Published in final edited form as:

DNA Repair (Amst). 2013 September ; 12(9): 723–732. doi:10.1016/j.dnarep.2013.05.005.

The HsRAD51B-HsRAD51C stabilizes the HsRAD51 nucleoprotein filament

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Abstract

There are six human RAD51 related proteins (HsRAD51 paralogs), HsRAD51B, HsRAD51C, HsRAD51D, HsXRCC2, HsXRCC3 and HsDMC1, that appear to enhance HsRAD51 mediated homologous recombinational (HR) repair of DNA double strand breaks (DSBs). Here we model the structures of HsRAD51, HsRAD51B and HsRAD51C and show similar domain orientations within a hypothetical nucleoprotein filament (NPF). We then demonstrate that HsRAD51B-HsRAD51C heterodimer forms stable complex on ssDNA and partially stabilizes the HsRAD51 NPF against the anti-recombinogenic activity of BLM. Moreover, HsRAD51B-HsRAD51C stimulates HsRAD51 mediated D-loop formation in the presence of RPA. However, HsRAD51B-HsRAD51C does not facilitate HsRAD51 nucleation on a RPA coated ssDNA. These results suggest that the HsRAD51B-HsRAD51C complex plays a role in stabilizing the HsRAD51 NPF during the presynaptic phase of HR, which appears downstream of BRCA2-mediated HsRAD51 NPF formation.

1. INTRODUCTION

Homologous recombination (HR) ensures genomic stability during DNA double strand breaks (DSBs) caused by ionizing radiation (IR) and stalled or collapsed replication machinery [1-3]. In eukaryotes, RAD51 is essential for HR and forms a nucleoprotein filament (NPF) that catalyzes homologous pairing and strand exchange (recombinase) [2-5]. However, unlike its robust bacterial counterpart RecA, RAD51 exhibits an inefficient recombinase activity that requires unusual cationic salts *in vitro* [4,6,7]. This observation would appear to necessitate the requirement of accessory proteins to facilitate efficient RAD51 mediated DSB repair by HR [4,8].

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In yeast, Rad55 and Rad57 display a limited homology to Rad51 (termed Rad51 paralogs; [2,3] and appear to have evolved from gene duplication events [9]. Mutations of either Rad55 or Rad57 lead to IR sensitivity that can be overcome by overexpression of Rad51 or expression of Rad51 gain-of-function mutants that possess a higher affinity for DNA [10-12]. The Rad55 and Rad57 proteins were shown to form a stable heterodimer [13]. Moreover, Rad55-Rad57 has been shown to regulate the stability of the Rad51 presynaptic NPF [14], and to relieve the inhibitory effect of RPA when Rad51 and RPA are introduced simultaneously with ssDNA during strand exchange reactions [13].

Five mitotic HsRAD51 paralogs, HsRAD51B, HsRAD51C, HsRAD51D, HsXRCC2 and HsXRCC3, have been identified in vertebrates that share 20-30% homology with HsRAD51 [15-20]. All five RAD51 paralogs contain canonical Walker A/B domains and all exhibit DNA dependent ATPase activities, although to a lesser extent compared to HsRAD51 [21-24]. These paralogs appear to exist in a variety of complexes *in vivo* and *in vitro* that include HsRAD51B-HsRAD51C, HsRAD51D-HsXRCC2, HsRAD51B-HsRAD51C-HsRAD51D-HsXRCC2 and HsRAD51C-HsXRCC3. Both HsXRCC3 and HsRAD51C have been shown to interact with HsRAD51 [25,26], although the interaction between HsRAD51C and HsRAD51 appears to be weak [26].

Mutations of most RAD51 paralog genes render cells susceptible to DSB causing agents. For example, Chinese hamster cell lines *irs1* and *irs1SF* defective in XRCC2 and XRCC3, respectively, are sensitive to IR and DNA crosslinking agents [26,27]. These cells also display chromosomal aberrations and missegregation [28,29]. Knock out of *Xrcc2*, *Rad51D* and *Rad51B* in mice lead to embryonic lethality [30-32]; a phenotype that is similar to Rad51 disruption [33,34]. Interestingly, a uterine leiomyoma contains a translocation in the intronic region of RAD51B that leads to premature termination of its transcript [35,36]. Six monoallelic mutations within RAD51C have been shown to confer susceptibility to breast and ovarian cancer [37], while one biallelic mutation caused abnormalities associated with Fanconi anemia (FA) [38,39]. The intricate involvement of RAD51C in the FA pathway led to its provisional assignment as FANCO, the 14th FA complementation group [40].

HsRAD51B-HsRAD51C, HsRAD51B-HsRAD51C-HsRAD51D-HsXRCC2 and HsRAD51C-HsXRCC3 complexes have been shown to bind ssDNA and dsDNA with 3'- or 5'-tails, dsDNA with gapped regions as well as branched DNA structures [23,41-43]. It has been previously demonstrated that HsRAD51B-HsRAD51C functions as a mediator during RAD51 catalyzed three-strand exchange assays by reducing the competition between RPA and HsRAD51 for ssDNA binding [23].

Both RecA and RAD51 NPF form an ATP cap that is located in the inter-subunit region of the adenosine nucleotide interface. RecA homologs contain a conserved Lys (K) residue in the ATP cap that contacts the ATP γ -phosphate, while RAD51 homologs contain a conserved Asp (D) residue that forms a salt-bridge with the ATP γ -phosphate [44-47]. Remarkably, substitution of the conserved Asp residue with Lys in human RAD51 [HsRAD51(D316K)] results in significant stabilization of the ATP-bound NPF as well as elimination of the unusual salt requirement which ultimately enhanced recombinase activity

[48]. These results have suggested that residues within the ATP cap may help to regulate the RAD51 NPF.

