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Functional Interactions of Meiotic Recombination Factors Rdh54 and Dmc1

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Abstract

Genetic studies in budding and fission yeasts have provided evidence that Rdh54, a Swi2/Snf2-like factor, synergizes with the Dmc1 recombinase to mediate inter-homologue recombination during meiosis. Rdh54 associates with Dmc1 in the yeast two-hybrid assay, but whether the Rdh54-Dmc1 interaction is direct and the manner in which these two recombination factors may functionally cooperate to accomplish their biological task have not yet been defined. Here, using purified *Schizosaccharomyces pombe* proteins, we demonstrate complex formation between Rdh54 and Dmc1 and enhancement of the recombinase activity of Dmc1 by Rdh54. Consistent with published cytological and chromatin immunoprecipitation data that implicate Rdh54 in preventing the non-specific association of Dmc1 with chromatin, we show here that Rdh54 mediates the efficient removal of Dmc1 from dsDNA. These functional attributes of Rdh54 are reliant on its ATPase function. The results presented herein provide valuable information concerning the Rdh54-Dmc1 protein pair that is germane for understanding their role in meiotic recombination. The biochemical systems established in this study should be useful for the continuing dissection of the action mechanism of Rdh54 and Dmc1.

1. Introduction

Homologous recombination (HR) is an important DNA repair tool, particularly in the elimination of DNA double-strand breaks (DSB)s and DNA inter-strand crosslinks. Furthermore, HR helps restart DNA replication forks that have stalled or are damaged [1-4],

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Conflict of interest The author declares that there are no conflicts of interest.

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and it provides a means of restoring the length of shortened telomeres independently of telomerase [5,6]. Accordingly, HR deficiency is associated with genome destabilization and cancer predisposition. HR plays an indispensable role in meiotic chromosome metabolism as well. Specifically, programmed DSBs that occur at meiotic chromosomal hotspots trigger recombination events that serve to tie homologues together, so as to ensure their proper disjunction in meiosis I [4,6-8].

In HR-mediated DSB repair, single-stranded DNA tails derived from nucleolytic processing of the break are bound by a recombinase enzyme to assemble as a right-handed protein filament, commonly referred to as the presynaptic filament. Assembly of the presynaptic filament requires ATP binding but not its hydrolysis [9-15]. The biochemical steps, including the engagement of the partner chromatid, the search for homology in the partner chromatid, and DNA joint formation upon location of DNA homology, occur within the confines of the presynaptic filament [16-18]. The DNA joint formed as a result of the invasion of a homologous chromatid by the initiating ssDNA substrate is called the displacement loop, or D-loop. The D-loop reaction can be examined using linear ssDNA and negatively supercoiled homologous duplex as substrates [16,19]. The vast majority of eukaryotes, including the budding yeast *Saccharomyces cerevisiae* and humans, possess two recombinases, Rad51 and Dmc1, that are capable of mediating the D-loop reaction [20,21]. Whereas Rad51 is expressed in both mitotic and meiotic cells and required for HR and DNA repair in both life states, the expression of Dmc1 is strictly restricted to meiosis. [8,15,22].

The Rad51-mediated D-loop reaction is enhanced by the Swi2/Snf2-like factors Rad54 and Rdh54 [23,24]. Rad54 and Rdh54 possess a robust dsDNA-dependent ATPase activity, and at the expense of ATP hydrolysis, these HR factors translocate on dsDNA, inducing dynamic topological changes in the DNA and the transient separation of the strands in the DNA duplex [24-29]. This DNA strand-opening activity of Rad54 and Rdh54 very likely facilitates DNA strand invasion during D-loop formation [24,30,31]. In addition, Rad54 and Rdh54, through their DNA translocase activity, remove Rad51 from dsDNA [26,32], remodel chromatin [33-36], and dissociate branched DNA structures that resemble HR intermediates [27,37]. The ability of Rad54 and Rdh54 to remove Rad51 from dsDNA is thought to prevent the non-specific association of this recombinase with bulk chromatin and to render the primer-template junction in the D-loop available for DNA repair synthesis [26,32].

