RAD51-associated Protein 1 (RAD51AP1) Interacts with the Meiotic Recombinase DMC1 through a Conserved Motif

Background: RAD51AP1 physically and functionally interacts with the RAD51 and DMC1 recombinases.

Results: Mutational analysis showed that the WWVP sequence in RAD51AP1 is part of the DMC1-specific interaction motif.

Conclusion: RAD51AP1 interacts with RAD51 and DMC1 through distinct motifs.

Significance: RAD51AP1 likely functions in meiotic homologous recombination by enhancing the recombinase activity of both RAD51 and DMC1.

Homologous recombination (HR) reactions mediated by the RAD51 recombinase are essential for DNA and replication fork repair, genome stability, and tumor suppression. RAD51-associated protein 1 (RAD51AP1) is an important HR factor that associates with and stimulates the recombinase activity of RAD51. We have recently shown that RAD51AP1 also partners with the meiotic recombinase DMC1, displaying isoform-specific interactions with DMC1. Here, we have characterized the DMC1 interaction site in RAD51AP1 by a series of truncations critical for DMC1 interaction but dispensable for RAD51 association. This RAD51AP1 motif is reminiscent of the FVPP motif in the tumor suppressor protein BRC2 that mediates DMC1 interaction. These results further implicate RAD51AP1 in meiotic HR via RAD51 and DMC1.

Homologous recombination (HR) helps ensure proper chromosome disjunction during the first meiotic division and represents an important pathway for eliminating chromosomal lesions, including DNA double-strand breaks (DSBs) and DNA interstrand cross-links (1). As such, HR impairment leads to genome instability and cancer predisposition and can also give rise to severe meiotic defects, including prophase arrest, apoptosis, and sterility (2, 3).

Meiotic HR is initiated when programmed DNA DSBs are introduced into chromosomes by the Spo11-associated protein complex (4) followed by nucleolytic resection of the DSBs to produce long single-stranded DNA tails (5). These single-stranded DNA tails are coated by either RAD51 or DMC1 recombinase to form a helical nucleoprotein filament, termed the presynaptic filament, that mediates DNA strand exchange with a homologous donor (6). Meiotic cells are more permissive than mitotic cells in the use of a non-sister chromatid as the information donor in DSB repair and much more adept at resolving the double Holliday junction arising from DNA strand exchange to yield crossover recombinants. These attributes of the meiotic HR machinery promote the linkage of homologous chromosome pairs to ensure their proper disjunction in meiosis I (7).

Although RAD51 is present in both mitotic and meiotic cells, DMC1 is expressed only during meiosis (8–10). RAD51 and DMC1 have been shown to colocalize in side-by-side foci on meiotic chromatin, suggesting their cooperative action during meiotic HR (11, 12). Despite the similarities in biochemical attributes and the site of operation of RAD51 and DMC1, genetic evidence from the budding yeast suggests that the two recombinases serve distinct roles in meiotic HR (13–15). For example, in the absence of Dmc1, the use of the sister chromatid as an information donor in HR becomes more prevalent (16). Moreover, the meiotic activity of Rad51 appears to be restrained by the Hed1 protein (17, 18), by the synaptonemal complex proteins Red1 and Hop1 (16), and by the Mek1-mediated phosphorylation of a key accessory factor Rad54 (19). These and other observations have suggested that Dmc1 works in conjunction with Rad51 in forming meiotic interhomologue crossovers, whereas Rad51 acting alone may be more adept at mediating DSB repair using the sister chromatid as an information donor (14).

Several accessory proteins of budding yeast Dmc1 have been identified, including Mei5–Sae3 (Sfr1–Swi5 in the fission yeast) (20–22), Hop2-Mnd1 (13, 23–25) and Rdh54 (12, 26, 27). These accessory factors stimulate the assembly of the Dmc1 presynaptic filament or the homologous DNA pairing activity of the presynaptic filament (20, 23, 25), whereas Rdh54 also appears to catalyze the removal of Dmc1 from double-stranded DNA (28). We have recently identified RAD51AP1 as a novel vertebrate accessory factor of DMC1 by showing that it stimulates the DMC1-mediated D-loop reaction. RAD51AP1 coop-
erates with the DMC1 presynaptic filament in the assembly of the synaptic complex, which harbors the duplex DNA partner held in homologous registry with the single-stranded DNA bound in the presynaptic filament (29). Interestingly, mutations in the extreme C terminus of RAD51AP1 that obliterate its interaction with RAD51 (30–32) do not affect DMC1 association (29). Furthermore, a naturally occurring spliced form (isoform 3) of RAD51AP1, although being perfectly capable of RAD51 interaction, fails to associate with DMC1 (29). We have also observed significant colocalization of RAD51AP1 with DMC1 on meiotic chromatin, consistent with the premise that they work together in meiotic DSB repair (29).

