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Research paper

Characterization of recombinase DMC1B and its functional role as Rad51 in DNA damage repair in *Giardia duodenalis* trophozoites

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ABSTRACT

Homologous recombination (HR) is a highly conserved pathway for the repair of chromosomes that harbor DNA double-stranded breaks (DSBs). The recombinase RAD51 plays a key role by catalyzing the pairing of homologous DNA molecules and the exchange of information between them. Two putative DMC1 homologs (DMC1A and DMC1B) have been identified in *Giardia duodenalis*. In terms of sequences, GdDMC1A and GdDMC1B bear all of the characteristic recombinase domains: DNA binding domains (helix-turn-helix motif, loops 1 and 2), an ATPcap and Walker A and B motifs associated with ATP binding and hydrolysis. Because GdDMC1B is expressed at the trophozoite stage and GdDMC1A is expressed in the cyst stage, we cloned the giardial dmc1B gene and expressed and purified its protein to determine its activities, including DNA binding, ATP hydrolysis, and DNA strand exchange. Our results revealed that it possessed these activities, and they were modulated by divalent metal ions in different manners. GdDMC1B expression at the protein and transcript levels, as well as its subcellular localization in trophozoites upon DNA damage, was assessed. We found a significant increase in GdDMC1B transcript and protein levels after ionizing radiation treatment. Additionally, GdDMC1B protein was mostly located in the nucleus of trophozoites after DNA damage. These results indicate that GdDMC1B is the recombinase responsible for DSBs repair in the trophozoite; therefore, a functional Rad51 role is proposed for GdDMC1B.

1. Introduction

Giardia duodenalis, also known as *Giardia lamblia* or *Giardia intestinalis*, is a flagellated protozoan parasite in the order Diplomonadida that, although there is nothing to indicate it, seems to belong to the group Metamonada within Excavata [1,2]. *Giardia* is the etiological agent of giardiasis, which is one of the most common gastrointestinal diseases among humans and other mammals in the world and can cause acute diarrhea. Giardia has a two-stage life cycle, consisting of the differentiation between the trophozoite (vegetative and replicative form) and the cyst (resistant and infective form). During its life cycle, several rounds of DNA replication, cytokinesis and nuclear division occur; therefore, the parasite is in constant flux in ploidy, and DNA repair is crucial for maintaining the integrity of its genome [3]. During its life cycle, the genome of *Giardia* can suffer ruptures due to endogenous or exogenous factors, and this damage can be repaired by different mechanisms. The DNA double-stranded breaks (DSBs), which are a particularly deleterious lesion, occur when replication forks collapse. In addition, DSBs are present at telomeres and are induced by ionizing radiation and other genotoxic agents (reviewed by Ref. [4]). Homologous recombination (HR) is a highly conserved pathway for the elimination of DSBs, particularly during the S phase of the cell cycle. In HR, a recombinase catalyzes the pairing and information exchange between homologous DNA molecules. The biochemical activities that have been ascribed to recombinases, e.g., RecA in bacteria and Rad51 in eukaryotes, include ATP hydrolysis, ssDNA and dsDNA binding, and the catalysis of DNA strand exchange [5–7].

Extensive evidence has indicated that *Giardia* possesses active HR machinery. For instance, the HR-mediated integration of

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exogenous DNA sequences, flanked by regions homologous to the locus of interest, in the *Giardia* genome has been well documented [8–11]. Additionally, the identification of DNA sequences that are likely of a recombinational origin [12] and the genetic exchange within and between assemblages [13] are also indicative of frequent HR events. Importantly, *Giardia* genes that encode conserved HR proteins have been identified using *in silico* approaches, and these genes include spo11, mre11, mnd1, rad50, hop1, hop2, and rad52, as well as two genes, dmc1a and dmc1b, that could encode recombinase proteins [14]. It is interesting that many of these genes have been linked to meiotic recombination in other organisms, while this mechanism has not been detected in *Giardia* [15]. In addition, low allelic heterozygosity has been reported in *Giardia*, in contrast to what is expected in asexual organisms [13,16,17]. Moreover, the proteins encoded by some of the aforementioned genes, including DMC1A (apparently expressed only in the cyst) and DMC1B (present in the cyst and the trophozoite) [18], are somewhat stage-specific. Consistently, putative recombinase transcripts have shown distinct profiles during encystation, suggesting that their expression is regulated during differentiation [19]. However, in a more recent study involving protein expression analysis by immunofluorescence, DMC1A and DMC1B proteins were found to be only expressed in the cyst and the trophozoite, respectively [15].

It has been reported that *Giardia* trophozoites can survive a gamma radiation dose of up to 1 kGy [20]. Because gamma-irradiation causes DSBs, it is evident that *Giardia* is able to repair these DSBs, and the participation of HR might be crucial in this regard. To understand the role of putative recombinase GdDMC1B in DSB repair, we expressed and purified its protein, and we present evidence here that it is indeed a *bona fide* recombinase because it performs the expected catalytic activities. To explore its response to DNA damage, we first determined the LD50 (lethal dose: radiation dose expected to kill 50% of a population) in Giardia trophozoites after gamma irradiation exposure to find a specific dose to analyze the expression of this recombinase in response to this treatment. DNA damage was verified by determining phosphorylated GdH2A histone levels by western blot and TUNEL (**T**dT-mediated d**U**TP-X **n**ick **e**nd **l**abeling) assay. giardial dmc1B-specific transcript and protein levels were analyzed on irradiated and untreated trophozoites, and we found GdDMC1B to be increased significantly at 1 h post-radiation. In addition, nuclear localization of the GdDMC1B protein was confirmed after DNA damage by immunofluorescence. We showed that GdDMC1B is present in the nuclei of trophozoites and is DNA damage-inducible.

