried out using a sequence located in the intergenic region between the two PDEs, 22 nucleotides before the initiation ATG from this second enzyme primer: Tc2B.1S (CGTACAGCGTTGTACGTGATTTATG) and TcPDE1igfin.AS (CAAACATAAATGGTGCCTTCGC).

Immunolocalization of PDEs in *T. cruzi* trypomastigotes: Cultured *T. cruzi* trypomastigotes were washed twice with phosphate buffered saline (PBS) and fixed with paraformaldehyde (3% w/v) in PBS pH 7.2 (Gibco) for 5 minutes, on glass slides. After 5 min, slides were washed with PBS-Tween (0.05%) and permeabilized with PBS plus 0.1% Triton X-100. After removal of permeabilizing solution and washing the slide with PBS for 5 min, samples were blocked with goat anti-rabbit serum (5% in PBS-0.05% Triton plus 1mg/ml bovine serum albumin) for 1 h. The primary antiserum (a 1:500 dilution of the NT+GAF A antiserum in PBS-Triton 0.05% plus 1 mg/ml bovine serum albumin) was incubated for another hour. Slides were washed with PBS, and incubated with Alexa488-conjugated goat anti-rabbit secondary antibody (Molecular Probes Inc., Catalog # A-11039) in the dark, for 1 h. After washing in PBS-Tween, samples were visualized using a confocal laser scanning Microscope (BIO-RAD) at an emission wavelength of 488 nm. Blocking experiments with 20 μg of the purified protein fragment in 250 μl PBS were also carried out, with the same antibody dilutions.

Results:

Amplification of open reading frames (ORF) encoding two different PDEs. Amplification of genomic DNA from *T. cruzi* CL Brenner with multiple oligonucleotides to the 3' and 5' flanking regions of the two expression sequence tags (ESTs) produced six PCR products ranging from 200 bp to 4.3 kbp (Fig. 1A). The sequence of clones 1 and 2a matched those of the ESTs found in the database. Clones 2b, 3, 4 and 5, corresponded to the

same stretch of DNA with the exception of their boundaries. The nucleotide sequence of the 4.3 kbp fragment (clone 3) was found to contain the 3´ end of a gene coding for one PDE, an intergenic region and the 5´ end of a second gene coding for a second putative PDE (Fig. 1B). In another approach, 14 positive cosmids were identified by hybridization of a *T. cruzi* library with the Tc13 probe (data not shown). One of the bands, designated J1929 had high homology to the N-terminal region of TbPDE2B. The 3' extension of clone J1929 by Gene Walking produced a nucleotide sequence that overlapped with the 5' end of the 4.3 kbp clone, and gave the ORF sequence of the first PDE which corresponded to TcrPDEB1. The second gene was amplified by Gene Walking, and corresponds to TcrPDEB2. However, while this work was in progress, this second gene was reported (1).
Only one positive signal corresponding to chromosomal band number VI, of 825-850 kbp was obtained when probe Tc13 was hybridized with genomic DNA from *T. cruzi* separated by pulse field gel electrophoresis (not shown).

Reference

1. D'Angelo, M. A., Sanguineti, S., Reece, J. M., Birnbaumer, L., Torres, H. N., and Flawia, M. M. (2004) *Biochem. J.* **378**(Pt 1), 63-72

Figure legend

Figure 1. Gene organization of TcrPDEBs.

A. (I) PCR amplification of genomic DNA from *T. cruzi* with oligonucleotides: 1: AQ44.1S/AQ44.1AS (491 bp); 2a y 2b: AA95.1S/AA95.1AS (226 bp and 4.3 kbp); 3: AQ44.mS/AQ44.mAS (4.3 kbp), 4: AQ44.1S/AA95.1AS (756 bp), 5: AA95.1S/AQ44.1AS (3.4 kbp). (II) Map of the fragments obtained with the different combination of primers. The arrow indicates the fragment selected for study. GA=GAF A, GB=GAF B; IG=intergenic region. Graphic is not in scale.

