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Trypanosoma cruzi MSH2: Functional analyses on different parasite strains provide evidences for a role on the oxidative stress response☆

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ABSTRACT

Components of the DNA mismatch repair (MMR) pathway are major players in processes known to generate genetic diversity, such as mutagenesis and DNA recombination. *Trypanosoma cruzi*, the protozoan parasite that causes Chagas disease has a highly heterogeneous population, composed of a pool of strains with distinct characteristics. Studies with a number of molecular markers identified up to six groups in the *T. cruzi* population, which showed distinct levels of genetic variability. To investigate the molecular basis for such differences, we analyzed the *T. cruzi* MSH2 gene, which encodes a key component of MMR, and showed the existence of distinct isoforms of this protein. Here we compared cell survival rates after exposure to genotoxic agents and levels of oxidative stress-induced DNA in different parasite strains. Analyses of *msh2* mutants in both *T. cruzi* and *T. brucei* were also used to investigate the role of *Tcmsh2* in the response to various DNA damaging agents. The results suggest that the distinct MSH2 isoforms have differences in their activity. More importantly, they also indicate that, in addition to its role in MMR, TcMSH2 acts in the parasite response to oxidative stress through a novel mitochondrial function that may be conserved in *T. brucei*.

1. Introduction

Trypanosoma cruzi is a protozoan parasite of great medical importance, since it causes Chagas' disease (or American Trypanosomiasis), a malady endemic throughout Latin America, with an estimated 50,000 deaths/year and 100 million people at risk (http://who.int/tdr/diseases/Chagas). *T. cruzi* belongs to the order Kinetoplastida, which is characterized by the presence of one flagellum and a single mitochondrion in which is situated the kinetoplast, a specialized DNA-containing organelle. Though separated by many millions of years of evolution, *T. cruzi* is related to another kinetoplastid parasite, *T. brucei* [1], which continues to afflict the economy and health of the people of sub-Saharan Africa.

T. cruzi has a heterogeneous population composed by a pool of strains that circulate in the domestic and sylvatic cycles involving humans, insect vectors and animal reservoirs. Based on studies with a number of molecular markers, the taxon was divided initially in two well-defined groups, so-called *T. cruzi* I and *T. cruzi* II lineages [2]. More recent studies proposed the existence of six discrete typing units named *T. cruzi* I to VI [3]. Although the *T. cruzi* population is predominantly clonal, a few hybrid lineages have been identified, including the clone CL Brener, chosen as the reference strain for the genome project [4].

Several reports highlighted the differential genetic diversity between *T. cruzi* I and II lineages and, more importantly, the intra-group variability observed within *T. cruzi* II. Phylogeny studies of DHFR-TS and TR sequences from 31 strains showed that all sequences from *T. cruzi* I strains are monophyletic, whereas sequences from *T. cruzi* II strains are paraphyletic and fall into 3 clades [5]. Analysis of *T. cruzi* multi-gene families showed that paralogous sequences encoding amastin, an amastigote surface glycoprotein, and TcAG48, an antigenic RNA binding protein, present higher intragenomic variability in *T. cruzi* II and CL Brener strains,

☆ Sequence data have been submitted to GenBank database under the accession number GQ869654.

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when compared with *T. cruzi* I strains [6]. Likewise, whereas sequences encoding the Tc52 immuno-regulatory protein are homogeneous in strains belonging to the *T. cruzi* I lineage, they show higher sequence polymorphisms in *T. cruzi* II strains [7].

Components of DNA repair pathways are key players in the processes that result in changes in genetic variability within any cell population. Germline mutations in DNA mismatch repair (MMR) genes are associated with susceptibility to hereditary nonpolyposis colorectal human cancer (HNPCC) [8]. Mutator populations of bacteria, yeast and mammalian cells with defects in mismatch repair have also been described in isolates showing increased frequency of drug resistance [9–11]. Post-replicative DNA mismatch repair promotes genetic stability by repairing DNA replication errors, inhibiting recombination between non-identical DNA sequences and participating in responses to DNA damage induced by genotoxic agents, such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) and cisplatin [12,13]. MMR components are also involved in the response to oxidative lesions in DNA such as 7,8-dihydro-8-oxoguanine (8oxoG) [14]. The initial steps of MMR are accomplished by heterodimers of MSH proteins, named MSH1 to MSH7 in eukaryotes. In *Saccharomyces cerevisiae,* the MSH2/MSH6 complex, or MutSα, recognizes nuclear base–base mismatches and small (1–2 bp) insertion–deletion (InDel) loops, while MSH2/MSH3 heterodimers, or MutSβ, recognizes a range of small and larger loop-outs. *S. cerevisiae* MSH1 protein is involved in mitochondrial genome stability [15].