2. Materials and methods

2.1. In silico analysis to identify giardial recombinases

DNA sequences of two genes from *Giardia* were identified *in silico* (GdDMC1A, GenBank: AY295089.1, *Giardia* genome database GL50803_13104; GdDMC1B GenBank: XM_001709949, *Giardia* genome database GL50803_13346) with *Saccharomyces cerevisiae* Rad51 (ScRad51, GenBanK: BAA00913.1) as a query against a giardial database (http://giardiadb.org/giardiadb) using the BLAST tool [21]. To identify the functional and structural motifs that are important for Rad51 homologs, sequence alignment of Rad51 homologs was performed with the ESPript program [22].

2.2. Cloning of the giardial dmc1B gene

The complete coding sequence of the dmc1B gene was amplified by PCR (restriction sites were added) and was cloned into the plasmid pCR2.1-TOPO. The primers used were B-F and B-R (Table 1). Later, the giardial dmc1b gene was subcloned in the vector pET32b (Novagen) for protein expression using the corresponding restriction enzymes.

2.3. Recombinant protein expression and purification

Plasmid for the expression of recombinant protein rGdDMC1B were transformed into the *Escherichia coli* strain BL-21 (DE3). An isolated colony was grown in LB media (supplemented with 100 μg/ml ampicillin) overnight at 37 °C. The culture was used to inoculate another flask to grow bacteria until the optical density reached 0.6. The protein was induced by adding 1 mM IPTG and incubating for 6 h at 37 °C; then, the culture was chilled, and the bacterial pellet was collected. Thereafter, each protein was purified as follows. All of the protein purification steps were performed at 4 °C.

2.4. Purification of rGdDMC1B protein

Forty grams of cell pellets from 10 L of culture were suspended in 200 ml of cell breakage buffer K and then were lysed by sonication. The crude lysate was clarified by centrifugation (100,000×*g*, 60 min), and the supernatant was incubated with 10 ml of nickel-agarose beads for 1 h with gentle mixing. The matrix was poured into a column and washed sequentially with buffer K with 300 mM KCl and 25 mM imidazole and with buffer K with 300 mM KCl and 50 mM imidazole. Protein elution was performed with buffer K with 300 mM KCl and 200 mM imidazole. The protein pool was then applied onto a column of Q-Sepharose (total 30 ml) and was eluted with a 120 ml gradient of 100–1000 mM KCl in buffer K. The peak of rGdDMC1B was diluted and fractionated in a column of macro hydroxyapatite (Bio-Rad). Eluted rGdDMC1B fractions were pooled and dialyzed against buffer K with 100 mM KCl and were applied to a Mono Q column. rGdDMC1B protein was concentrated in Centricon-30 microconcentrators and was stored at −80 °C.

Due to low protein yields after tag removal (data not shown) [23] and our being able to obtain in vitro catalytic activities, as observed when they were analyzed, purified double thioredoxin-H6-tagged recombinant proteins were used for in vitro activities.

2.5. ssDNA and dsDNA binding assay

Both ssDNA and dsDNA binding abilities were determined together for purified rGdDMC1B protein as follows: 1X Tris/DTT buffer [40 mM Tris-HCl pH 7.5, 1 mM DTT), 0.1 μg/μl BSA, 5 Mm $MgCl_2$, 0.3 ng/μl ssDNA (5′ ^{32}P-end labeled oligonucleotide A16(−)] and or 0.5 ng/μl dsDNA [5′ ^{32}P end labeled oligonucleotide A16(−) annealed with its complementary oligonucleotide A16(+)] [24], Table 1), 1 mM ATP and the purified recombinant protein rGdDMC1B (0, 0.05, 0.25, 0.55, 1, 2 and 4 μM). Oligo A16(−) was previously labeled with [γ-^{32}P] ATP and T4 PNK by conventional methods. The reactions were incubated at 37 °C for 1 h; also, SDS and proteinase K were added to a control sample (containing 4 μM protein) and were incubated for 2 min at 37 °C; then, the samples were transferred to ice. The samples were resolved by 10% native PAGE in TAE buffer (40 mM Tris-acetate, pH 7.5 and 0.5 mM ethylenediaminetetraacetic acid), and the reactions were performed in combination with ATP and $MgCl_2$ as indicated.

2.6. ATPase assay

Purified recombinant protein was quantified and used to conduct the ATP hydrolysis reaction as follows: rGdDMC1B (4 μM) was mixed in 50 Mm Tris-HCl (pH 7.5), 1 mM DTT, 5 mM $MgCl_2$, 0.1 mM ATP, [γ-^{32}P]ATP, 0.1 μg/μl BSA, 10 ng/μl of ssDNA or dsDNA