Here we demonstrate that the projected structures of HsRAD51, HsRAD51B and HsRAD51C appear capable of forming filaments with similar domain orientations. Furthermore, HsRAD51B-HsRAD51C heterodimer partially stabilizes the RAD51 NPF against the anti-recombinogenic activity of BLM. At sub-stoichiometric concentrations, HsRAD51B-HsRAD51C appears to enhance D-loop formation in the presence of RPA. Interestingly, while HsRAD51B-HsRAD51C appears to alleviate the inhibitory effect of RPA in strand exchange assays [23], the mechanism does not appear to be a function of facilitating HsRAD51 nucleation on an RPA coated ssDNA during D-loop formation *in vitro*. Our results are consistent with a role for HsRAD51B-HsRAD51C in stabilizing HsRAD51 on ssDNA by integrating into the NPF.

2. MATERIAL AND METHODS

2.1 Structure modeling and sequence alignments

Human RAD51, RAD51B and RAD51C structures were modeled using composite iterative threading assembly refinement software I-TASSER [49] without additional restraints and the optimized structures were superimposed on the *Methanococcus voltae* RadA (MvRAD51) crystal structure coordinates [50] (PDB ID: 1XU4) using Pymol molecular imaging software [51]. C-score, TM-score, normalized Z-score and RMSD are reported [49]. The normalized Z-score is equal to the Z-score of the alignment divided by a program-specific cut-off that has been determined based on large scale threading tests [49]. If most of the top threading alignments have a normalized Z-score >1, then the accuracy of the final model is considered high. Protein sequence alignments were done using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>).

2.2 Proteins expression and purification

HsRAD51B and HsRAD51C were cloned into pFast-Bac vector (Invitrogen) from cDNA. HsRAD51B carried a N-terminal His₆ tag. 2L of infected SF9 insect cells (Invitrogen) were harvested after 48 hours and resuspended in Buffer P containing 25 mM Tris-Cl (pH 8.0), 300 mM NaCl, 10mM imidazole, 10% glycerol and protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 µg/mL leupeptin, 1 µg/mL pepstatin, 1 µg/mL aprotinin). The resuspended pellet was flash frozen in liquid nitrogen and stored at -80 °C until purification.

The cell pellet was thawed on ice, sonicated and passed through a 21-gauge needle. The insoluble material was separated by centrifugation at 41000 rpm at 4 °C for 1 hr in a Beckman-Coulter Ti70 rotor. The soluble material was immediately loaded onto a NiSO₄ charged 3 mL Ni-NTA (Nitrilotriacetic acid) Superflow (Qiagen) column equilibrated with Buffer P. The column was washed with 10-column volumes of the same buffer followed by 10-column volumes of Buffer P containing 20 mM imidazole. The HsRAD51B-HsRAD51C complex was eluted in a 50 mL linear gradient from 20 mM - 250 mM imidazole in Buffer P. RAD51B-RAD51C containing fractions were dialyzed overnight into Buffer R containing

25 mM Tris-Cl (pH 8.0), 100 mM NaCl, 1 mM DTT, 0.5 mM EDTA and 10% glycerol. The dialyzed fraction was loaded onto a 10 mL SP-sepharose column (GE Healthcare) equilibrated with Buffer R and the flow through was collected and loaded onto a 10 mL Q-sepharose (GE Healthcare) column equilibrated in Buffer R. After a 5-column volume wash RAD51B-RAD51C was eluted in a linear gradient from 100 mM - 600 mM NaCl in Buffer R. Pooled fractions were dialyzed into Buffer R and loaded on a 10 mL Heparin sepharose (GE Healthcare) column equilibrated with Buffer R and washed with 5-column volumes of the same buffer. RAD51B-RAD51C was eluted in a linear gradient from 100 mM - 800 mM NaCl. Peak fractions containing RAD51B-RAD51C were concentrated using an Amicon ultra-15 (Millipore) centrifugal filter unit at 4 °C. The concentrated fraction was dialyzed into storage buffer containing 25 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM DTT, 0.1 mM EDTA and 10% glycerol and flash frozen in liquid nitrogen in small aliquots and stored in -80 °C.

HsRAD51 and RPA were expressed and purified as described [52]. BLM was purified as described [53] with an additional chromatographic step on S-sepharose (GE Healthcare).

2.3 DNA Substrates

For surface plasmon resonance (SPR) analysis, a 5'-biotinylated oligo dT₅₀ was used as ssDNA and for dsDNA 5'-biotinylated 50-mer 5'-TCG AGA GGG TAA ACC ACA-ATT ATT GAT ATA AAA TAG TTT TGG GTA GGC GA was annealed with its complement and purified by HPLC on a Gen-Pak FAX column (Waters). D-loop assay substrates were prepared as described [54]. ϕ X174 virion ssDNA and RFI dsDNA were purchased from NEB. RFI DNA was obtained by linearizing RFI ϕ X174 with ApaLI restriction enzyme (NEB).

2.4 SPR analysis

Biotinylated DNA was immobilized on a streptavidin-coated chip (GE Healthcare). Binding and dissociation were analyzed following the injection of the indicated amounts of protein at 25°C with a 5 μ L/min flow rate on a Biacore 3000 (GE Healthcare). Reactions were performed in Buffer containing 25 mM Tris-OAc (pH 7.5), 1 mM ATP, 1 mM Mg(OAc)₂, 2 mM DTT and 0.005% Tween-20.

