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ATP-Binding Cassette Properties of Recombination Mediator Protein RecF

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1. Introduction

1.1 Recombinational repair

*H*omologous *r*ecombination (HR) is essential for genetic diversity and genome stability. The conserved RecA-like recombinases promote pairing and consequent exchange of fragments between two homologous DNA molecules during conjugation in bacteria and meiotic recombination in eukaryotes. HR is a main DNA repair pathway particularly important in case of large-scale DNA damages, including chromosome or *d*ouble-*s*tranded (ds) *D*NA *b*reaks (DSBs) and long *s*ingle-*s*tranded (ss) DNA *g*aps (SSGs) (Cox, 1991; Kowalczykowski et al., 1994). The broken chain is paired with the intact DNA, which serves as a template for the synthesis of the damaged DNA. The same recombinases are also involved in the repair and origin-independent restart of stalled DNA replication, a frequently occurring event in every cell (Cox et al., 2000; Kowalczykowski, 2000; Kuzminov, 2001).

HR is initiated by the cooperative binding of RecA recombinase to ssDNA hundreds or thousands nucleotides long forming nucleoprotein filament, a so called presynaptic complex often designated as RecA*. The presynaptic complex can bind homologous dsDNA and exchange a DNA strands. RecA* has multiple activities beyond the strand invasion and exchange (Figure 1). Those include triggering DNA damage SOS response through stimulation of LexA autocleavage (Rehrauer et al., 1996) and activation of UmuD subunit of the error-prone DNA polymerase PolV important for translesion synthesis to bypass small-scale DNA errors (Jiang et al., 2009; Rajagopalan et al., 1992). RecA* was also suggested to stabilize and maintain stalled replication fork during DNA repair (Courcelle et al., 1997). Consequently, RecA binding to DNA is regulated at multiple levels (Cox, 2007).

1.2 Recombination mediator proteins

Transient ssDNA regions generated during replication are protected by ssDNA binding proteins like bacterial *ss*DNA *b*inding (SSB) protein and eukaryotic *r*eplication *p*rotein *A* (RPA), which prevent recombinase binding. Under DNA damage conditions, ubiquitous *r*ecombination *m*ediator *p*roteins (RMPs) overcome inhibitory effect of SSB and initiate presynaptic complex formation (Fig. 1)(Beernink and Morrical, 1999; Symington, 2002). RMPs are not directly involved in the repair of specific DNA damages, but they regulate initiation of multiple DNA repair pathways and damage response signaling cascades (Courcelle, 2005; Kowalczykowski, 2005; Lee and Paull, 2005; Moynahan et al., 2001;

Williams et al., 2007). In addition to presynaptic complex formation, many RMPs also promote DNA annealing (Luisi-DeLuca and Kolodner, 1994; Sugiyama et al., 1998). The importance of RMPs is reflected by the fact that recombination and repair pathways are often named after specific RMPs, e. g. RecF, RecBC, Rad52 pathways. RMPs include phage UvsY (Sweezy and Morrical, 1999), prokaryotic RecBCD and RecFOR proteins (Fujii et al., 2006; Kolodner et al., 1985; Lloyd and Thomas, 1983; Wang and Smith, 1983), and numerous eukaryotic members (Symington, 2002). Mutations of human RMPs are associated with cancer predisposition, mental retardation, UV-sensitivity and premature aging (Ouyang et al., 2008; Powell et al., 2002; Tal et al., 2009; Thompson and Schild, 2002).