Rdh54 associates with Dmc1 in the yeast two-hybrid system [38,39], providing the first clue for a functional relationship between these two HR factors. Interestingly, results from chromatin immunoprecipitation (ChIP) and cytological experiments have furnished evidence for a role of the Rdh54 DNA translocase activity in preventing the accumulation of Dmc1 on bulk chromatin [40,41]. The premise of Dmc1 and Rdh54 functionally interacting in the mediation of HR reactions is consistent with the role of Rdh54 in meiotic recombination [39, 40,42]. Here, using purified *S. pombe* proteins, we show a complex of Rdh54 and Dmc1 and functional synergy between these two HR factors in the D-loop reaction. Importantly, we provide evidence for an ability of Rdh54 to remove Dmc1 from dsDNA. The findings and biochemical systems described herein should form the basis for delineating the functional relationship between Rdh54 and Dmc1 in meiotic recombination.

2. Materials and methods

2.1. DNA substrates

For the D-loop assay, the 90-mer oligonucleotide D1 [43], being complementary to positions 1932 to 2022 of pBluescript SK DNA, was 5' end-labeled with T4 polynucleotide kinase (Promega) and [γ -³²P] ATP (Amersham Bioscience). The biotinylated 600 bp dsDNA used in the Rdh54-mediated Dmc1 removal experiment was prepared by PCR amplification of

pBluescript SK DNA using a biotinylated primer and a non-biotinylated primer, as described [26]. pBluescript SK DNA was made in *E. coli DH5α* and purified using the HiSpeed Plasmid Purification Kit (Qiagen). The biotinylated DNA was immobilized on streptavidin magnetic beads (Roche Molecular Biochemicals) to give a preparation that contained 50 ng of DNA per μ l of suspended volume of the beads, as described [26]. The pBluescript SK dsDNA included as the Dmc1 trap in the assay that measures the removal of Dmc1 from dsDNA was linearized by treatment with EcoRV.

2.2. Affinity pulldown using Affi-SpDmc1 beads

SpDmc1 or BSA was covalently coupled to Affi-Gel 15 beads (Bio-Rad) according to the manufacturer's instructions, to generate matrices containing 3 mg/ml SpDmc1 or 12 mg/ml BSA. To examine binding of Rdh54 to the Affi beads, 4.5 μ g of it was mixed with 8 μ l of Affi-beads, Affi-SpDmc1 or Affi-BSA beads in 30 μ l of buffer A (20 mM KH_2PO_4 pH 7.4, 10% glycerol, 0.5 mM EDTA, 0.01 % Igepal, 2 mM mercaptoethanol) containing 75 mM KCl. The reaction mixtures were incubated for 40 min on ice with gentle mixing every 30 seconds. The beads were collected by centrifugation, and the supernatant was removed. After being washed three times with 30 μ l of buffer A containing 120 mM KCl, the beads were treated with 20 μ l of 2% SDS to elute bound proteins. The supernatant, third wash, and SDS eluate, 8 μ l each, were subject to SDS-PAGE analysis with Coomassie Blue staining to determine their protein content.

2.3. D-loop assay

The ^{32}P -labeled 90-mer oligonucleotide substrate (2.4 μ M nucleotides) was incubated with SpDmc1 (0.8 μ M) in 10.5 μ l buffer C (35 mM Tris-HCl pH 7.5, 1 mM DTT, 50 mM KCl) containing 2 mM ATP, 5 mM MgCl_2 , 250 μ M CaCl_2 , 100 μ g/ml BSA and an ATP-regenerating system for 5 min at 37°C. The indicated amount of SpRdh54 was then added in 1 μ l, followed by a 1-min incubation at 23°C. The D-loop reaction was initiated by adding pBluescript SK DNA (35 μ M base pairs) in 1 μ l. The reaction mixtures were incubated for 10 min at 30°C, deproteinized, and subject to electrophoresis in 0.9% agarose gels in TAE buffer. The gels were dried onto a sheet of DEAE paper and the radiolabeled D-loop was visualized and quantified in the phosphorimager.