2.5 D-loop assays

RAD51 (1 μ M) was bound with RAD51B-RAD51C (at the indicated concentrations) on 90mer ssDNA (3 μ M nt) by incubating at 37 °C for 15 min in reaction buffer containing 25 mM Tris-OAc (pH 7.5), 1 mM ATP, 1 mM Mg(OAc)₂, 1 mM CaCl₂, 2 mM DTT and BSA (100 μ g/mL). After 15 min, RPA (200 nM) was added (as indicated) and D-loop formation was initiated by the addition of supercoiled dsDNA (50 μ M bp) and incubation at 37 °C for 15 min. D-loops were analyzed by electrophoresis on a 0.9% agarose gel after deproteinization. Gel was dried and exposed to a PhosphorImager (Molecular Dynamics) screen for quantification.

2.6 Inhibition of D-loop formation

RAD51 (1 μ M) filaments were formed with RAD51B-RAD51C (31.2 nM) when indicated, on 90-mer ssDNA (3 μ M nt) by incubating at 37 °C for 15 min in reaction buffer containing 25 mM Tris-OAc (pH 7.5), 1 mM ATP, 1 mM Mg(OAc)₂, 2 mM DTT, BSA (100 μ g/mL), 20 mM phosphocreatine and creatine phosphokinase (30 U/mL). After the 10 min incubation BLM at indicated concentrations and RPA (200 nM) were added and incubated for an additional 10 min at 37 °C. 1 mM CaCl₂ was then added and followed for another 10 min incubation at 37°C. D-loop formation was initiated by the addition of supercoiled dsDNA (50 μ M bp) and incubation at 37°C for 15 min. D-loops were analyzed by electrophoresis on a 0.9% agarose gel after deproteinization. Gel was dried and exposed to a PhosphoImager (GE Healthcare) screen for quantification.

2.7 ATPase assay

RAD51 (1 μ M) and RAD51B-RAD51C at the indicated concentrations with oligo dT₅₀ (3 μ M nt), ϕ X174 ssDNA (3 μ M nt) or RFIII ϕ X174 dsDNA (3 μ M bp) in reaction buffer containing 25 mM Tris-OAc (pH 7.5), 1 mM Mg(OAc)₂, 2 mM DTT, BSA (100 μ g/mL) and 200 μ M ATP supplemented with 200 nCi of [γ -³²P] ATP for 1 hr at 37°C. The reaction was stopped by the addition of 400 μ L 10% activated charcoal supplemented with 10 mM EDTA and incubated on ice for 2 hrs. Following centrifuging for 10 min, 50 μ L duplicate aliquots were taken for counting [³²P] free phosphate by Cerenkov method [55]. Hydrolyzed ATP percentage is indicated.

3. RESULTS

3.1 Homology based structural analysis of HsRAD51, HsRAD51B and HsRAD51C indicate similar functional domain orientations

The crystal structure of HsRAD51 and its paralogs have not been solved. Utilizing a composite iterative threading assembly refinement technique [49], the structures of HsRAD51, HsRAD51B and HsRAD51C were modeled based on the archaeobacterial RadA structure that was solved in the presence of potassium cation (Suppl. Table 1; [50]. Normalized Z-scores >1 of these structural alignments suggested relatively high confidence in the accuracy of the models (Suppl. Table 1; see Materials and Methods). A similar orientation of the ATP cap and the ssDNA binding L2 region was observed for the treaded models (Fig. 1). The RAD51B predicted structure contains an extra disordered region within L2 region (Fig. 1B and Suppl. Fig. 1). Interestingly, the HsRAD51(D316) residue of the ATP cap aligns in an orientation that seems to coordinate with the potassium cation that functions as a salt bridge between the ATP cap of RadA and the ATP γ -phosphate (Fig. 1A). However, the analogous residues to HsRAD51(D316), HsRAD51B(K324) and HsRAD51C(K328), align at a position that would allow direct interaction with the γ -phosphate group of the ATP similar to RecA (Fig. 1B, Fig. 1C and Suppl. Fig 1). These structural alignments are consistent with the hypothesis that HsRAD51B and HsRAD51C can form a functional NPF with HsRAD51 where the ATP cap Lys residues of the two paralogs may make direct contact with the ATP γ -phosphate.

3.2 RAD51B- RAD51C heterodimer forms stable complexes on ssDNA and dsDNA

HsRAD51, HsRAD51B, HsRAD51C and the HsRAD51B-HsRAD51C heterodimer complex have been shown to bind ssDNA, DNA substrates with tails and dsDNA [22,23,56]. The dsDNA binding of HsRAD51B and HsRAD51C appears less avid than HsRAD51 [22,23,56]. We expressed and purified the HsRAD51B-HsRAD51C heterodimer proteins (Suppl. Fig. 2) and examined the real-time DNA binding/dissociation kinetics using surface plasmon resonance (SPR). These studies were performed in 25mM KCl and 100mM KCl to determine the ionic conditions that allow HsRAD51B-HsRAD51C binding to DNA.