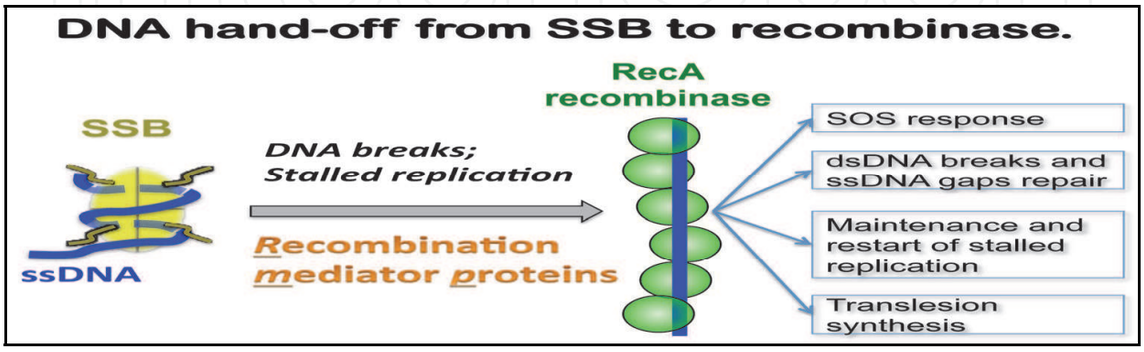

Fig. 1. The ssDNA hand-off from ssDNA binding proteins to RecA-like recombinase triggers multiple DNA damage response pathways important for DNA repair and origin-independent restart of stalled replication. Such DNA transaction is regulated by RMPs.

While ssDNA-binding proteins and RecA-like recombinases are well characterized, the mechanism of RMPs function remains poorly understood. Recent studies revealed a variety of RMPs structural domains. (Koroleva et al., 2007; Lee et al., 2004; Leiros et al., 2005; Makharashvili et al., 2004; Singleton et al., 2002; Yang et al., 2002). The diversity of RMPs structural domains reflects the plethora of different DNA damage response scenarios regulated by these proteins. The focus of this review is prokaryotic RMP RecF. Although a major bacterial recombination repair pathway is named after RecF, the mechanism of RecF activity and even its functional role remains one of the least understood and most controversial issues.

2. RecFOR recombination mediators

2.1 RecF pathway

The RecF was discovered as an alternative to RecBC pathway in genetic screens based on frequency of conjugation recombination in *E. coli*, and was found to be important for postreplication repair of extended SSGs (Horii and Clark, 1973; Lovett and Clark, 1983; Wang and Smith, 1984). Later, it was shown that *recF* mutants are even more hypersensitive to UV radiation than *RecBC,* that RecF pathway plays a major role in replication restart under UV damage conditions, and that RecF is involved in DSBs repair in the absence of RecBC and SbcBC (Clark, 1991; Courcelle, 2005; Courcelle et al., 1997; Ivancic-Bace et al., 2003; Kidane et al., 2004; Kusano et al., 1989; Whitby and Lloyd, 1995; Zahradka et al., 2006). Sequencing of new genomes revealed the ubiquitous nature of RecF pathway proteins found in most bacteria (Rocha et al., 2005), including the radiation resistant bacteria *Deinococcus radiodurans* (Bentchikou et al., 2010; Cox et al., 2010; Chang et al., 2010; Makarova et al.,

2001). RecF forms an epistatic group with RecO and RecR proteins (Asai and Kogoma, 1994; Courcelle et al., 1997; Courcelle and Hanawalt, 2003; Horii and Clark, 1973; Kolodner et al., 1985; Wang and Smith, 1984). All three proteins are equally important for recombinational repair in most genetic screens, although they do not form triple complex in solution. RecF and RecR genes are often located in DNA replication operons on chromosome, with the exception of extremophiles like *T. thermophiles* and *D. radiodurans* (Ream and Clark, 1983; Ream et al., 1980). In *E. coli*, RecF is co-transcribed with major subunits of replication machinery, DnaA and DnaN (Perez-Roger et al., 1991; Villarroya et al., 1998). RecF pathway proteins share either sequence or structural homology or functional similarities with eukaryotic proteins such as WRN, BLM, RAD52, and BRCA2, which are associated with cancer predisposition and premature aging when mutated (Karow et al., 2000; Kowalczykowski, 2005; Mohaghegh and Hickson, 2001; Yang et al., 2005).

