Full-length archaeal Rad51 structure and mutants: mechanisms for RAD51 assembly and control by BRCA2

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To clarify RAD51 interactions controlling homologous recombination, we report here the crystal structure of the full-length RAD51 homolog from *Pyrococcus furiosus*. The structure reveals how RAD51 proteins assemble into inactive heptameric rings and active DNA-bound filaments matching three-dimensional electron microscopy reconstructions. A polymerization motif (RAD51-PM) tethers individual subunits together to form assemblies. Subunit interactions support an allosteric 'switch' promoting ATPase activity and DNA binding roles for the N-terminal domain helix–hairpin–helix (HhH) motif. Structural and mutational results characterize RAD51 interactions with the breast cancer susceptibility protein BRCA2 in higher eukaryotes. A designed *P. furiosus* RAD51 mutant binds BRC repeats and forms BRCA2-dependent nuclear foci in human cells in response to γ -irradiation-induced DNA damage, similar to human RAD51. These results show that BRCA2 repeats mimic the RAD51-PM and imply analogous RAD51 interactions with RAD52 and RAD54. Both BRCA2 and RAD54 may act as antagonists and chaperones for RAD51 filament assembly by coupling RAD51 interface exchanges with DNA binding. Together, these structural and mutational results support an interface exchange hypothesis for coordinated protein interactions in homologous recombination.

Keywords: BRCA2/DNA repair/homologous recombination/RAD51/X-ray crystal structure

Introduction

DNA double-strand breaks (DSBs) may lead to programmed cell death, gross chromosomal rearrangements (GCRs) or chromosomal loss, resulting in diseases such as cancer (Khanna and Jackson, 2001; van Gent *et al.*, 2001). RAD51-mediated homologous recombinational repair (HRR) of DSBs uniquely retains genetic fidelity in both meiotic and mitotic cells, as HRR uses homologous DNA segments as replication templates (Khanna and Jackson, 2001; van Gent *et al.*, 2001; Symington, 2002).

Current views of eukaryotic HRR suggest that DSBs are processed to yield 3' single-stranded DNA (ssDNA) overhangs, which are then protected by replication protein A (RPA) (Sugiyama *et al.*, 1997). RAD52 is implicated in displacing RPA to aid RAD51 in binding ssDNA within a primary binding site, thus promoting nucleoprotein filament formation (Sung, 1997; Sugiyama and Kowalczykowski, 2002). In concert with other factors, the active RAD51 nucleoprotein filament binds the secondary double-stranded DNA (dsDNA) substrate, locates homology and exchanges DNA strands in an ATP-dependent manner (Benson *et al.*, 1994; Sugawara *et al.*, 2003). The RAD55/RAD57 dimer may aid both RAD51 ssDNA binding and formation of recombination-efficient joint DNA molecules (Sugawara *et al.*, 2003). Both ssDNA and dsDNA may occupy the primary RAD51 DNA binding site *in vitro*, where dsDNA binding inhibits strand exchange (Sung and Roberson, 1995). Though RAD54 binds and stabilizes RAD51 DNA nucleoprotein filaments (Mazin *et al.*, 2003), it may also remove dsDNA bound within the primary site before recombination or after strand exchange *in vivo* (Solinger *et al.*, 2002; Mazin *et al.*, 2003). The complexity of HRR suggests that orchestrated RAD51 protein interactions are required for ordered pathway progression, although the basis for this is unknown (Krejci *et al.*, 2001; Sugawara *et al.*, 2003).

Efficient HRR in higher eukaryotes requires the breast cancer associated protein, BRCA2. Cells harboring BRCA2 truncations display hallmarks of HRR deficiency: GCRs, DSBs and sensitization to genotoxic agents (V.P. Yu *et al.*, 2000). As with RAD51, BRCA2 deficiency is embryonic lethal (Lim and Hasty, 1996; Sonoda *et al.*, 1998). In the absence of DNA, RAD51 homologs form ring structures (Benson *et al.*, 1994; Komori *et al.*, 2000; S. Yang *et al.*, 2001b). Each of eight BRC repeats in BRCA2 can bind directly to RAD51, preventing polymerization of RAD51 into rings and nucleoprotein filaments *in vitro* (Davies *et al.*, 2001) and formation of RAD51 nuclear aggregates *in vivo* (Pellegrini *et al.*, 2002). The crystal structure of the *Homo sapiens* RAD51 ATPase domain (AD) fused to BRC repeat 4 (HsRAD51-AD:BRC4) suggests that BRC repeats control RAD51 disassembly by mimicking RAD51 inter-subunit interface

elements (Pellegrini *et al.*, 2002). However, the existence and nature of this interface are controversial (S.Yang *et al.*, 2001a). An atomic structure of a polymeric full-length RAD51 homolog is crucial for revealing atomic details of quaternary assembly, experimentally testing the proposed BRC-repeat-induced RAD51 disassembly mechanism and addressing the molecular mechanism for the orchestrated interactions of RAD51 in HRR.

Here we present the X-ray crystal structure of a polymeric full-length RAD51 homolog from the thermophilic archaeon *Pyrococcus furiosus* at 2.85 Å resolution. The structure reveals a polymerization motif (PM) involving an interdomain linker key for quaternary assembly. Structural and mutational results suggest how differences in RAD51 ring and helical nucleoprotein filament assemblies may allosterically regulate ATPase activity. A RAD51 filament assembly based on three-dimensional (3D) electron microscopy (EM) reconstructions (S.Yang *et al.*, 2001b) and crystallographic interfaces (Story *et al.*, 1992) suggests a novel role for RAD51 N-terminal domains (ND) in binding dsDNA within a large outer groove. By taking advantage of the simpler organization of archaeal recombination systems, our structural and mutational results in conjunction with HsRAD51-AD:BRC4 results (Pellegrini *et al.*, 2002) establish at the molecular level how BRC repeats disrupt RAD51 assembly and direct RAD51 to form foci in cells in response to DNA damage. These data provide insights into existing HRR mutational results (Aihara *et al.*, 1999; Krejci *et al.*, 2001; Fortin and Symington, 2002) and support a molecular mechanism for the ordered interactions of HRR partners BRCA2, RAD52, RAD54 and RAD55 by protein- and DNA-mediated exchanges of crystallographically defined RAD51 polymer interface elements.