2.4. Assay to monitor SpDmc1 removal from dsDNA

To assemble SpDmc1-dsDNA nucleoprotein filaments, SpDmc1 (3.8 μ M) was incubated with magnetic beads containing biotinylated dsDNA (15 μ M base pairs) in 18 μ l buffer C containing 2 mM ATP, 5 mM MgCl_2 and an ATP-regenerating system for 5 min at 37°C. After the incorporation of the indicated amount of Rdh54 in 1 μ l and a 3-min incubation at 23°C, the reactions were completed by adding linear pBluescript SK dsDNA (75 μ M base pairs) as SpDmc1 trap. Following a 25 min incubation at 30°C, the beads were captured with a Magnetic Particle Separator (Boehringer Mannheim), and the supernatants were set aside for further analysis. Bound proteins were eluted from the beads with 20 μ l of 2% SDS. The various supernatants and SDS eluates (8 μ l each) were analyzed by SDS-PAGE and Coomassie Blue staining to determine their content of proteins.

3. Results

3.1. Purification and characterization of SpRdh54

We expressed the SpRdh54 protein in *E. coli* and purified it to near homogeneity (Fig. 1A; Supplementary Materials and Methods). Like ScRdh54 [24,26], SpRdh54 binds dsDNA (Supplementary Fig. S1A), hydrolyzes ATP (Supplementary Fig. S1B), supercoils DNA (Supplementary Fig. S2A), and promotes DNA strand opening (Supplementary Fig. S2B).

3.2. Purification and characterization of SpDmc1

SpDmc1 was expressed in *E. coli* and purified to near homogeneity (Fig. 1A; Supplementary Materials and Methods). Consistent with the published studies [12,44], the purified SpDmc1 protein hydrolyzes ATP (Supplementary Fig. S3A), binds DNA in an ATP-dependent manner (Supplementary Fig. S3, A and B), forms extended filaments on DNA (Supplementary Fig. S3C) and induces topological changes in DNA (Supplementary Fig. S3D).

3.3. SpDmc1 and SpRdh54 physically interact

Genetic studies in *S. cerevisiae* and *S. pombe* have provided evidence for a role of Rdh54 in meiotic inter-homologue HR, a characteristic that has been ascribed to Dmc1 as well [4,39, 45,46]. Furthermore, yeast two-hybrid analyses have found that Rdh54 associates with Dmc1 [38,39]. For these reasons, we asked whether purified SpDmc1 and SpRdh54 proteins physically interact. For this, we covalently conjugated the purified SpDmc1 protein to Affi-Gel 15 beads (Affi-Dmc1) to use as affinity matrix in pulldown experiments with purified SpRdh54. We employed Affi-gel beads harboring covalently conjugated bovine serum albumin (Affi-BSA) as control in the pulldown experiment. As shown in Figure 1B, a significant fraction of the input SpRdh54 was retained on the Affi-Dmc1 beads, while, as expected, there was no binding to the Affi-BSA beads. Thus, in congruence with the yeast two-hybrid data [38,39], our results help establish a direct interaction between Dmc1 and Rdh54.

3.4. SpDmc1 and SpRdh54 functionally co-operate in the D-loop reaction

The homologous DNA pairing reaction mediated by various recombinases invariably requires ATP. A D-loop assay (see Fig. 1C, panel I for reaction schematic) was employed to assess the functional role of Rdh54 in the reaction. The results showed that SpDmc1 on its own has only a modest capability of making D-loops (Fig. 1C, panel II and panel III). Since ScRdh54 greatly stimulates the ScRad51-mediated D-loop reaction [24,26], we asked whether SpRdh54 can similarly enhance the D-loop forming ability of SpDmc1. Importantly, while SpRdh54 alone is devoid of homologous pairing activity, its addition to the Dmc1-mediated reaction led to a strong enhancement of the reaction efficiency (Fig. 1C). We note that the enhancement of the homologous pairing reaction occurs at amounts of SpRdh54 (150, 250, 380 nM) substoichiometric to that of SpDmc1 (800 nM) (Fig. 1C). Taken together, the results indicate that, as in the case with Rad51 [24,26], SpRdh54 functionally synergizes with SpDmc1 in the D-loop reaction.