Genetic studies demonstrated that RecF regulates several DNA repair and recombination pathways but is not directly involved in repair of specific DNA damage. For example, in R*ecF* mutants DNA lesions are removed with the efficiencies comparable to wild-type cells, while the UV resistance is strongly compromised (Courcelle et al., 1999; Rothman and Clark, 1977). RecF-mediated loading of RecA on ssDNA is required for the maintenance of arrested replication forks, fot the protection and processing of DNA ends to permit DNA repair and replication restart at the site of disruption.

The regulatory role of RecF in replication restart is further supported by examples where RecF impairs cell survival, like in thymine starvation experiments (Nakayama et al., 1982). Another example is revealed by genetic studies of DNA helicases UvrD and Rep (Petit and Ehrlich, 2002). Mutants lacking both helicases are not viable and *RecF* mutations suppress the lethality of the E. coli *Rep/UvrD* double mutant. UvrD helicase disassembles RecA* filaments, the reaction opposite to that of RecFOR, while Rep helicase promotes replication through transcription sites (Boubakri et al., 2010; Centore and Sandler, 2007; Heller and Marians, 2005; Lane and Denhardt, 1975; Veaute et al., 2005). The frequent pausing of the replication fork can potentially stimulate RecF-mediated initiation of RecA* filament formation leading to illegitimate recombination in the absence of UvrD (Mahdi et al., 2006).

2.2 Mechanism of RecOR activities

The involvement of all three RecF, -O and -R proteins in HR initiation is well documented by genetic studies. However, the mechanism of their activities in the initiation process remains poorly understood, particularly with respect to RecF. RecO and RecR alone are sufficient to promote formation of the RecA filament on SSB-bound ssDNA (Cox, 2007; Umezu et al., 1993). RecO binds DNA and the C-terminal tail of SSB and these interactions are critical for RecOR function, at least in the absence of RecF (Inoue et al., 2011; Manfredi et al., 2010; Ryzhikov et al., 2011; Sakai and Cox, 2009; Umezu and Kolodner, 1994). In addition, RecO anneals complimentary ssDNA strands protected by cognate SSB (Kantake et al., 2002; Luisi-DeLuca and Kolodner, 1994), resembling the properties of the eukaryotic RMPs, Rad52 and BRCA2 (Grimme et al., 2010; Mazloum et al., 2007; Sugiyama et al., 1998). RecR binds either RecO or RecF (Makharashvili et al., 2009; Umezu and Kolodner, 1994; Webb et al., 1995, 1997). Although *E. coli* RecR does not bind DNA at submillimolar concentrations, it significantly affects DNA binding properties of both RecO and RecF (Kantake et al., 2002; Makharashvili et al., 2009; Webb et al., 1999). RecR inhibits DNA annealing properties of RecO, even though RecOR complex binds both ss- and dsDNA. In

addition to initial loading of RecA, RecOR further stimulate homologous recombination by preventing the dissociation of RecA* filament from ssDNA in *E. coli* (Bork et al., 2001). Somewhat different properties were reported for *Bacillus subtilis* RecO, which does not require RecR for initiation of RecA* formation (Manfredi et al., 2008; Manfredi et al., 2010).
Crystal structures of all three proteins and of the RecOR complex from *D. radiodurans* have been reported (Koroleva et al., 2007; Lee et al., 2004; Leiros et al., 2005; Makharashvili et al., 2004; Timmins et al., 2007). RecR structure resembles that of a DNA clamp-like tetramer (Lee et al., 2004). However, the role of a potential DNA clamp in RMPs-mediated reaction is unknown. Moreover, in the crystal structure of RecOR complex RecO occupies large portion of the clamp inner space. Such conformation makes it challenging to predict functionally relevant interaction of the complex with DNA. Another intriguing fact is that the crystal structure of RecO did not resemble any structural features of its functional eukaryotic analog Rad52 (Leiros et al., 2005; Makharashvili et al., 2004; Singleton et al., 2002), which supports two identical reactions.