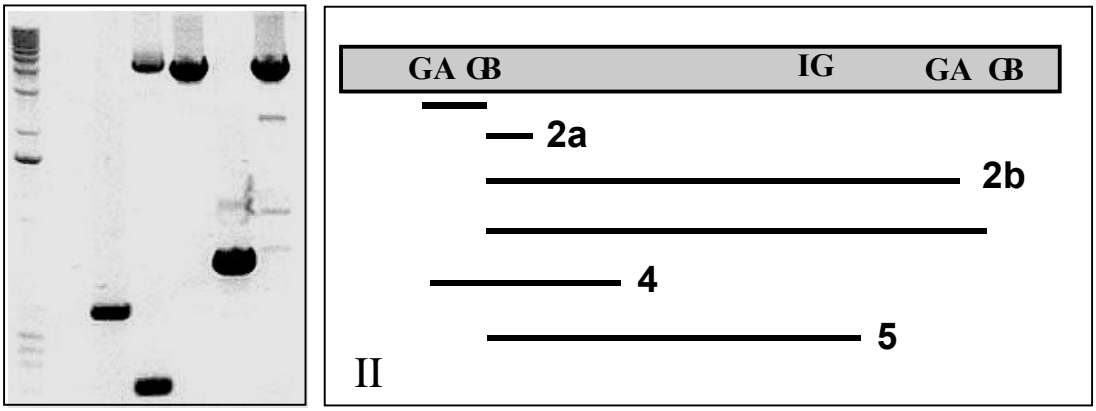
B. Restriction map of the 4.3 kbp clone. The fragment used as a probe (Tc13) is shown in a box. The broken arrow to the 5' end of the fragment indicates the direction of oligonucleotide AQ44mAS with respect to AQ44mS. CD= catalytic domain, IG= intergenic region. Graph is not in scale.

Figure 2.

Immunostaining of trypomastigote *T. cruzi* with the NT+GAF A antibody. Panel 1 shows a blocking control done using purified TbrPDEB2 NT+GAF A protein used to raise the antibody. Panel 2 shows staining, with predominant flagellar localization.

Figure 1 (Diaz Laxman supplement)

A



B

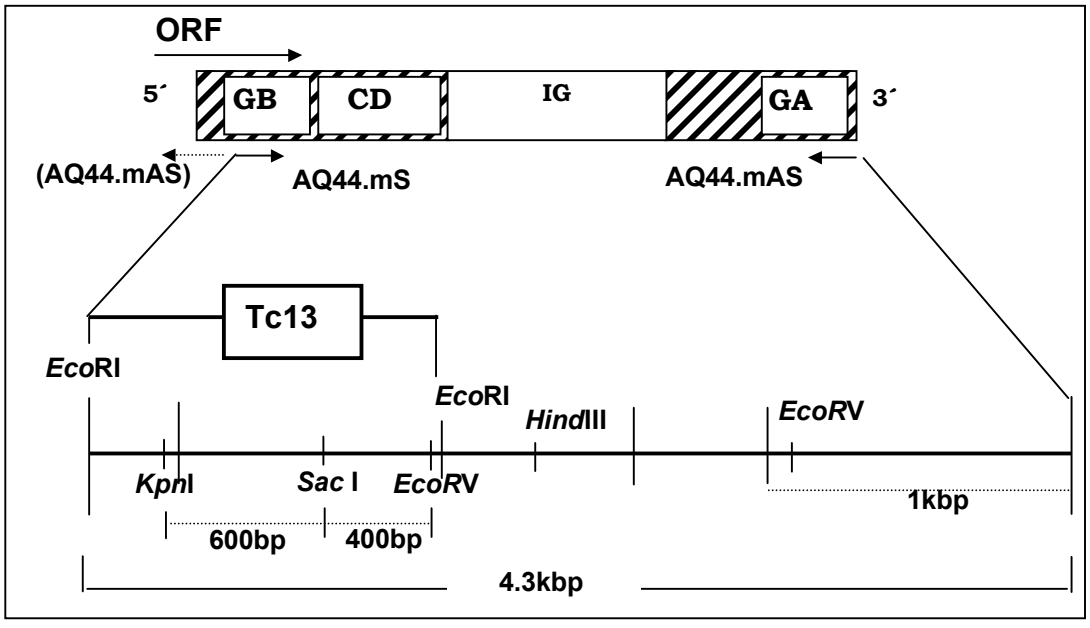


Figure 2 (Diaz Laxman supplement)