3.5. SpRdh54 removes SpDmc1 from duplex DNA

Biochemical studies with *S. cerevisiae* proteins have found that Rad54 and Rdh54 catalyze the removal of Rad51 from dsDNA [26,32,47,48]. That Rdh54 may also be capable of removing Dmc1 from dsDNA has been suggested from cytological and chromatin immunoprecipitation experiments done in meiotic *S. cerevisiae* cells [41]. We employed a previously devised assay system [26] to determine whether SpRdh54 can remove SpDmc1 protein from dsDNA. Briefly, SpDmc1 is preloaded onto a biotinylated duplex DNA molecule immobilized on streptavidin magnetic beads, before being incubated with SpRdh54 and a non-biotinylated DNA molecule that serves as the trap for SpDmc1 protein molecules dislodged from the biotinylated DNA (Fig. 2A). The supernatant containing SpDmc1 bound to the DNA trap and the SDS eluate of the magnetic beads harboring SpDmc1 that remains associated with the biotinylated DNA are analyzed by SDS-PAGE.

As shown in Figure 2B, SpRdh54 possesses the ability to remove SpDmc1 from DNA. To ascertain the role of the SpRdh54 ATPase activity, we assembled the SpDmc1 protein filament using AMP-PNP as nucleotide cofactor and then incorporated SpRdh54. In this case, little or no removal of SpDmc1 occurred. The results thus provided evidence that the dissociation of

SpDmc1 from dsDNA requires the ATP hydrolysis by SpRdh54. This premise was further supported by our observation with an ATPase defective variant of ScRdh54, to be presented below. Consistent with our published observation that ssDNA does not activate the Rdh54 ATPase function [24], SpRdh4 is incapable of dissociating the SpDmc1-ssDNA nucleoprotein filament (data not shown).

3.6. Relevance of the Rdh54 ATPase activity in Dmc1 removal from dsDNA

A high degree of sequence conservation exists between the *S. pombe* and *S. cerevisiae* Rdh54 orthologues [39]. Consistent with this, in an affinity pulldown experiment, ScRdh54 was efficiently retained on Affi-SpDmc1 beads but not on the control Affi-BSA beads, indicating that SpDmc1 is well capable of complex formation with ScRdh54 (Fig. 3A). Furthermore, we found that ScRdh54 can enhance the SpDmc1-mediated D-loop reaction (data not shown) and can also efficiently remove SpDmc1 from dsDNA (Fig. 3B).

The physical and functional interactions between SpDmc1 and ScRdh54 allowed us to utilize the ATP hydrolysis-defective K352R mutant variant of the latter protein [26] to ascertain the role of the Rdh54 ATPase activity in the the mediation of Dmc1 removal from dsDNA. We first verified, by affinity pulldown using Affi-SpDmc1 beads, that the *S. cerevisiae* rdh54 K352 mutant protein retains the ability to associate with SpDmc1 (Fig. 3A). Importantly, the results from the SpDmc1 removal assay provided unequivocal evidence that both the Dmc1-dsDNA nucleoprotein complex dissociative function of Rdh54 is completely reliant on its ATPase activity (Fig. 3B). As expected, the *S. cerevisiae* rdh54 K352 mutant is inactive in the D-loop reaction (data not shown).