2.3 Ambiguities of RecF function

In contrast to genetic data, initial biochemical studies did not reveal the function of RecF in recombination initiation (Umezu et al., 1993). RecF binds both ss- and dsDNA in the presence of ATP, and it is a weak DNA-dependent ATPase (Griffin and Kolodner, 1990; Madiraju and Clark, 1991, 1992). It interacts with RecR in the presence of ATP and DNA (Webb et al., 1999). Surprisingly however, RecF was initially shown to play an inhibitory role during RecOR-mediated loading of RecA on SSB-protected ssDNA (Umezu et al., 1993).
The UV-sensitivity of *RecF* mutant can be suppressed by RecOR overexpression, suggesting that RecF plays a regulatory role (Sandler and Clark, 1994). In agreement with this hypothesis, RecF dramatically increases the efficiency of RecOR-mediated RecA loading at ds/ssDNA junctions with a 3′ ssDNA extension under specific conditions (Morimatsu and Kowalczykowski, 2003). RecF was suggested to recognize specific DNA junction structure to direct RecA loading at the boundary of SSGs. While initial experiments demonstrated such a preference (Hegde et al., 1996), later work did not support the binding preference of RecF to DNA junction (Webb et al., 1999). Purified RecF tends to gradually aggregate in solution (Webb et al., 1999). Apparently, nonspecific high molecular weight RecF aggregates interact with DNA resulting in the inhibitory effect of RecF or false positive interactions of RecF with specific DNA substrates (Hegde et al., 1996). In addition, RecFR complex limits the extension of RecA* beyond SSGs, the observation indirectly supporting RecF specificity towards boundaries of SSGs while in complex with other proteins (Webb et al., 1997).
RecF is co-transcribed with the replication initiation protein DnaA and with the β-clamp subunit of DNA polymerase III DnaN. However, its open reading frame is usually shifted by one or two nucleotides relatively to that of DnaN (Villarroya et al., 1998). *E. coli RecF* gene also has multiple rear codons. Thus, expression of RecF is likely to be down regulated at translational level. Consequently, there are only a few copies of RecF in an *E. coli* cell.
How RecF promotes recombination remains an open question. The ability of RecFR complex to limit extension of RecA* filament beyond the SSGs suggests that the RecFR complex may specifically interact with RecA*. However, no direct observation of such interactions has been reported so far. RecF also binds RecX protein (Lusetti et al., 2006). RecX is a negative regulator of presynaptic complex formation, which inhibits filament extension by binding to RecA. RecF scavenges RecX from solution through direct interaction, thus diminishing negative regulatory effect of RecX (Drees et al., 2004; Lusetti et al., 2006). Additional

evidence of direct involvement of RecF in the initiation of RecA* filament formation was recently demonstrated in experiments with the SSB mutant lacking conserved C-terminus peptide. This SSB mutant inhibits RecOR-mediated recombination initiation, likely due to lack of interaction of SSB with RecO (Sakai and Cox, 2009). Surprisingly, RecF rescues the RecOR function with this SSB mutant, even on ssDNA plasmids without ds/ssDNA junction.

3. Structural studies of RecF

3.1 RecF is an ABC ATPase

The amino acid sequence of RecF contains three conserved motifs characteristic of ATP-binding cassette (ABC) ATPases: Walker A, Walker B, and a "signature" motif. Walker A, or P-loop, is a nucleotide binding site found in a variety of ATPases (Walker et al., 1982). Walker B motif provides acidic amino acids important for coordination of a water molecule and a metal ion during the hydrolysis of a triphosphate nucleotide bound to the Walker A motif. The signature motif is a unique feature of ABC ATPases, a diverse family of proteins ranging from membrane transporters to DNA-binding proteins (review in (Hopfner and Tainer, 2003). ATP-dependent dimerization is a common feature of this class of proteins. Signature motif residues interact with the nucleotide bound to an opposite monomer (Hopfner et al., 2000). This motif is important for both ATP-dependent dimerization and subsequent ATP hydrolysis. ABC ATPases are not motor proteins and utilize ATP binding and hydrolysis as a switch or sensor mechanism, regulating diverse signaling pathways and reactions.