HsRAD51 displayed comparable levels of ssDNA and dsDNA binding in both conditions (Fig. 2, time scale 100s-300s). However, HsRAD51 turnover from ssDNA increased at the higher salt concentration (Fig. 2A, time scale 300s-600s). The increased turnover can be attributed to the salt induced enhancement of the ATPase activity of the recombinase [6,7]; a well-known manifestation of the RecA/RAD51 family of proteins [4,57]. Interestingly, HsRAD51B-HsRAD51C binding to both ssDNA and dsDNA was significantly inhibited in 100 mM KCl (Fig. 2A and Fig. 2B). The inhibition reduced dsDNA binding to nearly background levels (Fig. 2B). However, an intriguing feature emerged from HsRAD51B-HsRAD51C dissociation pattern. Even though the maximum binding was comparably less than that of HsRAD51, the bound HsRAD51B-HsRAD51C appeared stably associated with the DNA as indicated by little or no turnover (k_{off} ; compare 300s-600s time scale of HsRAD51 with HsRAD51B-HsRAD51C in Fig. 2A and Fig. 2B). This feature was evident at both 25 mM and 100 mM salt on the ssDNA and at least 25 mM salt on the dsDNA (Fig. 2A and Fig. 2B). Collectively, these results indicate that HsRAD51B-HsRAD51C binds and forms unusually stable complexes on both ssDNA and dsDNA.

3.3 HsRAD51B-HsRAD51C stabilizes HsRAD51 on ssDNA

The stability of HsRAD51B-HsRAD51C on ssDNA suggested that it might stabilize the HsRAD51 NPF. We determined the stability of the HsRAD51 NPF on ssDNA in the presence of HsRAD51B-HsRAD51C by SPR (Fig. 2C and Fig. 2D). Our previous studies suggested that the rate of HsRAD51 binding to ssDNA was not a simple 1:1 interaction precluding an accurate determination of k_{on} and ultimately the calculation of K_D [48]. However, the dissociation rate (k_{off}) appeared to follow an uncomplicated single exponential decay that was indicative of the stability of the HsRAD51 NPF [48]. We found that increasing concentrations of HsRAD51B-HsRAD51C decreased the k_{off} of HsRAD51 in 25 mM KCl ($k_{off} \cdot \text{HsRAD51} = 0.005 \pm 0.001 \text{ s}^{-1}$; $k_{off} \cdot \text{HsRAD51} + \text{HsRAD51B-HsRAD51C}(1:1) = 0.0016 \pm 0.0003 \text{ s}^{-1}$; Fig. 2C, compare the dissociation curves at 250s-550s). Remarkably, at 100 mM KCl we observed little or no stabilization of the HsRAD51 NPF by HsRAD51B-HsRAD51C (Fig. 2D). These results suggest a significant stabilization of the HsRAD51 NPF by HsRAD51B-HsRAD51C that appears dependent on the ionic conditions.

3.4 HsRAD51B-HsRAD51C enhances HsRAD51 catalyzed D-loop formation in the presence of RPA at sub-stoichiometric amounts

We examined the effect of HsRAD51B-HsRAD51C on HsRAD51 and RPA catalyzed D-loop formation activity (Fig. 3A). Previous studies of HsRAD51 paralog stimulated strand exchange reactions catalyzed by HsRAD51 were observed when the paralogs were used at

sub-stoichiometric amounts [13,23]. A role for HsRAD51 paralogs in D-loop formation is unknown. For D-loop formation analysis we incubated HsRAD51 and varying amounts of HsRAD51B-HsRAD51C under traditionally low salt conditions. We incubated HsRAD51 and HsRAD51B-HsRAD51C with the ssDNA in D-loop buffer containing Ca^{++} , since this divalent cation appears to maintain HsRAD51 in a recombinase-catalytic ATP-bound form which is essential for D-loop formation [58]. HsRAD51B-HsRAD51C complex did not possess any D-loop forming ability alone (Fig. 3B, lanes 2 and 10). The co-incubation of HsRAD51B-HsRAD51C complex with HsRAD51 was necessary to see optimal D-loop formation compared to the addition of the paralog complex prior to HsRAD51 or after the formation of HsRAD51 nucleoprotein filament (data not shown). Purified human HsRPA (see Suppl. Fig. 2) was added prior to initiation of the D-loop reaction with addition of supercoiled dsDNA as previously described (Fig. 3B and Fig. 3C, lanes 11-17; [58]). We observed stimulation of the HsRAD51-catalyzed D-loop formation by HsRAD51B-HsRAD51C at sub-stoichiometric levels, but only in the presence of RPA (Fig. 3B and Fig. 3C; compare quantitated red bars -HsRPA with blue bars +HsRPA in Fig. 3C). D-loop formation was maximum at a molar ratio of ~1:30, HsRAD51B-HsRAD51C : HsRAD51 (Fig. 3B, lanes 11-13 and Fig 3C). Further increasing the concentration of HsRAD51B-HsRAD51C appeared to slightly decrease D-loop product formation in the presence of HsRPA and significantly decrease D-loop product formation in the absence of HsRPA.

To determine whether RAD51B-RAD51C directly interacted with RPA as a potential mechanism for D-loop stimulation, we performed pull-down studies with Ni-NTA beads exploiting the His-tag on RAD51B. However, we did not detect any direct physical interaction between RAD51B-RAD51C paralog complex and RPA (data not shown). Taken as a whole, these results are consistent with previous studies that suggest HsRPA enhances D-loop formation by sequestering and stabilizing the displaced ssDNA. In the absence of HsRPA we regard it likely that the higher concentrations of HsRAD51B-HsRAD51C compete with HsRAD51 binding on the invading ssDNA: making it less capable of a productive homology search. It is worth noting that HsRPA appears to significantly reduce such hypothetical competition ultimately enhancing D-loop catalytic activity nearly 2-fold.

3.5 HsRAD51B-HsRAD51C protects HsRAD51 nucleoprotein filament against the anti-recombinase function of BLM

The yeast Sgs1 and human BLM RecQ helicase family members as well as yeast Srs2 have been shown to induce anti-recombinase activity by dissociating HsRAD51 from ssDNA, thus preventing functional NPF formation for strand exchange [59-61]. We wondered whether the enhanced stability provided by HsRAD51B-HsRAD51C might influence BLM catalyzed dissociation of the HsRAD51 NPF and ultimately its recombinase functions?