Results

Structure determination and two domain architecture

To characterize RAD51 domain structure and polymeric assembly, we determined the crystal structure of the full-length, 349 residue RAD51 homolog from *P.furiosus* (Figure 1A and B). We used peak anomalous diffraction data from a selenomethionine (SeMet) substituted protein crystal to locate 21 selenium sites in the asymmetric unit and an additional three-wavelength dataset to improve experimental phases by multiwavelength anomalous diffraction (MAD). The final experimentally defined 1733-residue oligomeric structure was refined against over 100 000 measured reflections to 2.85 Å resolution (Table I). The archaeal and eukaryotic RAD51 homologs have nearly identical structures, reflecting their high 46% overall sequence identity (Figure 1A and D). Likewise, their dissimilar domain organization compared with bacterial RecA is also reflected in structure comparisons (Figure 1E and F). RecA also performs analogous strand exchange functions but shares homology in the ATPase domain only. *Escherichia coli* RecA (EcRecA) has 20% and 18.9% identity for the ATPase domains, but only 14.5% and 10.3% overall identity with the human and *P.furiosus* homologs, respectively (see Supplementary data available at *The EMBO Journal* Online). Thus we

will refer to the *P.furiosus* homolog as PfRad51, similar to the current terminology for the related archaeal protein *Archaeoglobus fulgidus* Rad51 (AfRad51), rather than RadA or RecA (Davies *et al.*, 2001).

This full-length PfRad51 structure reveals the spatial relationship between the smaller ~23 X 25 X 31 Å N-terminal domain (PfRad51-ND; Arg35–Leu94) and the larger ~40 X 44 X 56 Å ATPase domain (PfRad51-AD; Arg112–Asp349) (Figure 1B and C). Furthermore, the structure shows how these weakly interacting domains (~280 Å² buried surface) are attached by a protruding 17-residue interdomain linker (Gly95–Gly111) that is bent nearly 90° like an elbow at the β_0/α_5 junction.

PfRad51-AD closely resembles HsRAD51-AD (Pellegrini *et al.*, 2002), with a 1.0 Å root mean square deviation (RMSD) over 198 C α atoms (Figure 1A and D), and the *E.coli* RecA ATPase domain (EcRecA-AD) (Story *et al.*, 1992), with a 1.6 Å RMSD (Figure 1C and E). PfRad51-AD consists of a large twisted central β -sheet consisting of mixed parallel ($\beta_3\beta_2\beta_4\beta_5\beta_1\beta_6$) and antiparallel ($\beta_7\beta_8\beta_9$) β -strands, labeled according to the original EcRecA structure, sandwiched by α -helices on both sides (Figure 1B and C). The ATPase Walker A motif or phosphate binding loop (P-loop) lies between β_1 and α_7 (Figure 1A–C). The Walker B motif lies on β_4 and precedes α_{12} and a loop that corresponds to the disordered DNA-binding Loop 1 (L1) region of the EcRecA, *Mycobacterium tuberculosis* RecA (Datta *et al.*, 2000) and HsRAD51-AD structures. PfRad51 L1 contains a hairpin (Gly250, Arg251, Gly252) conserved between *P.furiosus*, *H.sapiens* and *Saccharomyces cerevisiae* that has multiple observed conformations but weak electron density. L1 precedes the second of two long and prominent α -helices, α_{11} and α_{13} (α_E and α_F in RecA), that separate PfRad51-ND from PfRad51-AD. The PfRad51 region analogous to EcRecA DNA-binding Loop 2 (L2, residues 287–301) was disordered, as in other RAD51/RecA structures.

PfRad51-ND forms a four-helix bundle ($\alpha_1\alpha_2\alpha_3\alpha_4$) (Figure 1A–D) that matches the topology of the HsRAD51 N-terminal domain (HsRAD51-ND) NMR structure (residues 18–97), including a disordered N-terminus (Aihara *et al.*, 1999). The EcRecA C-terminal domain (RecA-CD) and PfRad51-ND are both small and globular but lack structural similarity. A search using DALI (Holm and Sander, 1993) indicated that PfRad51-ND α_3 – α_4 forms an HhH motif, which acts in DNA phosphate backbone binding (Thayer *et al.*, 1995). Overall, the archaeal and human Rad51 proteins are close structural homologs that are distinct from RecA except for similarities within the conserved ATPase domain, implying that this domain forms the progenitor recombination unit, whereas the additional eukaryotic/archaeal RAD51-NDs and bacterial RecA-CD evidently evolved independently.

Quaternary assembly of PfRad51

Full-length RAD51 homologs have proven challenging to crystallize, likely due to flexibility in their two-domain structures and a tendency to form polymers. As crucial questions regarding RAD51's interactions within HRR cannot be addressed by structural analysis of single domains, we have employed PfRad51 for full-length and polymeric structural analyses. A heptameric ring in the

Fig. 1. Sequence, secondary structure, conservation, fold, residue function and domain architecture for the RAD51 protein family. (A) Alignment of RAD51 homologs from *P.furiosus* (PfRad51), *H.sapiens* (HsRAD51) and *S.cerevisiae* (ScRAD51). P and H under the sequence refer to PfRad51 and HsRAD51 key residues or mutations used in this study, while 2, 4 and 5 refer to ScRAD51 mutations that influence binding to ScRAD52, ScRAD54 or ScRAD55, respectively. B refers to HsRAD51 residues that bind BRC4. Triangles indicate contact residues between one subunit (blue) and its adjacent neighbor (black), or between heptamers (orange). (B) The N-terminal domain (ND, top) and ATPase domain (AD, bottom) of PfRad51 are connected by an elbow linker. Key motifs are colored according to the labels in (A). (C) Topology of a PfRad51 subunit shows conservation of the RecA-AD fold (Story *et al.*, 1992). (D) Overlay of PfRad51 (orange) with HsRAD51-ND (purple) and HsRAD51-AD (green) reveals strong structural conservation. (E) Overlay of PfRad51-AD (orange) and EcRecA-AD (green) reveals a conserved ATPase fold with additional PfRad51-ND (red) and EcRecA-AC (dark green) DNA binding domains positioned at opposite poles. (F) Organization of the recombinase family protein sequences. Regions of homology among RAD51-NDs, and RAD51-ADs and RecA-AD are colored red and yellow, respectively. Walker A and B motifs are green. Non-homologous regions are white or blue.

