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Characterization of a gene encoding alcohol dehydrogenase in benznidazole-susceptible and -resistant populations of *Trypanosoma cruzi*

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ABSTRACT

Alcohol dehydrogenases (ADH) are a class of oxidoreductases that catalyse the reversible oxidation of ethanol to acetaldehyde. In the human parasite *Trypanosoma cruzi* the *TcADH* gene was identified through microarray analysis as having reduced transcription in an *in vitro* induced benznidazole (BZ)-resistant population. In the present study, we have extended these results by characterizing the *TcADH* gene from 11 strains of *T. cruzi* that were either susceptible or naturally resistant to benznidazole and nifurtimox or had *in vivo* selected or *in vitro* induced resistance to BZ. Sequence comparisons showed that *TcADH* was more similar to prokaryotic ADHs than to orthologs identified *Leishmania* spp. Immunolocalisation using confocal microscopy revealed that TcADH is present in the kinetoplast region and along the parasite body, consistent with the mitochondrial localization predicted by sequence analysis. Northern blots showed a 1.9 kb transcript with similar signal intensity in all *T. cruzi* samples analysed, except for the *in vitro* selected resistant population, where transcript levels were 2-fold lower. These findings were confirmed by quantitative real-time PCR. In Western blot analysis, anti-TcADH polyclonal antisera recognised a 42 kDa protein in all *T. cruzi* strains tested. The level of expression of this polypeptide was approximately 2-fold lower in the *in vitro* induced benznidazole-resistant strain, than in the susceptible parental strain. The chromosomal location of the *TcADH* gene was variable, but was not associated with the zymodeme or with the drug resistance phenotype. The data presented here show that the TcADH enzyme has a decreased level of expression in the *in vitro* induced BZ-resistant *T. cruzi* population, a situation that has not been observed in the *in vivo* selected BZ-resistant and naturally resistant strains.

1. Introduction

The flagellate protozoan *Trypanosoma cruzi* (Kinetoplastida, Trypanosomatidae), the etiological agent of Chagas disease, currently affects some 13–15 million people in Latin America (World Health Organization, 2007). Two drugs, nifurtimox (4-[5-nitrofurfurylidenamino]-3-methylthiomorpholine-1,1-dioxide; NFX) and benznidazole (*N*-benzyl-2-[2-nitroimidazol-1-yl]acetamide; BZ), are presently utilised in the treatment of Chagas disease, but both have toxic side-effects and have a very low efficacy in treating the chronic phase of the disease (Urbina and Docampo, 2003). The 5-nitrofuran NFX acts by reducing nitro groups to unstable nitro anion radicals, which in turn react to produce highly toxic reduced-oxygen metabolites (i.e. superoxide anions and hydrogen peroxide) (Docampo et al., 1981). The mechanism of action of the 2-nitroimidazole BZ involves a process of reductive stress in which covalent modification of macromolecules, such as DNA, proteins and lipids, by the reduction of nitro intermediates leads to the inhibition of parasite growth (Diaz de Toranzo et al., 1988). Various strains of *T. cruzi* exhibit different levels of susceptibility to NFX and BZ, and this variability may partially explain the observed differences in effectiveness of chemotherapy involving the two drugs (Filardi and Brener, 1987).

The innovative DNA microarray technique is finding increasing application in many areas of microbiological research and has been employed in studies concerning the life cycle and development of parasites, parasite–host relationships, assessment of virulence in different organisms, design of vaccines and studies of responses to

Abbreviations: ADH, Alcohol dehydrogenase; BZ, Benznidazole; NFX, Nifurtimox; GST, Glutathione S-transferase; HGPRT, Hypoxanthine–guanine phosphoribosyltransferase; PCR, Polymerase chain reaction; PFGE, Pulsed field gel electrophoresis; TcADH, *Trypanosoma cruzi* alcohol dehydrogenase; Z, zymodeme.

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Table 1
Trypanosoma cruzi strains used in this study and the chromosomal location of the *TcADH* gene.

T. cruzi	Origin[a]	Host	Sus[b]	Zym[c]	Chromosomal location of *TcADH* (kb)
17WTS	Mex	Triatomine	S	Z1	1890; 1120
17LER	Mex	Triatomine	R	Z1	1890; 1120
Yuyu	BA	*Triatoma infestans*	R	Z1	1270; 1540
Quaraizinho	RGS	*Triatoma infestans*	S	Z1	1120
BZS	SP	Human (acute case)	S	Z2	1420
BZR	SP	Human (acute case)	R	Z2	1420
Berenice	MG	Human (chronic case)	S	Z2	1420
Ernane	GO	Human (chronic case)	S	Z2	1120; 1230
VL-10	MG	Human (chronic case)	R	Z2	1270
Buriti	RGS	*Triatoma infestans*	S	ZB	1270; 1540
CL Brener	RGS	*Triatoma infestans*	S	ZB	1270; 1540

[a] BA, Bahia; RGS, Rio Grande do Sul; SP, São Paulo; MG, Minas Gerais; GO, Goiás (States of Brazil) and Mex, Mexico.
[b] Sus, *in vivo* drug susceptibility as described by Filardi and Brener (1987); S, susceptible; R, resistant.
[c] Zym, zymodeme classification according to Murta et al. (1998).

infections by parasites and micro-organisms (Schena et al., 1995; Gobert et al., 2005). The methodology enables the collection of considerable amounts of valuable information concerning profiles of gene expression, and has been of particular value in studies of differentially expressed genes in *Leishmania* (Guimond et al., 2003) and *Candida albicans* (Rogers and Barker, 2002; Garaizar et al., 2006) correlated with the phenotype of resistance to drugs. With respect to *T. cruzi*, the methodology has been used to assess differential gene expression profiles during trypomastigote-amastigote transformation (Minning et al., 2003) and between *T. cruzi* groups I and II (Baptista et al., 2004), as well as for analysing host transcriptional responses to *T. cruzi* infection (Burleigh, 2004). In a previous study (S.M.F Murta et al., in preparation), we employed DNA microarray methodology and demonstrated the *ca*. 4-fold lower expression of a *T. cruzi* gene encoding for an alcohol dehydrogenase in an *in vitro* induced BZ-resistant population (17LER) than in its BZ-susceptible counterpart (17WTS).