The assay for HsRAD51 NPF stability was based on a previous approach to study the inhibitory effects of BLM on HsRAD51-mediated strand exchange activity [59]. First the HsRAD51 NPFs were formed either in the absence or presence of the HsRAD51B-HsRAD51C heterodimer in an ATP regeneration system that is required for recombinase NPF formation as well as for the subsequent BLM mediated step. The amount of HsRAD51B-HsRAD51C introduced into the reaction was based on the concentration

required for optimal D-loop formation (Fig. 3B). After the initial incubation, purified BLM (see Suppl. Fig. 2) and HsRPA were added, and the reactions were incubated further. HsRPA serves to prevent possible HsRAD51 rebinding events after it has been dislodged by BLM translocase activity on ssDNA [59] (See Fig. 4A for the reaction schematic). D-loop formation was initiated by addition of Ca^{++} and supercoiled DNA. BLM translocation is greatly attenuated in the presence of Ca^{++} [59], therefore it was necessary to add the divalent cation prior to the addition of supercoiled dsDNA. If BLM translocation dissociates HsRAD51 from the NPF, then that would lead to reduced D-loop formation. As expected, we observed a decreased HsRAD51 mediated D-loop formation with increasing concentration of BLM ($\text{EC}_{50} \cdot \text{BLM} = 64.0 \pm 2.4 \text{ nM}$; Fig. 4B and Fig. 4C). In contrast, the addition of HsRAD51B-HsRAD51C increased the amount of BLM required to inhibit HsRAD51 mediated D-loop formation by 50% ($\text{EC}_{50} \cdot \text{BLM} \cdot \text{HsRAD51B-HsRAD51C} = 93.1 \pm 1.5 \text{ nM}$; compare lanes 2-8 and lanes 9-15 of Fig. 4B and Fig. 4C). This result suggests that HsRAD51B-HsRAD51C at least partially stabilizes the HsRAD51 NPF during D-loop catalysis.

3.5 HsRAD51-HsRAD51C does not alter the ATPase activity of the HsRAD51

Both HsRAD51 and HsRAD51B-HsRAD51C are DNA stimulated ATPases [23,55,56]. The ATPase activity of HsRAD51B-HsRAD51C, derives from both constituent monomers HsRAD51B and HsRAD51C [22]. Since we observed increased D-loop formation with sub-stoichiometric amounts of HsRAD51B-HsRAD51C, and because the recombinase activity of HsRAD51 can be enhanced by suppressing its ATP hydrolysis, we determined whether the paralog complex altered the HsRAD51 ATPase activity [58].

We examined the ATP hydrolysis activity of the HsRAD51 / HsRAD51B-HsRAD51C mixed filament in varying ratios with an oligo dT50, ϕ X174 ssDNA and RFIII ϕ X174 dsDNA substrates (Fig. 5). As expected, HsRAD51 indicated the highest ATP turnover rate with the oligo dT50 substrate (Fig. 5). Collectively, the ATP turnover rates of the mixed filament increased with the concentration of the paralog complex, even at ratios that induced enhanced D-loop formation (compare Fig. 5 with Fig. 3C). This suggests that the stimulatory effect of HsRAD51B-HsRAD51C on HsRAD51 mediated strand exchange activity is not linked to altered HsRAD51 ATPase activity.

3.6 RAD51B-RAD51C does not alleviate inhibitory effects of RPA to promote RAD51 mediated D-loop formation

RPA binds to ssDNA during DSB repair once the 5'-ends are resected to prevent formation of secondary structures [62]. RPA is eventually replaced by HsRAD51 nucleation mediated by either Rad52 in yeast or BRCA2 in vertebrates [63-67]. During recombinase assays *in vitro*, pre-incubation of RPA and ssDNA leads to greatly reduced strand exchange activity, due to its strong affinity for the ssDNA. Previously, it was shown that HsRAD51B-HsRAD51C heterodimer functions as a presynaptic mediator in a three-strand exchange assay [23]. We determined whether HsRAD51B-HsRAD51C could alleviate the inhibitory effect of HsRPA without the requirement of BRCA2 during HsRAD51 catalyzed D-loop formation.

In this approach we pre-incubated ssDNA with the indicated amounts of HsRPA and then HsRAD51 or HsRAD51 with HsRAD51B-HsRAD51C was added (Fig. 6). Pre-addition of HsRPA lead to significantly reduced product formation compared to HsRPA addition after HsRAD51/HsRAD51B-HsRAD51C NPF had formed (compared lanes 2 and 6, and lanes 7 and 11 in Fig. 6A and Fig 6B). We did not observe any alleviation of HsRPA inhibition by HsRAD51B-HsRAD51C (Fig. 6A and Fig 6B). This observation appeared even more evident at higher concentrations of RPA.

4. DISCUSSION

4.1 Evolution of RAD51 paralogs in eukaryotes

Gene duplication allows one copy of the gene to retain the existing function while the other evolves to gain a new function by mutations [9,68]. The process also leads to separation of ancestral function [68]. Phylogenetic analyses indicate that the RecA/RAD51 family of genes could be divided into three subfamilies; RAD α , RAD β and RecA that have all evolved from a common ancestral RecA [9]. RAD α contains genes of highly conserved function that include RAD51 and DMC1. RAD β includes genes of divergent functions that include RAD51 paralogs RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3 [9]. RecA is found in eubacteria [9]. The *S.cerevisiae* Rad51 paralogs, Rad55 and Rad57, appear to be orthologous to vertebrate XRCC2 and XRCC3, respectively [9]. Even though RAD51 paralogs share only 20-30% homology with RAD51, they are well conserved within vertebrate cells [69,70].