Table 1. X-ray crystallographic and solution small-angle X-ray scattering data collection and analysis

X-ray crystallographic data				
X-ray source	Stanford Synchrotron Radiation Laboratory (SSRL) beamline 9-2, Stanford, CA			
Space group	C222 ₁			
Unit cell dimensions (Å)	SeMet1: $a = 144.2$, $b = 193.1$, $c = 176.9$; SeMet2: $a = 145.0$, $b = 193.7$, $c = 177.7$			
Dataset	SeMet1	SeMet2- λ_1	SeMet2- λ_2	SeMet2- λ_3
Wavelength (Å)	0.979029	0.979126	0.911620	0.979413
Data range (Å) (last shell)	40–2.85 (2.95–2.85)	40–2.95 (3.06–2.95)	40–3.1 (3.21–3.10)	40–3.1 (3.21–3.10)
Observations (unique)	462 283 (57 867)	289 368 (52 910)	243 283 (45 729)	239 395 (45 397)
Completeness (%) (last shell)	97.1 (89.3)	98.9 (98.1)	99.2 (99.5)	99.0 (96.9)
R_{sym}^a (last shell)	0.094 (0.37)	0.090 (0.335)	0.084 (0.346)	0.083 (0.363)
$I/\sigma I$ (last shell)	20 (3.2)	19 (3.3)	22 (3.6)	23 (3.0)
X-ray structure refinement				
Resolution	40–2.85			
Reflections $F > 0$ (cross-validation)	106 150 (5206)			
Amino acids (solvent molecules)	1733 (96)			
Non-hydrogen atoms (solvent molecules)	13 594 (262)			
Average B value main (side chain)	76.2 (83.7)			
Ramachandran allowed residues	1499 (99.1%)			
Non-glycine and non-proline residues	1514			
R_{cryst}^b (R_{free}^c)	0.257 (0.307)			
RMS bond length (Å)	0.0085			
RMS bond angle (°)	1.39			

^a R_{sym} is the unweighted R value on I between symmetry mates.

^b $R_{\text{cryst}} = \sum_{hkl} \|F_{\text{obs}}(hkl) - |F_{\text{calc}}(hkl)|\| / \sum_{hkl} F_{\text{obs}}(hkl)$

^c R_{free} is the cross-validation R factor for 5% of reflections against which the model was not refined.

asymmetric unit forms a dimer of heptamers (biheptamer) by crystallographic 2-fold symmetry. To identify PfRad51's oligomeric state in solution, we used dynamic light scattering, which revealed an assembly consistent with the 38.4 kDa protein forming a 493–531 kDa complex of 13–14 subunits (data not shown). EM revealed ring-like structures ($\sim 110 \pm 5$ Å diameter) that are similar to those observed previously (Komori *et al.*, 2000), and that match the dimensions of the crystal structure (~ 118 Å diameter and ~ 105 Å height) (Figure 2A). We sorted 617 ring images into 10 classes and found consistent 7-fold symmetry. 3D EM reconstructions of the *Sulfolobus solfataricus* Rad51 homolog were reported as octamers (S.Yang *et al.*, 2001b). Therefore, the oligomerization state may differ from species to species, or the admitted bias imposed from the octameric yeast DMC1 protein starting model affected the *S.solfataricus* EM reconstruction outcome.

Solution small-angle X-ray scattering (SAXS) data are consistent with the crystallographically defined PfRad51 dimer of heptamers (Figure 2B and C), whose average radius of gyration (R_g) of ~ 57.6 Å is not significantly altered by ADP or ATP analogs (see Supplementary data). Thus, there are insignificant conformational changes associated with nucleotide exchange or the ring blocks conformational changes or it precludes tight nucleotide binding, consistent with the absence of electron density for ATP γ S in our crystal conditions.

The PfRad51 heptamer consists of ATPase domains arranged as a ring of pie-shaped wedges with a central ~ 21 Å diameter hole lined by the L1 hairpin (Figure 2D and E). Inter-subunit contacts are made by L1 and α_{13} from one subunit and α_{12} , L1 and the β_3/α_{11} turn of the adjacent subunit. However, the most prominent interface feature is the polymerization motif 95-GTFMRADE-102, which contains two distinct elements. The first is a

β -zipper involving elbow linker residues Met98 and Ala100 (β_0) contacting Ile200 and Val202 (β_3), which extends the central β -sheet through main-chain hydrogen bonds (Figures 1A–C, and 2D and G). Notably, an analogous intermolecular β -sheet is seen in the helical EcRecA X-ray crystal structure by β_0 contacting β_3 (Story *et al.*, 1992). Therefore the elbow linker is expected to be flexible because it protrudes from surrounding domains, leaving PfRad51-ND well ordered in only one subunit via stabilizing crystal contacts. Also, it is involved in the assembly of both rings and filaments. The second polymerization motif element is the insertion of conserved elbow linker residue Phe97 into a hydrophobic pocket formed by residues of β_2 (Ile169, Ile171), β_3 (Tyr201, Ala203) and α_{11} (Leu214, Ala218, Lys221) of an adjacent subunit, similar to a ball and socket (Figure 2G). This gives Phe97 the largest buried surface area (~ 102 Å²) of any interface residue. Conserved Ala100 C β is also buried within α_9 (Phe177, Pro179, Leu197) and β_3 (Ile200). Interestingly, inter-subunit β -sheet extension following a ball-and-socket interaction was also observed in the RAD52 structure (Kagawa *et al.*, 2002; Singleton *et al.*, 2002). Most contacts between heptamers (~ 2072 Å² buried surface) involve polar side-chain interactions about a 2-fold symmetry axis, indicating that the dimer of heptamers may readily disassociate through interactions with DNA or mediator proteins (Figures 1A and 2F).