The interconversions of alcohols, aldehydes and ketones are essential metabolic processes in both prokaryotes and eukaryotes. Alcohol dehydrogenases (ADH; EC 1.1.1.1) comprise a class of oxidoreductases that catalyses the reversible oxidation of ethanol to acetaldehyde with the concomitant reduction of NAD (Reid and Fewson, 1994) and they may be categorised as NAD(P)-dependent, NAD(P)-independent or FAD-dependent enzymes. Of the three categories, the NAD(P)-dependent enzymes have been most fully characterized and further subdivided, according to structure and catalytic activity, into groups I and II comprising, respectively, long-chain and short-chain zinc independent ADHs, and group III containing the iron-activated ADHs (Jörnvall et al., 1987). Iron-dependent dehydrogenases are present in various micro-organisms such as *Escherichia coli* and *Clostridium perfringens*, as well as in protozoan pathogens such as *Leishmania, Trichomonas vaginalis, Giardia lamblia*, and *Entamoeba histolytica*. Remarkably, the lack of this type of enzyme in vertebrates makes it an important target for antimicrobial chemotherapy (Chen et al., 2004).

Few ADHs have been described for members of the Trypanosomatidae (Molinas et al., 2003). The presence of ADH (also known as NADP-aldehyde-reductase) in *T. cruzi* was first described by Arauzo and Cazzulo (1989) who partially purified the enzyme and characterized its activity. Recently the complete genome of *T. cruzi* has been sequenced revealing that this parasite contains a gene (*TcADH*) encoding a group III iron-activated ADH (El-Sayed et al., 2005).

In the present paper we report the first characterization of the *ADH* gene in *T. cruzi*. Initially, a phylogenetic analysis was carried out by comparing the amino acid sequence of the TcADH protein with those of ADHs from other organisms. Immunolocalisation assays of the protein inside the parasite were conducted through confocal microscopy. Subsequently, the levels of mRNA, the copy numbers and the chromosomal location of *TcADH* gene and the level of TcADH protein were determined in 11 BZ-susceptible and -resistant populations of *T. cruzi*.

2. Material and methods

2.1. Strains of T. cruzi

The 11 strains of *T. cruzi* employed in this study are listed in Table 1. The BZ-resistant *T. cruzi* population (17LER) derived from the Tehuantepec cl2 susceptible wild-type strain (17WTS) (Nirdé et al., 1995) was obtained *in vitro* by increasing in a stepwise manner the concentration of benznidazole (BZ) (*N*-benzyl-2-nitro-1-imidazolacetamide, Rochagan, Roche Co.). The 17LER parasites are resistant to 220 μM BZ. The BZ-resistant population BZR was derived in a previous study (Murta and Romanha, 1998) from the susceptible Y strain following *in vivo* selection after 25 successive passages in mice treated with a single high dose of BZ (500 mg/kg body weight). The *in vivo* susceptibility to BZ and NFX of the remaining seven strains of *T. cruzi* had been characterized previously (Filardi and Brener, 1987). Of these strains, five were susceptible and two were naturally resistant to both drugs. All *T. cruzi* strains had been classified previously as zymodemes Z1, Z2 or ZB according to their isoenzyme patterns (Murta et al., 1998).

2.2. Phylogenetic analysis

On the basis of one nucleotide (GenBank accession no. XM814171) and two amino acid (GenBank accession no. XP819264; GenBank accession no. XP821876) sequences of *T. cruzi* ADH that were available in GenBank, orthologous genes were identified in *Leishmania major* (GenBank accession no. LmjF30.2090), *L. infantum* (GenBank accession no. LinJ30.2440) and *L. braziliensis* (GenBank accession no. LbrM30v2.2040). The following amino acid sequences were also employed: *Candida albicans* (GenBank accession no. EAK99442), *Chromobacterium violaceum* (GenBank accession no. NP902398), *Clostridium perfringens* (GenBank accession no. NP561365), *Drosophila melanogaster* (GenBank accession no. NP477209), *Entamoeba histolytica* (GenBank accession no. XP652262), *Escherichia coli* (GenBank accession no. NP756272), *Giardia lamblia* (GenBank accession no. XP770830), *Shewanella amazonensis* (GenBank accession no. ZP00585549) and *Trichomonas vaginalis* (GenBank accession no. AAO21494). Selected sequences were compared using the Basic Local Alignment Search Tool (BLAST) provided by the National Center for Biotechnology Information (NCBI), and WU-BLAST software from The Institute of Genomic Research (TIGR). Both nucleotide (BLASTX) and amino acid (BLASTP) sequences of TcADH were compared with non-redundant sequences of proteins deposited in GenBank and the *Leishmania* database at Genedb (www.genedb.org). The sequences obtained