TcMSH2 was the first component of the MMR machinery described in *T. cruzi* [16]. Analyses of the *TcMSH2* single copy gene present in different strains of the parasite demonstrated the existence of three isoforms of this protein, named MSH2A, B and C, which correlate with the division in three phylogenetic *T. cruzi* lineages [17]. A role of MMR in creating differences in genetic variability among *T. cruzi* strains was initially unveiled by studies showing that strains representative of *T. cruzi* II displayed allelic variation of nuclear microsatellite *loci* induced by hydrogen peroxide, the same not occurring in a strain belonging to the *T. cruzi* I lineage [18]. Moreover, parasites from MSH2 haplogroups B and C were more resistant to cisplatin treatment, as previously reported in other MMR-deficient cells [13]. These studies suggest that, at least under genotoxic stress conditions, strains belonging to the *T. cruzi* I lineage (presenting the A isoform of *TcMSH2* gene) have a more efficient MMR activity than *T. cruzi* II strains [18]. They also support the hypothesis that the distinct genetic diversity found in the two *T. cruzi* lineages might be consequence of different levels of MMR efficiency. Therefore, to better understand the mechanisms involved in generating genetic diversity in *T. cruzi* and, more precisely, the role of the MMR pathway, we investigated the response of DNA damaging agents in strains representative of the two main lineages and the activities of different isoforms of *T. cruzi* MSH2 proteins. Using gene deletion analysis to examine the response to oxidative damage, we uncovered a novel function for MSH2, related to mitochondrial DNA repair, which is conserved in both *T. cruzi* and *T. brucei*.

2. Materials and methods

2.1. Strains and growth conditions

Five cloned *T. cruzi* strains (Col1.7G2, Silvio X-10 cl1, Esmeraldo cl3, JG and CL Brener) were used. Genotyping of each strain using several markers demonstrated that Col1.7G2, Silvio X-10cl1 belong to *T. cruzi* I lineage whereas Esmeraldo cl3 and JG are representatives of *T. cruzi* II lineage [19]. CL Brener is a hybrid strain (*T. cruzi* II/III), reference for the genome sequencing project [4] and it was selected for the knockout experiments. Epimastigotes were maintained as described [20]. *T. brucei* wild type and *MSH2* deletion mutants were grown as previously described [21].

2.2. TcMSH2 amino acid sequence analysis and northern blot assays

TcMSH2 sequences from CL Brener were retrieved from GeneDB (accession numbers Tc00.1047053509643.80 and Tc00.1047053507711.320), whereas the Col1.7G2 sequence was obtained from a cloned PCR amplified fragment as described [22]. Deduced amino acid sequences were aligned using ClustalW version 1.8 and all protein sequences were run against the Pfam [23], Prosite [24] and InterPro [25] databases. For Northern blot analysis, total RNA (25 μg) was separated on formaldehyde agarose gels, blotted onto nylon membranes, cross-linked through UV irradiation and probed with [α-^{32}P] labeled *TcMSH2* as previously described [19]. The probe was labeled according to the Megaprime DNA labeling (GE Healthcare) protocol and the signals were quantified using the ImageJ program (http://rsbweb.nih.gov/ij/).

2.3. Treatment of the strains with genotoxic agents

Cultures with 10^7 parasites per mL were incubated in 24-well plates with different concentrations of MNNG (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine) (provided by Dr. Álvaro Augusto da Costa Leitão, Instituto de Biofísica Carlos Chagas Filho, RJ), cisplatin (Quiral Química do Brasil S/A), or H_2O_2 (Merck), in the presence or absence of 3 μM of the MMR inhibitor cadmium chloride, as indicated. Previous studies have shown a wide range for the H_2O_2 IC_{50} using various *T. cruzi* strains, varying from 98 to 190 μM [26,27]. After incubation for 3 or 5 days, cell densities were measured with a haematocytometer using Erythrosin B exclusion.

2.4. Measurement of 8-oxoguanine accumulation

Two different protocols were used to assess the 8-oxoG accumulation in *T. cruzi* DNA strains. In the *in situ* experiment, a protocol adapted from Struthers et al. [28] was used. Epimastigotes were incubated in the presence of 200 or 300 μM hydrogen peroxide for 20 min at 28 °C, washed twice with PBS and fixed with 4% paraformaldehyde. Aliquots (20 μL) of the cell suspension were distributed into wells of 8-wells chambered-slides. After 1 h of incubation at 4 °C, cells were permeabilized with 0.2% Triton X-100, treated with 100 μg/mL RNase A and incubated with FITC-conjugated avidin (5 μg/mL final concentration) for 1 h at room temperature in the absence of light. After washing with PBS and mounted with a solution of 9:1 Glicerol:Tris–HCl, pH 9.0, the slides were visualized under a fluorescence microscope in a 100× oil immersion. Pre-incubation of FITC-conjugated avidin with 0.5 mM of 8oxodG results in 80% decrease in the fluorescence signal, whereas pre-incubation with dGTP has no significant effect on parasite labeling. Fluorescence intensities were averaged with the ImageJ program and plotted as fluorescence arbitrary units (average fluorescence intensity measured in 100 cells after subtracting the average background intensity). Background signals were measured in 100 fields, randomly chosen on the slides.

The 8-oxoG accumulation was also assessed by HPLC-electrochemical detection. Cells (10^9/mL) were treated with 20 mM H_2O_2 for 1 h at 28 °C, washed with PBS, and the DNA was isolated by the chaotropic NaI method [29] in the presence of 0.1 mM desferroxamine. DNA samples (100 μg) were treated with nuclease P_1 and alkaline phosphatase and analyzed by HPLC. Samples (100 μg) of digested DNA were injected into the HPLC/electrochemical detection system consisting of a Shimadzu model LC-10AD pump connected to a Luna C_{18} (Phenomenex, Torrance, CA, USA) reverse-phase column (250 mm × 4.6 mm ID, particle size 5 μm). The flow