The ATPase active site

Overall, the PfRad51 and HsRAD51-AD ATPase active site features are conserved, but a sulfate ion, likely scavenged from the PfRad51:ATP γ S cocrystallization solution, is bound within the active site in lieu of ATP γ S at the expected β -phosphate position. To determine whether the inability to observe ATP γ S is related to ring properties, we made comparisons with the EcRecA and

Fig. 2. PfRad51 polymeric assembly and polymerization motif. (A) Electron micrographs of PfRad51 reveal heptameric ring structures (scale bar, 20 nm). (B) SAXS intensities from PfRad51 (circles) plotted against the momentum transfer Q and calculated profiles for heptameric (dotted line) and biheptameric (thin curve) models indicate a biheptameric ring assembly. Rigid-body refinement of two heptamers improved the fit into the experimental data (thick curve). (C) Electron pair distribution functions $P(r)$ for the SAXS data (circles) and for the heptamer (dotted line), biheptamer (thin curve) and rigid-body refined biheptamer (thick curve) models support biheptameric assembly. (D) Interface between two adjacent ATPase domains oriented similarly to the boxed region in (E) showing the β_0/β_3 inter-subunit β -sheet. The N-terminal domains have been removed for clarity. (E and F) Single PfRad51 heptamer (E, top view) and biheptamer (F, side view) models show 7-fold symmetric assembly. Sulfates (balls) denote the ATPase active site. (G) A polymerization motif is formed by β_0 (98-MRA-100) of the inter-subunit β -sheet and buried Phe97 and Ala100 side chains. The adjacent subunits are yellow and white and in an orientation similar to that in (D). Composite omit $2F_o - F_c$ density is contoured at 2σ (purple) and 4σ (pink) and hydrogen bonds are shown as dashed lines.

EcRecA:ADP helical filament crystal structures (Story and Steitz, 1992; Story *et al.*, 1992). The sulfate likely leaves the phosphate-binding pocket intact; however, conformational differences in the ribose- and base-binding regions of the ATP site evidently disfavor nucleotide binding in the PfRad51 ring (Figure 3A and B). Residues of the elbow linker (Ala100) and α_5 (Tyr103, Leu104, Arg107) of the adjacent subunit pack against α_9 and the preceding loop (Phe177, Pro179, Glu180). These interactions position the α_9 Glu180 carboxylate near α_5 Arg107 N ϵ of the adjacent subunit, which may prevent Glu180 from contacting the nucleotide exocyclic amine analogous to EcRecA Asp100. Arg181 is positioned over the adenine base, similar to EcRecA Tyr103, but is moved away from the nucleotide. Additionally, β_8 Ile342 is positioned to sterically hinder binding of the nucleotide ribose, as opposed to EcRecA Ile262.

The PfRad51-AD and HsRAD51-AD domains are nearly identical, with the exception that the HsRAD51-AD active site is more closed, possibly due to a smaller Cl⁻ ion in the active site or active site destabilization from missing adjacent subunits. The HsRAD51-AD P-loop cap, Phe129, is moved inward ~ 3 Å compared with the corresponding PfRad51 Phe140 residue, which holds the P-loop open via interactions between Phe140 and Arg178 with Pro331 and His332 of the adjacent subunit. These

structures suggest that more efficient nucleotide binding and subsequent hydrolysis may require an allosteric switch from apo assemblies to DNA-bound filament forms, and that ring structures may be biologically relevant RAD51 storage forms that decrease futile cycling of ATP.

Coupling of ATPase and strand-exchange activities

The ATPase activity of RAD51 proteins is stimulated by DNA (Komori *et al.*, 2000; Tomblin and Fishel, 2002). Conserved Arg251 resides in L1 on the inner face of the ring crystal structure and filament model discussed below (Figures 1B and 2D), where it likely comes in contact with ssDNA or dsDNA bound in the primary site. The L1 linkage to the Walker B motif through α_{12} also suggests a possible role in modulating ATPase activity (Figure 1B and C). Therefore we generated R251A and R251E mutants and investigated their effect on ATPase and strand-exchange activities. Without DNA, little ATP is hydrolyzed by wild-type or mutant PfRad51 (Figure 3C). With ssDNA, the ATPase activity of all three proteins increases significantly, but surprisingly wild-type activity is only slightly higher than that of mutant. dsDNA stimulates to a lesser extent, with wild-type activity ~ 2 -fold higher than mutant activity. This suggests that primary DNA binding may not involve major contacts with Arg251, but that other elements, such as the adjacent

Fig. 3. The ATPase active site and mutational decoupling of ATPase and strand exchange activities. **(A and B)** Comparisons of the ATP active sites of the PfRad51 ring (yellow) and EcRecA helical filament (green) suggest a mechanism for conformation-induced allostery for ATP binding. Key side chains and ADP (purple) from the RecA structure are shown as balls and sticks. In the PfRad51 ring, hydrophobic interactions between α_5 and α_9 of adjacent subunits pull Arg181 (α_9) up and away from the nucleotide base (A). This arrangement may also allow PfRad51 Ile342 (β_8) to sterically hinder nucleotide binding (B). In helical EcRecA, the absence of Pro101 hydrophobic contacts with the adjacent subunit allows Tyr103 to stack with the nucleotide base (A). **(C)** Analysis of ATPase activity of wild-type PfRad51 and L1 region mutants, R251A and R251E, with no DNA, ssDNA or dsDNA reveals that all proteins hydrolyze ATP in a DNA-dependent manner. **(D)** The ability to form joint molecules between circular ssDNA and linear dsDNA is greatly diminished for the PfRad51 R251A and R251E mutants compared with wild-type protein, despite their ability to hydrolyze ATP. **(E)** The more robust activity of AfRad51 wild-type protein confirms the results in (D) by comparison with the activity of an analogous AfRad51 R228A mutation.

L2 region, may be involved. The increased surface of dsDNA relative to ssDNA may account for greater contact with Arg251 from an adjacent site, producing the corresponding small effect on ATPase activity.