Here, we have explored the DMC1 interaction region in RAD51AP1 by deletional and mutational analysis. Importantly, we have found that a conserved WVPP motif in RAD51AP1, which is reminiscent of the FVPP motif of BRCA2 previously shown to be important for DMC1 but not RAD51 binding (33), is indispensable for physical and functional interactions with DMC1. These results thus support the premise that RAD51AP1 serves as a cofactor of both RAD51 and DMC1 and show that it does so via a physical interaction with the two recombinases being mediated by distinct motifs.

**EXPERIMENTAL PROCEDURES**

**Construction of RAD51AP1 Fragments or Mutants**—All the numberings of RAD51AP1 residues are based on the coding frame of isoform 2 (Fig. 6A and Ref. 34). The RAD51AP1 fragment 1 (F1, residues 1–94) was generated by inserting a stop codon in the RAD51AP1 coding frame in the *Escherichia coli* expression vector pET24a. The DNA sequences that code for RAD51AP1 fragment 2 (F2, residues 95–187), fragment 3 (F3, residues 188–335), the C40 fragment (residues 295–335), and the C60 fragment (residues 275–335) were also introduced into the C-terminal His6 affinity tags. The primers used in the interaction with RAD51 (31, 32). DMC1 with an N-terminal His6- tag was expressed in Hi5 insect cells and purified as described previously (35).

**Affinity Pull-down Assay**—MBP-tagged RAD51AP1 isoform 2, isoform 2 fragments, isoform 2 full-length or fragment mutants, and GST-tagged isoform 3 (5 μg) were incubated with DMC1 (5 μg in Figs. 1D, 4E, and 6C but 10 μg in other experiments) in 30 μl of buffer A (25 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 50 mM KCl) at 4 °C for 30 min. Then, 20 μl of amylase resin (New England Biolabs) or glutathione resin (Amersham Biosciences) was added, followed by gentle mixing at 4 °C for 30 min. After washing the resin three times with buffer A, bound proteins were eluted with 20 μl of 2% SDS. The supernatant (S) containing unbound proteins, wash (W), and the SDS eluate (E), 10 μl of each, were analyzed by 10% SDS-PAGE and Coomassie Blue staining.

**D-loop Assay**—Unless stated otherwise, all the steps were conducted at 37 °C. DMC1 (0.8 μM) was incubated with the radiolabeled 90-mer oligonucleotide 1 (2.4 μM nucleotides, see supplemental Table 2 for the sequence) in 9.5 μl of buffer R (25 mM Tris-HCl (pH 7.5), 50 mM KCl, 4 mM MgCl2, 2 mM ATP, 1 mM DTT) for 5 min to assemble the presynaptic filament. Then the indicated amount of RAD51AP1, RAD51AP1 fragment, or mutant was incorporated in 2 μl, followed by a 5-min incubation, before pBluescript replicative form I DNA (35 μM base pairs) was added in 1 μl. After a 10-min incubation, reaction mixtures were treated with 0.5% SDS and 0.5 mg/ml proteinase K for 20 min and then subjected to agarose gel electrophoresis in TAE buffer (40 mM Tris, 20 mM NaOAc (pH 7.4), 2 mM EDTA) at 25 °C. Gels were dried and analyzed in a Personal Molecular Imager FX (Bio-Rad), with quantification done using the Quantity One software (Bio-Rad).

**Assay for Synaptic Complex Formation**—Unless stated otherwise, all the steps were conducted at 37 °C. The assay was performed as described in our published study (29). Briefly, DMC1 (4 μM) was incubated with the 60-mer oligonucleotide 2...
acts with DMC1 with an affinity comparable with full-length RAD51AP1 but that F1 and F2 are unable to associate with DMC1 (Fig. 1D). These observations thus indicate that the DMC1 binding region lies between residues 188 and 335 of RAD51AP1.