4. Discussion

In meiosis, inter-homologue HR events lead to the formation of chromosomal arm crossovers that link the homologue pairs, so as to orchestrate chromosome segregation during the first meiotic division. Genetic, cytological, and yeast two-hybrid studies have provided evidence that Dmc1 and Rdh54 function together in the promotion of inter-homologue recombination. However, to date, there has been no biochemical study directed at delineating the manner in which Dmc1 and Rdh54 physically and functionally interact to promote HR. In our effort to help fill this knowledge gap, we have devised protein expression vectors and procedures for the purification of SpDmc1 and SpRdh54 to near homogeneity and have carried out a variety of biochemical studies with these proteins. Our results confirm that SpRdh54 has similar biochemical attributes as its *S. cerevisiae* counterpart. Importantly, we have presented results to reveal that SpRdh54 and SpDmc1 interact directly, and that the two HR factors functionally co-operate in the catalysis of D-loop formation.

By ChIP and cytological analyses, Dmc1 has been found to localize to Spo11-made DSBs. Interestingly, in the *spo11Δ rdh54Δ* double mutant, foci of Dmc1 assemble spontaneously [40,41], and results from ChIP experiments have strongly suggested that these aberrant Dmc1 foci reflect non-specific association of Dmc1 with bulk chromatin [41]. Analysis of the *rdh54 K352A* mutant has yielded evidence that ATP hydrolysis by Rdh54 is indispensable for the avoidance of Dmc1's non-specific association with chromatin [41]. Consistent with the *in vivo* observations, the use of highly purified SpRdh54, ScRdh54, and also the K352R mutant variant of ScRdh54 has allowed us to demonstrate that Rdh54 removes Dmc1 from dsDNA in a manner that requires its ATPase activity. This function of Rdh54 likely ensures the timely delivery of Dmc1 to meiotic DSBs to initiate repair by HR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. SpDmc1 binding and promotion of the D-loop reaction by purified SpRdh54

(A) Purified SpRdh54 and SpDmc1 (1 μ g) were analyzed by SDS-PAGE with Coomassie Blue staining.

(B) SpRdh54 was mixed with Affi-Gel 15 beads or beads containing covalently conjugated BSA or SpDmc1. After washing, the bound SpRdh54 was eluted with SDS (E) and analyzed along with the supernatant (S) and wash (W) by SDS-PAGE and Coomassie Blue staining.

(C) The reaction schematic of the D-loop reaction is shown in panel I. In panel II, the ability of SpDmc1 to form D-loop was tested in the absence or presence of SpRdh54 (150 and 250 nM in lanes 4 and 5, and 380 nM in lanes 2, 6, 7, and 9) or ATP, as indicated. The results were plotted in panel III.

Figure 2. Dissociation of the SpDmc1-dsDNA complex by SpRdh54

(A) The reaction schematic is shown.

(B) Nucleoprotein complexes of SpDmc1 and magnetic bead-bound DNA were assembled with either ATP or AMP-PNP as nucleotide cofactor and then incubated without or with SpRdh54 (200 nM in lane 3, 400 nM in lanes 4 and 8, 600 nM in lane 5, and 750 nM in lanes 6 and 9). The supernatant and bead fractions were analyzed by SDS-PAGE and Coomassie Blue staining for their protein content. CK, creatine kinase used in the ATP regenerating system. The results were plotted.

Figure 3. Dependence of functional interactions on the Rdh54 ATPase activity

(A) ScRdh54 or its K352R variant was mixed with Affi-Gel 15 beads containing covalently conjugated BSA or SpDmc1. After washing, the bound Rdh54 or rdh54 K352 protein was eluted with SDS (E) and analyzed along with the supernatant (S) and wash (W) by SDS-PAGE and Coomassie Blue staining.

(B) The ability of ScRdh54 (120, 240, and 360 nM in lanes 3-5, respectively) or its K352R variant (120, 240, and 360 nM in lanes 7 to 9, respectively) to dissociate the SpDmc1-dsDNA nucleoprotein complex, as in Figure 2. The results were plotted.