Wild-type PfRad51 performed Mg- and ATP-dependent strand exchange between circular ssDNA and linear dsDNA substrates to form a joint molecule product (Figure 3D). However, strand exchange was not detected for either Arg251 mutant. As the control protein AfRad51 had more robust activity in our assays, we tested strand exchange of an analogous AfRad51 L1 mutant, R228A, and obtained identical results (Figure 3E). Together, the decrease in strand-exchange activity and retention of ATPase activity for the mutants suggest that L1 (Arg251) couples these activities and acts in DNA pairing in response to ATPase activity, rather than acting in direct primary ssDNA binding.

Structure-based helical filament models

To gain insight into the nature of the assembly of the helical RAD51 nucleoprotein filament, we modeled PfRad51 as a filament based on the EcRecA crystal structure (Figure 4A and B). A small rotation of each PfRad51 subunit or movement of the flexible linker region, as shown in Figure 4A, preserves the intermolecular β -sheet extension and the Phe97 ball and socket. The secondary structures composing the subunit interface are conserved between the PfRad51 and RecA helical models. Moreover, 11/20 of the PfRad51 residues making significant contacts in the ring are retained in the filament, and 9/20 are invariant between PfRad51, HsRAD51 and ScRAD51. The β_7/β_8 loop that once formed the biheptameric interface now contacts β_3 and α_9 of an adjacent

subunit. Helical assembly removes L1 interactions with α_{12} and α_{13} of adjacent subunits, releasing the Walker B motif and key ATPase active site residues Asp238 and *cis*-Ser239. This arrangement is similar to the catalytically active T7 gp4 ATPase ring structure, which shares the RecA fold (Singleton *et al.*, 2000). VanLoock and colleagues interpret RecA EM reconstructions with an assembly different from the crystal structure, placing ATP sites at the subunit interfaces to facilitate ATPase cooperativity (VanLoock *et al.*, 2003). RAD51 lacks cooperative ATP hydrolysis (Tomblin and Fishel, 2002), consistent with our placement of ATP away from the interfaces.

Our filament model matches EM data demonstrating that RAD51 and RecA nucleoprotein filaments possess a large outer groove with one smooth face and one lobed face (S.Yang *et al.*, 2001a,b; X.Yu *et al.*, 2001; VanLoock *et al.*, 2003). When the EcRecA-AD crystal and HsRAD51-ND NMR structures were docked into HsRAD51 nucleoprotein filament 3D EM reconstructions, HsRAD51-ND was placed against the C-terminal end of EcRecA-AD (S.Yang *et al.*, 2001a). However, EcRecA-CD and PfRad51-ND lie on opposite sides of their respective ATPase domains and thus the polarity of these lobes must be switched in the context of the filament (Figures 1E and 4A and B).

We strengthened this model with computational docking of our PfRad51 structure into 3D EM reconstruction density of the archaeal *S.solfataricus* Rad51 homolog (Figure 4C). In comparing models depicted in Figure 4A and C, we found that all significant interface contacts and basic domain orientations were retained. Surprisingly, docking resulted in a small PfRad51-ND rotation relative

to PfRad51-AD (5.0 Å C α RMSD) and translation between adjacent subunits (3.8 Å RMSD). We also found that torsional collapse of the filament into a ring is consistent with a heptamer. Our analysis of PfRad51 in the context of the *E.coli* crystal and *S.solfataricus* EM filament structures indicates that ring and filament assemblies may involve structural features conserved across kingdoms. Interestingly, a difference in the polarity of the RAD51 and RecA extended domains may account for some of the observed *in vitro* differences in strand-exchange polarity between these proteins (Sung and Robberson, 1995; Holmes *et al.*, 2002).

Structural and computational analysis for DNA binding and strand exchange

RAD51 activity requires simultaneous sequence-independent binding of both ssDNA and dsDNA, likely through sugar-phosphate interactions, to allow homology search reactions. Therefore we used comparative structure analysis and computational approaches to determine potential DNA binding sites. Positive electrostatic potential for binding DNA phosphates maps to the filament interior, L1 and HhH motifs and the smooth surface of the outer groove (Figure 4D and E). The L2 region may also contribute to primary ssDNA binding within the filament but is disordered, hindering electrostatic potential calculations. However, fitting the RecA-bound extended ssDNA structure (Nishinaka *et al.*, 1997) near visible L2 components of adjacent subunits parallel to the outer groove is consistent with RAD51 binding three ssDNA nucleotides per subunit (Sung and Robberson, 1995).

Although NMR studies show that HsRAD51 HhH residues Ala61–Glu69 may bind dsDNA (Aihara *et al.*, 1999), the secondary site remains to be unambiguously identified. Therefore we investigated structural modes of dsDNA binding to the HhH motif by modeling a PfRad51:DNA complex after DNA-bound HhH motifs from DNA polymerase β (Sawaya *et al.*, 1997) and RuvA (Ariyoshi *et al.*, 2000) cocrystal structures (Figure 4F). In this dsDNA binding mode, the positively charged dipoles of α_1 and α_4 helices each point toward the phosphate backbone of one DNA strand (Figure 4E and F). This arrangement places the dsDNA in the wide (~ 30 Å) outer

groove of the protein filament. Additional DNA contacts would include residues of the β_6/β_7 hairpin, β_8 , β_9 and α_6 on the smooth side of the groove. This dsDNA position complements a RecA model, whereby RecA-CDs are believed to bind secondary dsDNA (Aihara *et al.*, 1997). The role of RPA in binding and preventing ssDNA from occupying the secondary DNA binding site is also consistent with this model (Van Komen *et al.*, 2002). This model is further corroborated by our mutagenesis data, which suggest that DNA pairing may involve L1 moderation of contacts between ssDNA within the filament and dsDNA outside the filament through channels (Figure 4D and F).