To investigate the functional significance of the DMC1-RAD51AP1 F3 pair, we performed two distinct biochemical assays to interrogate the homologous DNA pairing process. The first was the D-loop assay that monitors the formation of the final product of homologous DNA pairing (Fig. 2A). DMC1 alone converted approximately 4% of the input single-stranded DNA oligo into D-loops (Fig. 2B, lane 3). As expected from published studies (29), the addition of RAD51AP1 greatly stimulated this reaction up to 28% of D-loop formation (Fig. 2B, lanes 4–6). Neither F1 nor F2 stimulated D-loop formation (Fig. 2B, lanes 7–12), whereas F3 was almost as effective as full-length RAD51AP1 in stimulating the reaction up to 24% of product formation (lanes 13–15).

Next, we tested the RAD51AP1 fragments in an assay that examines the formation of the synaptic complex by probing for the protection of a homologous target in linear duplex DNA against restriction enzyme digestion (Fig. 2C). We note that, as determined by Adzuma (36), the structure of the synaptic complex bears resemblance to the D-loop. Following incubation of the DMC1 presynaptic filament with RAD51AP1, linear duplex DNA that harbors a homologous target, including an embedded SspI restriction site, is added. Formation of the synaptic complex, wherein the presynaptic filament is homologously paired to the target duplex region, results in protection of the duplex DNA against digestion by the restriction enzyme SspI (25, 29, 37). As reported before (29), neither the DMC1 presynaptic filament nor RAD51AP1 afforded significant protection against SspI (Fig. 2D, lanes 2–3). However, as we had observed before (29), a large fraction of the double-stranded DNA was protected from SspI digestion by the combination of the DMC1 presynaptic filament and RAD51AP1 (Fig. 2D, lanes 4–5). When the RAD51AP1 fragments were tested with the DMC1 presynaptic filament, we found that F1 and F2 are not able to protect the DNA from SspI digestion but that, importantly, F3 is nearly as effective as the full-length protein in this regard (Fig. 2D, lanes 6–9 and lanes 10 and 11, respectively).

Taken together, the results from the above assays indicate that the C-terminal portion of RAD51AP1 encompassing residues 188–335 mediates the interaction with DMC1 and that it is capable of promoting synaptic complex assembly and D-loop formation in conjunction with the DMC1 presynaptic filament. We note that this same C-terminal region is similarly capable of binding RAD51 and stimulating RAD51-mediated D-loop formation (31)

Evidence for Distinct DMC1 and RAD51 Interaction Epitopes in RAD51AP1—The extreme C terminus of RAD51AP1 appears to be critical for RAD51 interaction (30–32). We wondered if it might also be required for DMC1 binding. Therefore, we constructed truncation mutants of RAD51AP1 F3 that lack the C-terminal 7, 12, or 17 residues (Fig. 3A), expressed these

3 M. H. Dunlop and E. Dray, unpublished data.
truncation mutants in *E. coli*, and purified them for biochemical testing with DMC1 and RAD51 (Fig. 3B). By affinity pull-down that made use of the MBP tag on the various F3 variants, we found that the F3C/H9004, F3C/H900412, and F3C/H900417 truncation mutants are all proficient in DMC1 interaction (Fig. 3C) but devoid of the ability to associate with RAD51 (supplemental Fig. 1A).

We next tested the F3 truncation mutants in the D-loop assay (Fig. 3D). Importantly, we observed that none of the three C-terminal truncations impairs the ability of F3 to promote DMC1-mediated D-loop formation but that all three mutants are defective in the RAD51-mediated reaction (supplemental Fig. 1B).

Thus, the deletion of as few as seven of the C-terminal residues of RAD51AP1 impairs its ability to undergo physical and functional interactions with RAD51, but even deleting as many as 17 of the C-terminal RAD51AP1 residues does not seem to affect the ability to bind DMC1 or to enhance the recombinase function of DMC1. Taken together, these results strongly suggest that RAD51AP1 employs distinct regions to mediate its physical and functional interaction with DMC1 and RAD51, a premise that is validated by additional analyses, as documented below.

**A WVPP Motif Is Critical for DMC1 Interaction**—In our effort to more precisely demarcate the DMC1 interaction domain, we expressed the last 40 or 60 residues of RAD51AP1, the C40 and C60 fragments, in *E. coli*, purified them (Fig. 4A and B), and tested them in the affinity pull-down assay for DMC1 binding. The results showed that C60, but not C40, is capable of DMC1 interaction (Fig. 4C), whereas both C40 and C60 are able to associate with RAD51 (supplemental Fig. 2A). These findings again strengthen the deduction that separate
domains in RAD51AP1 are involved in DMC1 or RAD51 interaction and also provide evidence that the region between 60 and 40 residues from the C terminus of RAD51AP1, corresponding to residues 275–295 in isoform 2, may be critically important for DMC1 interaction.