DNA-binding ABC ATPases include DNA mismatch and nucleotide excision repair enzymes (Ban and Yang, 1998; Junop et al., 2001; Obmolova et al., 2000; Tessmer et al., 2008), structural maintenance of chromosome (SMC) proteins cohesin and condensin (Strunnikov, 1998), and DSBs repair enzyme Rad50 (Hirano et al., 1995). SMCs and Rad50 are characterized by the presence of a long coiled-coil structural domain inserted between N- and C-terminal halves of the globular head domain (Haering et al., 2002). RecF lacks a coiled-coil region, but it does exhibit an ATP-dependent DNA binding and a slow DNA-dependent ATP hydrolysis activity (Hegde et al., 1996; Madiraju and Clark, 1992; Webb et al., 1995). However, the SMC-like properties of RecF and their role in recombinational repair have not been addressed. Previously, only Walker A motif has been shown to be critical for RecF function (Sandler et al., 1992; Webb et al., 1999). All known ABC-type ATPases function as a heterooligomeric complexes in which a sequence of inter- and intra-molecular interactions is triggered by the ATP-dependent dimerization and the dimer-dependent ATP hydrolysis (Deardorff et al., 2007; Dorsett, 2011; Hopfner and Tainer, 2003; Junop et al., 2001; Moncalian et al., 2004; Smith et al., 2002). Thus, RecF may function in recombination initiation through a multistep pathway of protein-protein and DNA-protein interactions regulated by ATP-dependent RecF dimerization.

3.2 Structural similarity of RecF with Rad50 head domain

The diversity of ABC ATPases makes it difficult to predict to which subfamily RecF belongs to based on sequence comparison. RecF is a globular protein lacking long coiled-coil domains of Rad50 and SMC proteins. However, it does not have significant sequence similarity beyond three major motifs with globular DNA binding proteins like MutS. We crystalized and solved a high resolution structure of RecF from *D. radiodurans* (DrRecF) (Fig.

2) (Koroleva et al., 2007). The structure was solved with resolution of 1.6 Å using native and selenomethionine protein derivative crystals. The structure is comprised of two domains. The ATPase domain I is formed by two β-sheets wrapped around central α-helix A and is similar to the corresponding subdomain of the Rad50 head domain (Figure 2, right). Structures of nucleotide-binding domains are similar in all ABC ATPases. In contrast, structure of subdomain containing signature motif (Lobe II in Rad50) is highly diverse among even DNA binding ABC ATPases. However, all structural elements presented in RecF domain II are present in Rad50 Lobe II subdomain and these domains are structurally more similar than ATP-binding domains. The only difference is two long α-helixes of RecF which are connected at the apical part of this "arm-like" domain. In Rad50 analogous α-helixes are extended into an extremely long coiled-coil structure, absent in RecF. High degree of structural similarity unequivocally puts RecF in the same family together with Rad50 and SMC proteins. Therefore, RecF represents the only known globular protein with a structure highly homologous to that of the head domains of Rad50, cohesin and condensin.

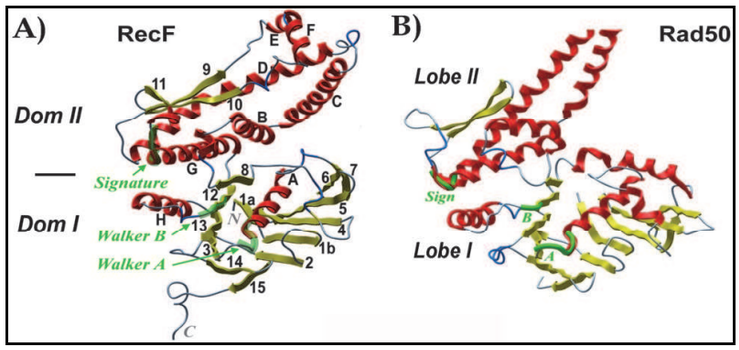

Fig. 2. Cartoon representation of **A**) RecF and **B**) Rad50 head domain structures. α-helixes are shown in red and β-sheets in yellow. In RecF, α-helixes are lettered and β -strands are numbered. Walker A, B, and signature motifs are highlighted in green and labeled. In RecF, ATP-binding domain is designated as Domain I and signature motif domain as Domain II. In Rad50 corresponding domains are referred as Lobe I and Lobe II subdomains.