The homologous recombination mechanism appears largely conserved in prokaryotes and eukaryotes. The structural and functional properties of the bacterial RecA and eukaryotic RAD51 recombinases are similar [56,71,72]. However, compared to RecA, RAD51 possesses a greatly reduced recombinase function [4,6,7]. This would seem to necessitate the requirement of other recombination mediator to promote efficient recombinational repair in more complex genomes.

4.2 Stabilization of the nucleoprotein filament

We previously reported that the ATP cap is involved in regulating the turnover of HsRAD51, thereby affecting the recombinase function [48]. The HsRAD51(D316K) ATP cap mutation attenuates the ATPase activity and leads to formation of a stable NPF that results from slow turnover of the protein-DNA complex. This in turn, promotes an enhanced recombinase activity. In our modeled structures, the ATP cap residues RAD51(D316), RAD51B(K324) and RAD51C(K328) are positioned in a similar orientation. It is tempting to speculate that the slow protein turnover observed for RAD51B-RAD51C-DNA interaction may be mediated by the ATP cap residues of the paralog complex (Fig. 2). However, RAD51B-RAD51C complex does not possess any recombinase function (Fig. 3 and [23]. Also, addition of HsRAD51B-HsRAD51C does not reduce the amount of HsRAD51 required for strand exchange functions (data not shown; [23]. In fact, increasing the amounts of HsRAD51B-HsRAD51C in the reactions leads to a reduction of HsRAD51 recombinase activity (Fig. 3). In our SPR studies we observed a reduction in the k_{off} when mixed HsRAD51/HsRAD51B-HsRAD51C NPFs were formed (Fig. 2). These results are

consistent with the stabilization of the NPF we observe against the anti-recombination function of BLM (Fig. 4). Taken as a whole, these observations appear to suggest that one of the functions of HsRAD51B-HsRAD51C is to protect and stabilize the HsRAD51 NPF during homologous recombination. However, further structural studies are necessary to reveal any arrangement of RAD51B-RAD51C within the NPF.

HsRAD51 must be in the ATP-bound active state to catalyze D-loop formation *in vitro*. Such a state occurs in the presence of Ca^{++} [58]. In the cytosol, Ca^{++} ions are tightly regulated as they function as secondary messengers and they do not appear in concentrations appreciable for D-loop formation [73]. One hypothesis for the role of HsRAD51B-HsRAD51C might be as auxiliary proteins in stabilizing the ATP-bound HsRAD51 NPF. However, our ATPase data suggest that RAD51B-RAD51C stabilization of the HsRAD51 NPF does not occur by reducing ATP hydrolysis (Fig. 5). Structurally, such an ATPase suppression mechanism seems unlikely as both RAD51B and RAD51C paralogs contain Walker A box domains that hydrolyze ATP (Suppl. Fig. 1).

So how does HsRAD51B-HsRAD51C stabilize the HsRAD51 NPF, and how does this stabilization block BLM-catalyzed dissociation of the HsRAD51 NPF? One possibility is that HsRAD51-HsRAD51C is physically bound to the ssDNA at intervals within the HsRAD51 NPF ultimately producing roadblocks to the translocation of BLM. This idea is consistent with the titration results that demonstrate significant NPF stabilization beginning at a ratio of 8:1 HsRAD51:HsRAD51B-HsRAD51C, with maximal stabilization at a 1:1 ratio (Fig. 2C) However, we cannot rule out the possibility of post-translational modifications or the involvement of other RAD51 paralogs that might alter and/or improve the stabilization effect conferred by RAD51B-RAD51C complex *in vivo*.

RAD51 foci formation during IR is the initial step of HR repair [74,75]. BRCA2 is involved in transporting HsRAD51 to the nucleus, as the latter lacks a nuclear localization signal (NLS) as well as initiating recombinase nucleation on ssDNA [76]. The BRC repeats of BRCA2 stabilize HsRAD51 by suppressing its ATPase activity and facilitates nucleation on ssDNA while inhibiting HsRAD51 filament formation on dsDNA [63,77,78]. However, in pancreatic epithelial tumor derived CAPAN-1 cells that contain a C-terminal truncated BRCA2 lacking the NLS [79], it was found that IR-induced HsRAD51 foci formation occurs during S phase (albeit to a lesser extent), in a BRCA2 independent manner [5,76]. Surprisingly, BRCA2 independent HsRAD51 nuclear localization was attributed to HsRAD51C, which contains a functional NLS [76]. Therefore, HsRAD51B-HsRAD51C mediated formation and stabilization of the NPF against the RecQ family of anti-recombinases might be critical in the event of BRCA2 absence.

