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Peptide aptamer mimicking RAD51-binding domain of BRCA2 inhibits DNA damage repair and survival in *Trypanosoma brucei*

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ABSTRACT

The eukaryotic DNA recombination repair protein BRCA2 is functional in the parasitic protozoan *Trypanosoma brucei*. The mechanism of the involvement of BRCA2 in homologous recombination includes its interaction with the DNA recombinase proteins of the RAD51 family. BRCA2 is known to interact with RAD51 through its unique and essential BRC sequence motifs. *T. brucei* BRCA2 homolog (TbBRCA2) has fifteen repeating BRC motifs as compared to mammalian BRCA2 that has only eight. We report here our yeast 2-hybrid analysis studies on the interactions of TbBRCA2 BRC motifs with five different RAD51 paralogues of *T. brucei*. Our study revealed that a single BRC motif is sufficient to bind to these RAD51 paralogues. To test the possibility whether a single 44 amino acid long repeating unit of the TbBRCA2 BRC motif may be exploited as an inhibitor of *T. brucei* growth, we ectopically expressed this peptide segment in the procyclic form of the parasite and evaluated its effects on cell survival as well as the sensitivity of these cells to the DNA damaging agent methyl methane sulfonate (MMS). Expression of a single BRC motif led to MMS sensitivity and inhibited cellular proliferation in *T. brucei*.

1. Introduction

Homologous recombination (HR) of DNA is an essential component of any form of life [1–3]. It is particularly important to the parasitic protozoan *Trypanosoma brucei* because through HR this extracellular parasite can switch its cell surface antigen coat and survives from humoral immunity of its mammalian hosts [4–11]. Parasites of the *T. brucei* group, often known as African trypanosomes, can successfully proliferate in the mammalian bloodstream due to a sophisticated strategy of antigenic variation of a homogeneous Variant Surface Glycoprotein (VSG) coat [7–11]. Once an infected mammalian host mounts the appropriate antibody response, it can effectively clear a given VSG variant via antibody mediated lysis [11]. However, VSG switch variants are continuously being generated causing temporary escape from immuno-destruction [4–7]. Individual trypanosomes have many hundreds of VSG genes and thus cyclical waves of parasitemia make up a chronic infection which can persist for years [4–11]. One of the major pathways that *T. brucei* accomplish these antigenic variations is through gene conversion which uses the HR machineries of the cell [11,12].

Another potential involvement of HR in trypanosome digenetic life cycle is the stress management phase when the parasite switches hosts from the insect vector to mammal and vice versa during transmission [13–15]. The heat and oxygen level shocks experienced by the parasite cells during these transmission stages may induce DNA damage and understandably the parasite must have fully functional HR mechanisms to cope with such stresses to survive [13–15].

One of the critical proteins in the HR pathway in many eukaryotes is the multifunctional scaffolding protein BRCA2 [1–3]. BRCA2 is widely expressed in eukaryotes with the exception of yeast and few other cells. It encodes a large nuclear protein localized to the nucleus of S-phase cells. BRCA2 has been implicated in processes fundamental to all cells including RAD51-mediated recombination repair [16,17]. Mouse and human BRCA2-deficient cells accumulate spontaneous chromosomal aberrations during cell division in culture, implicating BRCA2 in the maintenance of genome stability [16]. BRCA2-deficient cells are hypersensitive to genotoxic agents that have the potential to cause DNA double-strand breaks (DSBs), implicating BRCA2 in cell cycle signaling and/or DSB repair [16,17].

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Mitotic cells can repair DNA DSBs by two major recombination mechanisms, nonhomologous end joining (NHEJ) and homologous recombination [18]. In NHEJ, DNA ends are joined with little or no base pairing at the joining site and the end-joining product can suffer insertion or deletion mutations [19]. In contrast, DSB repair by homologous recombination requires the presence of an intact DNA duplex with extensive homology to the region flanking the break to serve as a repair template. The preferred template for homologous recombination repair is the sister chromatid [20]. A key step in DSB repair by homologous recombination is the invasion of a 3′ single-strand DNA (ssDNA) end into the intact template. RAD51 protein carries out this reaction. RAD51 functions as a polymer, made up of hundreds of monomers that coat ssDNA and form a nucleoprotein filament that catalyzes the strand invasion reaction, which is followed by new DNA synthesis [21]. The resulting intermediate can either disassemble (i.e., the newly synthesized strand can be displaced and anneal with the non-invading 3′-ssDNA end to elicit non-crossover gene conversion only) or be processed to a Holliday junction intermediate to yield gene conversion with or without crossover [22,23]. Homologous recombination is considered to be error free when it involves sister chromatids [20], but it can also be deleterious when it takes place between repetitive sequences, and in excess, it can promote genome instability and cause diseases [24–26]. The first evidence linking BRCA2 to homologous recombination was its direct interaction with RAD51. The interaction is mediated by six of eight internal BRC repeats (BRC1-BRC4, BRC7, and BRC8) that are encoded by BRCA2 exon 11 and are highly conserved among mammals [21]. BRCA2 and RAD51 colocalize to subnuclear foci following DNA damage and during the S and G2 phases of the cell cycle [27]. Structural, cell biological, and biochemical evidence indicates that BRCA2 regulates RAD51 assembly at sites of DNA damage [28]. Direct evidence of a role for BRCA2 in assisting RAD51-mediated chromosomal repair was provided by the demonstration that with a chromosomal DSB in direct repeats, gene conversion was decreased by >100-fold in the BRCA2-deficient human cancer cell line CAPAN-1 and 4- to 6-fold in BRCA2-deficient mouse cells compared with wild-type cells [29,30]. The 4-fold decrease in gene conversion was accompanied by a 2- to 3-fold increase in deletion events, suggesting that DSB repair by error-prone mechanisms predominates in BRCA2-deficient cells [30]. It also indicates that chromosomal instability provoked by BRCA2 deficiency is the result of incorrect routing of DSB processing down error-prone pathways because error-free processing by homologous recombination is unavailable [30,31]. Unlike its mammalian hosts, *T. brucei* apparently lacks any NHEJ mechanisms [32,33]. Thus, any DNA damage events requiring double-stranded DNA break repair should be lethal to the cells if BRCA2 is impaired in these cells.