Fig. 4. PfRad51 and EcRecA helical filament models and the implied Rad51 HhH DNA binding site. (A) PfRad51 filament assembly with PfRad51-ADs (alternating in orange and green) placed by superposition with EcRecA-ADs from X-ray crystallography (B) (Story *et al.*, 1992). (B) The helical EcRecA structure with EcRecA-ADs alternating orange and green with C-terminal domains (yellow) and ADP molecules (balls). The PfRad51-NDs (A; yellow) within the groove have opposite polarity to the EcRecA-CDs. (C) Independent rigid body docking of the PfRad51 crystal structure into 3D EM reconstruction density of the *S.solfataricus* Rad51 homolog bound to DNA retained the basic features of the model presented in (A) that was based upon crystallographic polymers. (D) Positive charges [2.0 kT/e $^-$ (blue) to -2.0 kT/e $^-$ (red)] for the L1 region implicated in DNA interaction are contributed largely by Arg251 residues (center). Asymmetry reflects L1 region flexibility. (E) The PfRad51 helical filament model has positive electrostatic potential for DNA binding within the ssDNA binding interior and for the HhH motifs. (F) A dsDNA model fits into the large outer groove of the PfRad51 filament when guided by HhH-containing protein:DNA X-ray cocrystal structures. This suggests a method for RAD51 to bind ssDNA internally and dsDNA externally for homology search reactions, in which pairing may occur within channels.

Fig. 5. Molecular basis for BRCA2 regulation of RAD51 function. (A) PfRad51 with adjacent subunit interface polypeptide (green), showing the key subunit interface polymerization motif elements β_0 and Phe97. (B) Superposition of HsRAD51-AD:BRC4 (HsRAD51-AD not shown) onto the PfRad51 structure (ribbons) reveals that the BRC4 repeat (green) occupies the same area as PfRad51-ND (not shown) and mimics β_0 to disrupt the inter-subunit β -sheet between RAD51 subunits. (C) The PfRad51 van der Waals surface (blue) with the foremost subunit (coil) overlaid with BRC4 (orange) from HsRAD51-AD:BRC4 shows how RAD51-ND displacement and disassembly of the ring involves intercalation of the BRC repeat between RAD51 subunits. (D) Critical components of the BRC4 repeat (orange) for mimicry of the RAD51 polymerization motif. Phe1524 and Ala1527 match the adjacent PfRad51 subunit residues Phe97 and Ala100 (blue). (E) Binding of HsRAD51, PfRad51 and the PfRad51 E219S/D220A/D267M mutant to BRCA2 BRC3/BRC4 repeats establishes the additional critical components for RAD51:BRC repeat binding. In each triplet, we show the amount of input protein (first lane), a negative control of GST alone (second lane) and binding to a GST-BRC3/4 fusion protein (third lane). (F and G) BRC-dependent disassembly and targeting of mutant PfRad51 shown by fluorescence. A GFP-PfRad51 E219S/D220A/D267M mutant is targeted to dsDNA breaks in human 293T cells forming nuclear foci following γ -irradiation (F). The ability to form foci is abolished in the presence of BRC repeats 3 and 4 (G).

Control of RAD51 by BRCA2

To determine how BRCA2 operates as a binding antagonist for RAD51 polymerization and a chaperone for foci formation and DNA targeting, we examined how the BRC4 peptide from the HsRAD51-AD:BRC4 structure (Pellegrini *et al.*, 2002) would affect a polymeric full-length RAD51 structure. The PfRad51 sequence 95-GTFMRAD-102, equivalent to 85-GFTTATE-91 of HsRAD51, forms the RAD51-PM by key inter-subunit contacts between β_0/β_3 and insertion of Phe97 and Ala100 (HsRAD51 Phe86 and Ala89) into neighboring hydrophobic cavities (Figures 1A and 5A and D). These interactions resemble those made by BRCA2 1523-GFHTASG-1529 in HsRAD51-AD:BRC4 (Figure 5B and D). This comparison establishes that an ancient polymerization motif, RAD51-PM, in a large family of recombinases may have become incorporated into the more recently evolved protein BRCA2.

Despite the similarity between these interfaces, PfRad51 does not bind to human BRC repeats 3 and 4 (Figure 5E). Therefore additional binding elements may be critical for stable RAD51:BRC repeat interactions. Comparison of Figure 5A and B shows that, in the presence of a full-length HsRAD51 molecule, BRC repeat residues C-terminal to the polymerization motif would likely bind HsRAD51-ND instead of HsRAD51-AD, where the observed binding may be an artifact of covalent attachment of BRC4 to HsRAD51 missing HsRAD51-ND (Pellegrini *et al.*, 2002), or HsRAD51-ND would be displaced during binding. The latter would allow BRC repeat Val1542–Gln1551 to prevent RAD51 self-association by removing contact of adjacent HsRAD51-NDs and HsRAD51-ADs, and may provide a method to open the RAD51:RAD51 interface for BRC mimicry by unzipping the polymerization motif. Therefore we searched for additional binding elements by structure comparison. BRC4 Leu1545 and Phe1546 side

chains form a hydrophobic wedge that projects towards the middle of HsRAD51-AD α_4 and α_5 (full-length PfRad51 α_{11} and α_{13}). Phe1546 is buried within the HsRAD51-AD:BRC4 interface and forms van der Waals contacts with HsRAD51-AD Met251 and Tyr205, which in the absence of BRCA2 would be largely solvent exposed. In PfRad51, hydrophilic Asp267 and Gln216 occupy their equivalent respective positions. The BRC repeat wraps around HsRAD51-AD α_4 , employing residues with small side chains, such as Ser208 and Ala209, on the penultimate turn. PfRad51 has larger charged Glu219 and Asp220 at these positions.

To test the biochemical basis for BRC interactions with PfRad51, we created a PfRad51 mutant in which Glu219, Asp220 and Asp267 were replaced with the equivalent residues from HsRAD51-AD (PfRad51 E219S/D220A/D267M). In contrast with wild type, this PfRad51 mutant showed significant binding to the BRC repeats (Figure 5E). We also tested whether the BRC repeats disassemble mutant PfRad51 in cells. Upon transfection of GFP-tagged PfRad51 mutant protein into transformed human 293T cells, we found that the PfRad51 mutant forms nuclear foci in response to γ -irradiation-induced DNA damage, similar to GFP-HsRAD51 (Figure 5F) (Pellegrini *et al.*, 2002), but that formation of nuclear foci is prevented in the presence of coexpressed BRC3/4 (Figure 5G). Combined, these structural, biochemical and cellular biological results argue that the BRC repeats likely bind and disassemble RAD51 polymers via the crystallographically defined interfaces and furthermore promote RAD51 nuclear foci formation in response to DNA damage.

