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Characterization of the *Trypanosoma cruzi Rad51* gene and its role in recombination events associated with the parasite resistance to ionizing radiation☆

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Abstract

The *Rad51* gene encodes a highly conserved enzyme involved in DNA double-strand break (DSB) repair and recombination processes. We cloned and characterized the *Rad51* gene from *Trypanosoma cruzi*, the protozoan parasite that causes Chagas disease. This gene is expressed in all three forms of the parasite life cycle, with mRNA levels that are two-fold more abundant in the intracellular amastigote form. The recombinase activity of the *TcRad51* gene product was verified by an increase in recombination events observed in transfected mammalian cells expressing *TcRad51* and containing two inactive copies of the neomycin-resistant gene. As a component of the DSB repair machinery, we investigated the role of *TcRad51* in the resistance to ionizing radiation and zeocin treatment presented by *T. cruzi*. When exposed to gamma irradiation, different strains of the parasite survive to dosages as high as 1 kGy. A role for *TcRad51* in this process was evidenced by the increased expression of its mRNA after irradiation. Furthermore, transfected parasites over-expressing *TcRad51* have a faster kinetics of recovery of the normal pattern of chromosomal bands after irradiation as well as a higher resistance to zeocin treatment than do wild-type cultures.

Keywords: Recombination; *Trypanosoma cruzi*; RAD51; Ionizing radiation

1. Introduction

Trypanosoma cruzi is a human parasite that has a preferential asexual reproduction with rare events of sexual exchange [1,2]. Because of the accumulation of mutations, sexual reproduction in diploid taxa should eventually result in high levels of heterozygosity [3]. However, studies of genomic sequences have shown that in most strains, the *T. cruzi* genome is highly homozygous [4,5]. This is in contrast with the high heterozygosity found in the genome of the clone CL Brener, the reference strain chosen for the genome sequencing project [6]. The two distinct haplotypes found throughout most of the CL Brener genome is consistent with data from several laboratories indicating that some *T. cruzi* strains contain, in fact, hybrid genomes. On the other hand, the high level of homozygozity found in the vast majority of *T. cruzi* strains can be explained by the occurrence of highly active mitotic recombination and/or genetic conversion events. The characterization of genes involved in homologous recombination (HR) may help us to better understand the *T. cruzi* population structure and the role of DNA recombination machinery in the turnover of the parasite genome. The present study on the characterization of the *T. cruzi Rad51* gene represents the first step towards this goal.

☆ *Note:* Nucleotide sequence data reported in this paper are avaiable in the GenBank™ database under the accession number DQ166205.

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The protein RAD51 has a pivotal function in homologous recombination and repair of DNA double-strand breaks (DSB). Studies with the *Escherichia coli* homologue (RecA), show that, similarly to eukaryotic RAD51, it binds cooperatively to DNA to form helical nucleoprotein filaments that mediate pairing and strand exchange between homologous DNA molecules. The involvement of yeast and mammalian RAD51 in DSB repair has been evidenced in studies of resistance to ionizing radiation (IR), a DSB inducer [7]. Homologous recombination is the most conservative of the three processes responsible for DSB repair (which also includes non-homologous end joining and single-strand annealing) and seems to be more predominant in lower eukaryotes [8]. The DSB repair process begins when the ends of the break point are processed in the 5′ to 3′ direction leaving a protruding 3′ end ssDNA. The single-strand binding protein RPA coats this ssDNA. Invasion of a homologous duplex DNA is promoted by RAD51 after its association with RPA-coated DNA [7].

The characterization of the *Rad51* gene in *Trypanosoma brucei* demonstrated the importance of homologous recombination in antigenic variation, a mechanism that allows the parasite to escape immune killing mediated by antibodies directed against the variant surface glycoproteins, or VSGs [9]. Similar to other unicellular organisms, the disruption of *Rad51* in *T. brucei* is not lethal, but results in a higher sensitivity to genotoxic agents that cause DSB and in an impaired ability to undergo VSG switching [9]. The *Rad51* gene has also been identified and characterized in *Leishmania major*. Its product is able to bind to DNA and exhibit DNA-stimulated ATPase activity. It has also been shown that the levels of LmRAD51 increase after the treatment with genotoxic agents [10].