Even though HR is an error-free repair pathway to repair DSBs, unregulated HR could lead to gross chromosomal rearrangements [80]. Therefore, cells have evolved counter mechanism to suppress HR at untimely events. Both cellular and biochemical data indicate that the yeast Srs2 helicase disrupts Rad51-ssDNA filaments to suppress HR [60,61]. Overexpression of the RecQ homolog Sgs1 in Srs2 deleted strains suppresses the hyper-recombinogenic phenotypes [81,82]. The human BLM and RECQL5 helicases have been reported to suppress HsRAD51 mediated strand exchange activity by dissociating

HsRAD51-ssDNA filaments [59,83]. Recently, yeast Rad55-Rad57 heterodimer complex was shown to stabilize the Rad51 NPF and counteract the anti-recombinogenic activity of the Srs2 helicase by a direct protein-protein mediated interaction [14]. Furthermore, a genetic study determined that the yeast Shu complex, containing Shu1, Shu2, Csm2 and Psy3 (Shu1 and Psy3 being Rad51 paralogs), were involved in suppressing the anti-recombinase activity of Srs2 by physically interacting with the helicase [84]. A similar direct interaction between human HsRAD51B-HsRAD51C paralog complex with the helicase BLM that might inhibit the anti-recombinase function is unknown. However, BLM has been shown to physically interact with HsRAD51D of the HsRAD51D-HsXRCC2 complex [85].

4.3 Enhanced D-loop formation in the presence of HsRPA

HsRAD51C possesses an ability to destabilize dsDNA that might facilitate DNA strand exchange activity [22]. In our D-loop assays the HsRPA induced stimulation appears more apparent in the presence of HsRAD51B-HsRAD51C (Fig. 3). We could rationalize this observation as HsRPA capturing the displaced strand that has been destabilized by HsRAD51C of the HsRAD51B-HsRAD51C complex to facilitate HsRAD51 catalyzed D-loop formation. In fact, previous studies have shown the RPA facilitating the strand exchange activity by binding to the displaced ssDNA [54,86]. Intriguingly, the highest stimulation of RAD51B-RAD51C mediated D-loop formation in the presence of RPA, occurred when the paralog heterodimer and the ssDNA oligo were at a 1:1 molar ratio (Fig. 3). This co-incidental stoichiometry suggests that RAD51B-RAD51C might facilitate RAD51 loading onto ssDNA. Although, an interesting hypothesis, our data demonstrate that it is still not sufficient to overcome the inhibitory effect of RPA to promote D-loop formation (Fig. 6), consistent with recent observations that RAD51 paralogs are likely to function downstream of BRCA2 [87,88].

Our studies are consistent with a model in which the HsRAD51 paralog complex HsRAD51B-HsRAD51C regulates of the stability of the HsRAD51 NPF by acting as a pro-recombinase against anti-recombinogenic factors downstream of BRCA2 functions (Fig. 7). It is likely that the dynamic property afforded to the HsRAD51 NPF by these recombination accessory proteins is required for a highly regulated HR mechanism in the cell.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to thank Jeremy Keirsey for help with purified BLM. This work was supported by the National Institute of Health grant GM080176 (RF), CA117898 (JG) and The Bloom's Syndrom Foundation (RF, JG).

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HIGHLIGHTS

1. Threaded structure of human HsRAD51B and HsRAD51C proteins suggests they are capable of integrating into the HsRAD51 NPF.
2. Purified HsRAD51B-HsRAD51C shows extremely stable binding to ssDNA and dsDNA.
3. HsRAD51B-HsRAD51C enhances the stability of the HsRAD51 NPF.
4. HsRAD51B-HsRAD51C enhances HsRAD51 catalyzed D-loop formation at substoichiometric concentrations.
5. HsRAD51B-HsRAD51C protects the HsRAD51 NPF from dissociation by the RecQ family helicase BLM.

Figure 1. Homology based modeled structures of HsRAD51, HsRAD51B and HsRAD51C indicate similar domain orientations

(A) HsRAD51 structure was modeled using homology based modeling software I-TASSER [49] and the optimized structures were superimposed on the *Methanococcus voltae* RadA (MvRAD51) crystal structure coordinates (PDB ID: 1XU4) using Pymol. The non-hydrolysable ATP analog AMP-PNP is bound at the interface between MvRAD51 top monomer (cyan) and the bottom monomer (wheat). The Walker A subunit of the bottom monomer is indicated in grey. L2 ssDNA binding regions of MvRAD51 and HsRAD51 are highlighted in salmon and brown colors, respectively. The ATP caps of MvRAD51 and HsRAD51 are indicated in red and magenta colors, respectively. D316 of HsRAD51 is indicated. Two potassium (K^+) ions at the interface of MvRAD51 monomers are also illustrated. Modeled and superimposed structures of HsRAD51B in Blue (B) and Human HsRAD51C in Green (C). The domain color assignments are similar to A.

Figure 2. HsRAD51B-HsRAD51C heterodimer forms stable complexes on DNA

(A) ssDNA binding and dissociation curves obtained from surface plasmon resonance (SPR). The SPR curves correspond to 800nM concentration of either HsRAD51 or HsRAD51B-HsRAD51C heterodimer. DNA binding corresponds to the timescale 100s-300s and dissociation from 300s-600s. Binding response is given in Response Units. DNA binding and dissociation were analyzed in 25mM Tris-OAc (pH 7.5), 1mM ATP, 1mM Mg(OAc)₂, 2mM DTT and 0.005% Tween-20. Control (without protein) curve is red, HsRAD51B-HsRAD51C binding at 25mM K⁺ (KCl) curve is black, HsRAD51B-HsRAD51C binding at 100mM K⁺ curve is pink, HsRAD51 binding at 25mM K⁺ curve is blue and HsRAD51 binding at 100mM K⁺ curve is green. (B) dsDNA binding and dissociation of HsRAD51B-HsRAD51C heterodimer and HsRAD51 analyzed by SPR. The reaction conditions and the binding curve descriptions are same as in A. (C) ssDNA binding and dissociation curves for HsRAD51 in the presence if indicated concentrations of HsRAD51B-HsRAD51C at 25mM K⁺ (KCl). (D) ssDNA binding and dissociation curves

for HsRAD51 in the presence of indicated concentrations of HsRAD51B HsRAD51C at 100mM K⁺ (KCl).