Discussion

Our structural and mutational results shed light on the structurally implied synergistic interactions among RAD51, BRCA2, RAD52, RAD54, RAD55 and DNA substrates in eukaryotic HRR and support an interface exchange hypothesis. Specifically, our results identify the RAD51 polymerization motif and associated polymeric interface as a probable platform for the choreography of interacting multiprotein:DNA complexes by facilitating exchange reactions acting at each HRR step (Figure 6).

The two-domain RAD51 structure, with its flexible elbow linker, provides the moving parts to facilitate exchanges by interactions with DNA and other mediator proteins. After DNA damage, BRC repeats may depolymerize and bind the RAD51 ring by unzipping the RAD51-PM to open the RAD51 interface, mimicking these elements and clamping HsRAD51-AD α_4 (α_{11} in PfRad51) (Figures 5 and 6). A single BRCA2 molecule may bind multiple RAD51 molecules by using its BRC repeats, and the recent crystal structure of the BRCA2 DNA binding domains (BRCA2DBD) implicates BRCA2 in displacing RPA and binding DNA by its three oligonucleotide/oligosaccharide (OB) folds (H.Yang *et al.*, 2002). BRCA2 may recognize the dsDNA/ssDNA junction via its DNA binding helix–turn–helix (HTH) motif and ssDNA binding by the OB folds, or template homologous dsDNA might be sequestered by the HTH. The positively charged BRC repeat ‘helical arches’ (Lys1530, Lys1531, Lys1533, Lys1536, Lys1543, Lys1544 and Lys1549 in BRC4) opposite the RAD51 binding surfaces (Figure 5B) are

Fig. 6. Proposed model for BRCA2 coordination of RAD51 activities in HRR. (1) BRCA2 binds to RAD51 subunits within the ring (AD, brown; ND, red; elbow linker/ β_0 , yellow arrow; β_3 , brown arrow) via BRC repeat mimicry of the RAD51 polymerization motif (β_0 mimic, blue arrow). (2) BRC repeats disassemble the ring. (3) The RAD51:BRCA2 complex is recruited to a DSB. (4) BRCA2 helps displace RPA and binds the primary ssDNA substrate by its OB folds (5), and loads RAD51 onto DNA. The handoff reactions might be facilitated by attraction of DNA by the positively charged BRC repeat helical arches. The BRCA2 HTH domain (red) may bind dsDNA *in cis* at the ssDNA/dsDNA intra-DNA junction (3) or *in trans* to the dsDNA that later serves as the homologous DNA template (6) and the positively charged arch may also help to attract the dsDNA template.

positioned for possible electrostatic guidance to deliver RAD51 to DNA (Figure 6). The BRC repeat may then be unzipped from RAD51 by DNA interactions, allowing RAD51 to bind DNA.

The results here further identify putative BRCA2 binding determinants within RAD51 proteins from higher eukaryotes. Thus the set of four amino acids equivalent to Tyr205, Ser208, Ala209 and Met251 of HsRAD51 among RAD51 homologs could be exploited to explore possible BRCA2-like binding and therefore BRCA2-like function in other organisms. According to this criterion, *Xenopus laevis* is expected to possess a BRCA2-like protein, as all four XIRAD51 residues (Tyr202, Ser205, Ala206, Met248) are identical with the human set. The existence of a BRCA2 function also appears possible in *Drosophila melanogaster* (Gln202, Ala205, Gly206, Met248) and *Caenorhabditis elegans* (Ile221, Gly224, Ala225, Cys267), but unlikely in the lower eukaryotes *S.cerevisiae* (Asp263, Ala266, Gln267, Ala309) and *Schizosaccharomyces pombe* (Gln227, Ala230, Asn231, Thr273).

Based on structural homology, RAD51 mutations that affect RAD52, RAD54 and RAD55 binding map to RAD51 interface elements, similar to BRCA2. This correspondence suggests that the molecular mechanisms controlling HRR may involve DNA- and protein-mediated RAD51 interface exchanges to choreograph HRR pathway progression. The ScRAD51 mutant A248T, which decreases both RAD52 and RAD54 binding, would lie on β_3 of the RAD51 β -zipper interface (Figure 1A) (Krejci *et al.*, 2001). Several ScRAD51 mutants that decrease RAD54 binding also map to RAD51 interface elements: S231P (α_9), intermolecular connection to P-loop C377Y (β_7/β_8 loop) and β -zipper T146A (β_0). Others lie at the HsRAD51-AD:BRC4 interface: M269V (α_{11}) and L310S

(α_{13}) and thus identify an oligomerization motif, RAD51:BRC-OM. Finally, some ScRAD51 mutants that modulate RAD52 and RAD54 binding map to the implied dsDNA binding region: Y388H, G393S/D and S231P. ScRAD51 mutant L119P, which suppresses the radiation sensitivity of RAD55/RAD57 mutants, lies in α_3 of the HhH motif (Fortin and Symington, 2002).

RAD52 may aid in both nucleation and further extension of the RAD51 filament by interactions with RPA and RAD51 β_3 (Sung, 1997; Krejci *et al.*, 2001; Sugiyama and Kowalczykowski, 2002). Recent evidence suggests that ScRAD52 targets ScRAD51 to DSBs in yeast (Sugawara *et al.*, 2003). Interestingly, ScRAD52 contains a putative polymerization motif 315-TFVTAKA-321, which is similar to the respective ScRAD51-PM (143-GFVTAAD-149) and PfRad51-PM (96-TFMRAD-102) sequences, which would complement binding β_3 , as evidenced by the A248T mutant (Krejci *et al.*, 2001). RAD54 binds RAD51 nucleoprotein filaments, stimulating DNA pairing activity (Mazin *et al.*, 2003). The ability of RAD54 to bind the RAD51 elbow linker (β_0), established by our data and mutational results (Krejci *et al.*, 2001), is in agreement with RAD54 protection of the DNA exposed in the large groove adjacent to the RAD51 HhH motif.