When we analyzed the region that harbors residues 275–295 of RAD51AP1 from various species, we found a cluster of four highly conserved residues, WVPP, that resembles the FVPP motif in the tumor suppressor BRCA2 (Fig. 4 D and Ref. 33), known to be involved in complex formation with DMC1. To test the relevance of this RAD51AP1 sequence in DMC1 binding, we changed Trp-287 to alanine within the context of RAD51AP1 F3. The W287A mutant was expressed in E. coli and purified (Fig. 4, A and B). Biochemical testing revealed that F3 W287A is defective in DMC1 binding (Fig. 4 E) and, accordingly, incapable of enhancing the DMC1-mediated D-loop reaction (Fig. 5 A) or synaptic complex assembly (B). In sharp contrast, the F3 W287A mutant remains proficient in RAD51 interaction and fully functional in the RAD51-mediated D-loop reaction (supplemental Fig. 2, B and C).

To further investigate the functional significance of the Trp-287 residue, we introduced the W287A mutation into full-
The mutant protein was expressed in E. coli and purified for testing in the affinity pull-down, synaptic complex assembly, and D-loop reactions with DMC1 and RAD51 (Fig. 6, B). Importantly, the results from this endeavor showed clearly that Trp-287 is important for binding and stimulating DMC1 (Fig. 6, C–E) but dispensable for RAD51 (supplemental Fig. 3, A and B). As expected, RAD51AP1 isoform 3 (29), which lacks the WVPP motif, showed no activity in the DMC1-mediated reactions (Fig. 6, C–E).

Recently provided evidence to implicate RAD51AP1 as a novel partner of the meiotic recombinase DMC1 (31, 32, 34) in that it physically interacts with and enhances the recombinase activity of DMC1. By cytological analysis, RAD51AP1 was seen to colocalize with DMC1 on meiotic chromatin as frequently as it does with RAD51 (29).

At first glance, the attribute of RAD51AP1 as a DMC1 cofactor resembles what has been reported for RAD51 (31, 32, 34). However, studies documented here and elsewhere have unveiled an important distinction between the RAD51AP1-RAD51 and RAD51AP1-DMC1 pairs. Specifically, as we have documented here and elsewhere (29), RAD51AP1 employs sep-
DMC1 Interaction Motif in RAD51AP1

arate epitopes to interact with RAD51 and DMC1. Importantly, our biochemical mapping studies have led us to identify a conserved WVPP motif in RAD51AP1 that is reminiscent of the FVPP motif in BRC2A2, as previously shown by Thorslund et al. (33), to be important for the interaction of BRC2A2 with DMC1 but dispensable for its association with RAD51. Indeed, although the W278A mutation in RAD51AP1 ablates DMC1 interaction, it has little or no effect on complex formation with RAD51. Our results, together with those reported by Thorslund et al. (33), help uncover the W/FVPP sequence as part of a conserved DMC1 interaction motif. Importantly, our studies involving different RAD51AP1 isoforms and the W278A mutant (Ref. 29 and this work) have provided compelling evidence that complex formation between RAD51AP1 and DMC1 is a prerequisite for functional cooperation of these HR factors in the assembly of the synaptic complex and D-loop reaction. Although RAD51 and DMC1 share a great deal of amino acid sequence homology, the fact that RAD51AP1 possesses distinct epitopes for mediating RAD51 and DMC1 interactions implies that divergent domains in RAD51 and DMC1 are responsible for complex formation with RAD51AP1.

On the basis of the colocalization of DMC1 and RAD51 foci on meiotic chromatin of yeast, lily, and mouse (11, 38–40), and the fact that the two recombinases appear to cooperate functionally in crossover formation (14, 41, 42), it has been suggested that the two recombinases work together at the same DSB repair centers. It is possible that RAD51AP1, with its separate epitopes for binding DMC1 and RAD51, may simultaneously associate with both recombinases to serve as a molecular bridge to bring the two recombinases together in these repair centers. This would be an interesting topic for future investigations.

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J. Biol. Chem. 2011, 286:37328-37334.
doi: 10.1074/jbc.M111.290015 originally published online September 8, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M111.290015

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