Homologous recombination is largely driven by RAD51 [2]. *T. brucei* was shown to encode six RAD51-related proteins (TbRAD51.1, TbRAD51.2, TbRAD51.3, TbRAD51.4, TbRAD51.5 and TbRAD51.6) [34]. In addition to TbRAD51.1, two of these RAD51-related proteins, TbRAD51.3 and TbRAD51.5 were shown to contribute to DNA repair, homologous recombination and RAD51 function in the cell [34]. Surprisingly, however, only TbRAD51.3 contributes to VSG switching [34]. TbRAD51.2, a homolog of mammalian meiosis-specific RAD51 DMC1 [35], also has been studied in detail in *T. brucei* and was found not to act in DNA recombination, repair or antigenic variation in bloodstream stage cells [36].

T. brucei BRCA2 (TbBRCA2) is essential for HR and VSG switching and structurally distinct from mammalian BRCA2 [37]. Thus, it could be a target for pharmacological intervention. To explore this possibility, we studied the molecular biology of TbBRCA2 BRC repeats and their interactions with different major RAD51 paralogues of the parasite. Here we report that a single BRC motif is sufficient to bind to these proteins. We show evidence that a single 44 amino acid long repeat unit of the TbBRCA2 BRC motif may be exploited as an inhibitor of *T. brucei* growth as the ectopically expressed peptide segment shunts the proliferation of the parasite cells *in vitro* and increased the sensitivity of these cells to the DNA damaging agent methyl methane sulfonate (MMS).

2. Materials and methods

2.1. Trypanosome strains, media, and transfection

The procyclic form of *T. brucei* 427 double resistant cell line (29-13) expressing the tetracycline repressor gene (TetR) and T7RNA polymerase (T7RNAP) were grown in SDM-79 medium (JRH Biosciences, Lenexa, KS) containing 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and appropriate antibiotics (hygromycin: 50 μg/ml; G418: 15 μg/ml) at 27 °C [38,39]. Bloodstream form cells were maintained in HMI-9 medium with G418 (2.5 μg/ml) at 37 °C. Procyclic cells were transfected by electroporating 3×10^7 cells with 20 μg of Not I linearized plasmid DNA construct that had been phenol/chloroform treated and ethanol precipitated. Electroporation was performed in 0.5 ml of Zimmerman Post-Fusion medium (132 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.5 mM magnesium acetate, 0.09 mM calcium acetate at pH 7.0), with a BTX Electro Cell Manipulator 600 set at 1.6 kV and 25 μF capacitance [35,36]. Cells were allowed to recover for 24 h before transfectants were selected for with 2.5 μg/ml phleomycin. Expression of constructs was induced with 1 μg/ml doxycycline.

2.2. RNA isolation, DNase treatment, and real-time RT-PCR

Total RNA was isolated from the bloodstream and procyclic trypanosomes using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. All primers used for amplification and real-time PCR are listed in Table 1. Total RNA was DNase I treated using RQ1 (Promega, Madison, WI) following the protocol provided by the supplier. cDNA was synthesized from total RNA using the iScript cDNA synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Real-time PCR was done using the IQ SYBR Green Supermix Kit (Bio-Rad Laboratories). Beta-actin was used as the endogenous control for normalization of the real-time PCR data.

2.3. Southern blot analysis

Southern blot analysis was performed to determine the gene copy number for BRCA2 in *T. brucei*. Genomic DNA (5–10 μg) was digested with different restriction endonucleases. The restriction endonucleases chosen do not have a site in the DNA used as probe. The digests were resolved on a 0.8% agarose gel and transferred to nylon membrane in denaturing buffer (Schleicher and Schuell, Keene, New Hampshire) following standard protocols. The blots were probed with a ^{32}P-labeled BRCA2 ORF fragment (+3705 to +4305 of TbBRCA2 ORF with the first nucleotide of the ORF as +1) amplified from genomic DNA with the primer set 5′-CACCATGGC CATCGATTTTGCTG GCTTGTTCG-3′ and 5′-CTATACCTGTTCTTCTTC ACTGCTTAAGG-3′ following standard protocols.

2.4. DNA cloning and sequence analysis

All primers used for amplification are listed in Table 1. For the evaluation of protein–protein interactions by yeast two hybrid analysis, the open reading frame (ORF) of TbRAD51.1 (XP_828893), TbRAD51.2 (XP_827266), TbRAD51.3 (XP_828338), TbRAD51.4 (XP_828775), and TbRAD51.5 (EAN78580) and the BRC repeat