The nucleotide-dependent movement of RAD51-NDs and the filament expansion and contraction seen in EM reconstructions (X.Yu *et al.*, 2001) may advance DNA within the large outer groove during homology search activity. Residues of α_{11} contact the linker region and PfRad51-ND, which may hold PfRad51-ND relative to the ATPase and relay these observed nucleotide-induced conformational changes. Positively charged conserved residues (316-RKGKGGK-323, β_6/β_7 hairpin) create a flexible loop, implied by weak electron density. This region lies on the smooth side of the groove and separates the putative dsDNA binding site and P-loop, which may also provide a connection between ATP hydrolysis and DNA binding. Once homology is found, aided by HhH, C-terminal loop and L1 interactions, the strand-exchange reaction may take place, with geometric selection within the RAD51 filament aiding complementarity. As RAD54 interactions with RAD51 may be analogous to those of the BRC repeat interface, the molecular basis for how RAD54 stabilizes the filament (Mazin *et al.*, 2003) and paradoxically acts in its disassembly (Solinger *et al.*, 2002) may be explained by analogous exchanges of RAD51 interface elements. Therefore our results, in conjunction with other studies, support the interface exchange hypothesis whereby complexes of interacting RAD51 partners and DNA exchange interfaces to promote and coordinate HRR pathway progression.

Materials and methods

Cloning, expression and protein purification

The *radA* genes corresponding to *A.fulgidus* and *P.furiosus* Rad51 proteins were cloned into the pET21b expression vector (Novagen). The *HsRAD51* gene and the segment encoding BRC repeats 3 and 4 (residues 1338–1617) from *HsBRCA2* were cloned into the pGEX-2TK and pGEX-4T3 GST-fusion vectors (Amersham), respectively. PfRad51 mutants (R251A, R251E and E219S/D220A/D267M) were generated using QuickChange (Stratagene). Corresponding protein was expressed and purified from *E.coli*. Met auxotrophs were cultured in the presence of

50 mg/l DL-selenomethionine (Sigma) for SeMet-protein. HsRad51 was cleaved from GST using thrombin. See Supplementary data for details.

X-ray crystal structure determination

SeMet-PfRad51 protein (51 mg/ml) was crystallized by sitting-drop vapor diffusion. Redundant X-ray diffraction data for one crystal at the peak of the Se K absorption edge (Table I) and a second three-wavelength dataset were collected at SSRL beamline 9–2 to determine phases by MAD. Data processing, phasing, model building, refinement and other details are described in the Supplementary data. The final model consists of residues 35–286 and 302–349 for subunit 1, residues 96–286 and 302–349 for subunits 2–6 and residues 96–286 and 303–349 for subunit 7. Structure coordinates have been deposited in the Protein Data Bank with the accession code 1PZN.

Electron microscopy

Carbon-coated copper grids were glow-discharged and then floated for 2 min on 5 μ l drops containing 0.2 mg/ml PfRad51 protein, 1 mM ATP γ S and 20 mM MgCl₂, previously heated at 65°C for 10 min. Grids were stained with 3% uranyl acetate and imaged on a Philips CM120 microscope at 100 kV in low-dose mode. Rings were divided into 10 classes and analyzed using EMAN (Ludtke *et al.*, 1999) to determine 7-fold symmetry. Docking of our *P.furiosus* crystal structure using N- and C-terminal domain rigid bodies into 3D reconstruction EM density of homologous archaeal *S.solfataricus* protein filaments (S.Yang *et al.*, 2001b), a gracious gift from Edward Egelman, was performed using COAN (Volkman and Hanein, 1999) at 25 Å resolution (see Supplementary data).

Solution small-angle X-ray scattering

Sixteen X-ray PfRad51 protein-scattering datasets (35, 8, 4, 2 and 1 mg/ml) in the presence and absence of 2.5 mM ATP γ S or ADP were collected at SSRL beamline 4–2. The detector channel numbers were converted to momentum transfer $Q = 4\pi\sin(\theta)/1.3806$ Å, where θ is a scattering angle, by recording the (100) reflection from cholesterol myristate powder (see Supplementary data).

ATPase assays

Duplicate PfRad51 wild-type and R251A and R251E mutant proteins (2 μ M) were mixed with 200 μ M ATP and 0.05 μ Ci/ μ l [γ -³²P]ATP with or without 12 μ M DNA (ϕ X174 virion ssDNA or Pst I-digested ϕ X174 RF I dsDNA; New England Biolabs) and incubated at 70°C in 20 mM Tris-HCl pH 7.5, 6 mM MgCl₂, 0.1 mM dithiothreitol (DTT) and 50 mM NaCl. Aliquots were removed at various time points, and reactions were terminated and [³²P] counted as described (Tomblin and Fishel, 2002).

Strand-exchange assays

PfRad51 and AfRad51 wild-type and mutant (PfRad51 R251A and R251E, and AfRad51 R228A) proteins (16 μ M) were mixed with 3 mM ATP, 0.05 mg/ml bovine serum albumen and ϕ X174 virion ssDNA (47.5 μ M nucleotide) and incubated at 70°C for 20 min in 20 mM Tris-HCl pH 7.5, 4 mM Mg acetate, 50 mM NaCl and 2 mM DTT. PstI-digested double-stranded ϕ X174 RF I DNA (46.2 μ M nucleotide) was added to the mixture and either not incubated or incubated at 70°C for 60 min. Reactions were deproteinized and DNA products were separated by 1.0% agarose gel electrophoresis (see Supplementary data).

BRC repeat binding assays

Purified HsRAD51, PfRad51 or PfRad51 E219S/D220A/D267M mutant protein (200 ng each) was incubated with 2 μ g GST-HsRAD51, GST-BRC3/4 or GST alone in 500 μ l of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 1 mM phenyl-methylsulfonyl fluoride, 10 mM Na vanadate, Complete protease inhibitor (Roche) and glutathione Sepharose for 20 min at 25°C. The beads were washed and proteins were eluted and resolved by 12% Tris-glycine PAGE. Western blots were performed with commercial antibodies (see Supplementary data).

Nuclear targeting and competition in human cells

The *PfRad51 E219S/D220A/D267M* gene was fused to GFP in vector pGFP-C1 (Clontech). Assembly of PfRad51 mutant protein in the presence and absence of BRC3/4 repeats in γ -irradiated 293T cells was monitored as previously described (Pellegrini *et al.*, 2002) (see Supplementary data).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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