In this paper, we described the cloning and characterization of the *T. cruzi Rad51* gene and investigated its role in DNA recombination. We amplified a DNA fragment containing the *TcRad51* open reading frame (ORF) and tested its activity *in vivo* using CHO cells containing a recombinogenic substrate. By over-expressing *TcRad51* in *T. cruzi* epimastigotes, we also found that the activity of TcRAD51 in DSB repair may be responsible for the parasite resistance to ionizing radiation.

2. Materials and methods

2.1. Parasite cultures, γ-irradiation and zeocin treatment

All *T. cruzi* strains or cloned stocks were provided by Dr. Égler Chiari, Departamento de Parasitologia da Universidade Federal de Minas Gerais. Epimastigote cultures were maintained at 28 °C, pH 7.3, in BHI medium (33 g/L brain–heart infusion, 3 g/L tryptose, 0.02 g/L hemin, 0.4 g/L KCl, 4 g/L Na_2HPO_4 and 0.3 g/L glucose) supplemented with complement-inactivated 10% fetal bovine serum, streptomycin sulfate (0.2 g/L), and penicillin (200,000 units/L). Cultures with 3×10^5 parasites were exposed to a cobalt (^{60}Co) irradiator with a dose of 1578 Gy/h or treated with different concentrations of zeocin (Invitrogen). After cultivating the treated cultures for various periods, cells were counted in a cytometric chamber.

2.2. DNA and RNA purification

Total DNA was prepared from *T. cruzi* epimastigotes by the proteinase K-phenol/chloroform extraction method according to previously described protocols [11]. Parasite RNA was purified from the different developmental stages [obtained as described [11]] using the RNAeasy kit (Qiagen), according to manufacturer's instructions.

2.3. Cloning procedures

The proofreading Pfu DNA polymerase (Stratagene) was used in PCR amplifications of the *TcRad51* gene using 5′ (Tcrad51.10; ATGAACACCCGCTCCAAGAG) and 3′ (TAGATCAATCCCTTGCATCCCCAA) primers flanking the *Rad51* ORF. Amplicons were cloned into pGEM-T Easy vector (Promega) and the recombinant plasmids were recovered by alkaline lysis using the Wizard Plus SV Miniprep kit (Promega).

2.4. DNA sequencing and analyses

DNA sequencing reactions were prepared using the dye terminator kit and generated using the MegaBace 1000 automated sequencer (GE Healthcare). Sequence alignments and homology searches were performed using the BLAST [12] programs as well as the MultAlin interface [13].

2.5. Northern and Southern blot analysis

For Northern blot analysis, 10 μg of RNA were size-fractionated in 1.2% agarose gels containing 5% formaldehyde, blotted onto a Hybond-N^+ membrane (GE Healthcare) by capillary transfer and fixed through UV irradiation. A cDNA probe corresponding to the *TcRad51* gene was PCR amplified, gel purified and labeled with [α-^{32}P]dCTP using the Megaprime™ DNA labeling protocol from GE Healthcare. The membrane was hybridized in a 50% formamide buffer for 18 h at 42 °C and washed with 2 × SSC/0.1% SDS at 60 °C, as previously described [11].

For Southern blot hybridization, total DNA of *T. cruzi* purified from epimastigote cultures was digested with the restriction endonucleases *Eco*RI, *Hin*dIII and *Pst*I (New England Biolabs), size-fractionated by electrophoresis in 0.8% agarose gel and transferred to a Hybond-N^+ membrane (GE Healthcare). The membrane was hybridized to the same [α-^{32}P]dCTP (GE Healthcare) labeled cDNA probe. Hybridization and washing steps were carried out as described for Northern blot analysis.

2.6. Real-time RT-PCR analyses

One microgram of total *T. cruzi* RNA, quantified by capillary electrophoresis (RNA 6000 nanochips kit, Agilent), was used in first strand cDNA synthesis reactions using the SuperScript™ II reverse transcriptase kit (Invitrogen) and oligo$(dT)_{12–18}$ primer, following the manufacturer's instructions. Control reactions without reverse transcriptase were also performed to determine