Figure 3. HsRAD51B-HsRAD51C enhances HsRAD51 catalyzed D-loop formation in the presence of HsRPA

(A) Schematic diagram of the experimental approach. Asterisk indicates the [^{32} P] label on ssDNA. Utilized proteins and DNA substrates are illustrated with labels. B-C, HsRAD51B-HsRAD51C heterodimer. HsRAD51 (1μM) filaments were formed with HsRAD51B-HsRAD51C at the indicated concentrations (if added), on 90mer ssDNA (3μM nt) by incubating at 37°C for 15min in reaction buffer containing 25mM Tris-OAc (pH 7.5), 1mM ATP, 1mM Mg(OAc)₂, 1mM CaCl₂, 2mM DTT and BSA (100μg/mL). After 15min, RPA

(200nM) was added (if indicated) and D-loop formation was initiated by the addition of supercoiled dsDNA (50 μ M bp) and incubation at 37°C for 15min. D-loops were analyzed by electrophoresis on a 0.9% agarose gel after deproteinization. Gel was dried and exposed to a PhosphoImager screen for quantification. (C) Quantification of D-loops formed in **B**. Error bars indicate S.D.

Figure 4. HsRAD51B-RAD51C protects HsRAD51 nucleoprotein filament against anti-recombinase function of BLM

(A) Experimental scheme. Asterisk denotes the [^{32}P] label on ssDNA. Utilized proteins and DNA substrates are illustrated with labels. B-C, HsRAD51B-HsRAD51C heterodimer. (B) HsRAD51 (1 μM) filaments were formed with HsRAD51B-HsRAD51C (31.2 μM) if indicated, on 90mer ssDNA (3 μM nt) by incubating at 37°C for 15min in reaction buffer containing 25mM Tris-OAc (pH 7.5), 1mM ATP, 1mM Mg(OAc) $_2$, 2mM DTT, BSA (100 $\mu\text{g}/\text{mL}$), 20mM phosphocreatine and creatine phosphokinase (30U/mL). After the

10min incubation BLM at indicated concentrations and HsRPA (200nM) were added and incubated further for 10min at 37°C. 1mM CaCl₂ was then added and followed by another 10min incubation at 37°C. D-loop formation was initiated by the addition of supercoiled dsDNA (50μM bp) and incubation at 37°C for 15min. D-loops were analyzed by electrophoresis on a 0.9% agarose gel after deproteinization. Gel was dried and exposed to a PhosphoImager screen for quantification. (C) Quantification of relative amounts of D-loops formed in **B**. Error bars indicate S.D.

Figure 5. HsRAD51B-HsRAD51C does not suppress the overall ATPase activity of the HsRAD51/HsRAD51B-HsRAD51C nucleoprotein filament

ATPase assays were performed by incubating HsRAD51 (1μM) and HsRAD51B-HsRAD51C at the indicated concentrations with oligo dT₅₀ (3μM nt), ϕX174 ssDNA (3μM nt) or ϕX174 dsDNA (3μM bp) in reaction buffer containing 25mM Tris-OAc (pH 7.5), 1mM Mg(OAc)₂, 2mM DTT, BSA (100μg/mL) and 200μM ATP supplemented with 200nCi of [γ -³²P] ATP for 1hr at 37°C. The reaction was stopped by the addition of 400μL 10% activated charcoal supplemented with 10mM EDTA and incubated on ice for 2 hrs. Following centrifuging for 10 min, 50μL duplicate aliquots were taken for counting [³²P] free phosphate by Cerenkov method. ATP hydrolyzed percentage is indicated.

Figure 6. HsRAD51B-HsRAD51C does not alleviate inhibitory effects of HsRPA to promote HsRAD51 mediated D-loop formation

(A) HsRPA 200nM, 400nM, 800nM and 1600nM (lanes 2-5 and lanes 7-10) was incubated with 90mer ssDNA (3 μ M nt) at 37°C for 10min in reaction buffer containing 25mM Tris-OAc (pH 7.5), 1mM ATP, 1mM Mg(OAc)₂, 1mM CaCl₂, 2mM DTT and BSA (100 μ g/mL) for 5min. After 5min, HsRAD51 (1 μ M) and HsRAD51B-HsRAD51C (32.5 nM) if indicated, was added and incubation was continued for another 15min at 37°C. Reaction in lane 6, RAD51 was incubated with 90mer ssDNA (3 μ M nt) at 37°C for 10min in reaction buffer. Reaction in lane 11, HsRAD51 (1 μ M) and HsRAD51B-HsRAD51C (32.5nM) incubated with 90mer ssDNA (3 μ M nt) at 37°C for 10min in reaction buffer, prior to

addition of RPA and further incubation at 37°C for 5min. D-loop formation was initiated by the addition of supercoiled dsDNA (50μM bp) and incubation at 37°C for 15min. D-loops were analyzed by electrophoresis on a 0.9% agarose gel after deproteinization. Gels were dried and exposed to PhosphoImager screens for quantification. **(B)** Quantification of D-loops formed in **A**. Error bars indicate S.D.

Figure 7. Proposed model for HsRAD51B-HsRAD51C mediated stabilization of the HsRAD51 nucleoprotein filament

HsRAD51 mediated D-loop formation that can be enhanced by HsRAD51B-HsRAD51C paralogs. The stable nucleoprotein filament that forms could antagonize the action of anti-recombinases, a property that may be enhanced by additional cofactors. Proteins are illustrated with labels. B-C denotes HsRAD51B-HsRAD51C heterodimer.