3.3 The model of ATP-dependent dimer suggests mechanism of DNA binding

RecF was crystallized as a monomer. ATP-dependent dimer was modeled based on known intersubunit interactions conserved in ABC ATPases and, specifically, based on a known structure of Rad50 dimer (Fig. 3)(Hopfner et al., 2000). In all proteins of this family, a conserved serine of the signature motif interacts with a γ-phosphate group of ATP. The ATP bound to Walker A motif was modeled accordingly to its highly conserved conformation in all Walker A and B containing structures. These constrains unambiguously dictate a single conformation of the potential RecF dimer (Fig. 3A). The model suggests a potential DNA binding site located on the top of two nucleotide-binding domains, in a conformation similar to the proposed DNA binding site of Rad50 (Figs. 3B-D). The resulting RecF dimer forms a semi-clamp or a symmetrical crab-claw with two arms extending in the directions similar to those of coiled–coil regions of Rad50 dimer (Hopfner et al, 2001). The claw structure contains sufficient space to accommodate and cradle dsDNA. In this model, the majority of conserved residues map to the dimerization interface and pocket region of the claw, where DNA binding is expected to occur.

The proposed model explains an ATP-dependence of RecF DNA binding. First, it is an acidic protein with mostly negatively charged surface area. In the model of an ATP-dependent dimer, small patches of positively charged surface area are aligned on the top of the dimer, creating the extended basic surface area. Second, the arms of domain II form a deep cleft, sufficient to engulf a DNA helix. The constrains of a signature motif interaction with a γ-phosphate group of ATP does not allow to alter the distance between these arms in the model without significant structural clashes of surface exposed residues of the two monomers. Thus, the ATP-dependent dimerization leads to favorite juxtaposition of the surface charges and to surface complementarity, which stimulate DNA binding.

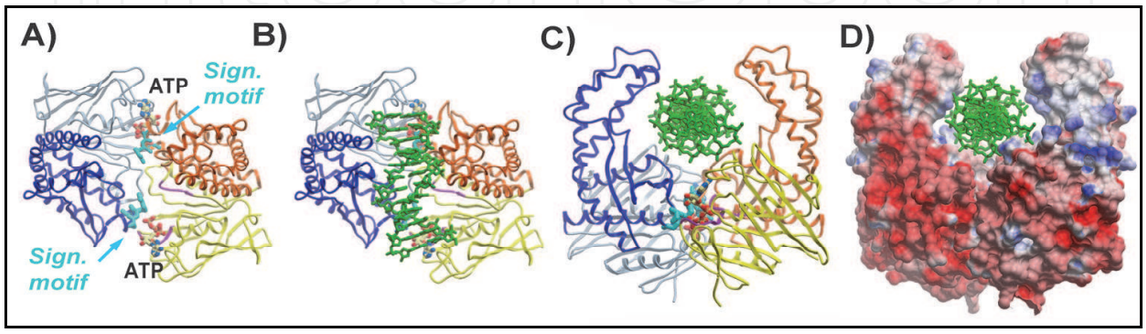

Fig. 3. A model of RecF dimer. **A**) Domains I and II of one RecF monomer are color-coded in yellow and orange, and of the other monomer in grey and blue. Signature motif residues are shown by stick representation in cyan and ATP by stick representation with nitrogen, oxygen, carbon and phosphate atoms are colored in blue, red, yellow and orange, correspondingly. **B**) The same dimer representation with bound dsDNA shown by stick representation in green. **C**) Orthogonal view of the dimer shown in B). **D**) Surface representation of DrRecF dimer in same orientation as in C) color-coded according to the surface electrostatic potential.

Proving ATP-dependent dimerization of RecF in solution was quite challenging due to poor solubility and a tendency of RecF to form nonspecific soluble aggregates (Webb et al., 1999). Initial attempts with size *e*xclusion *c*hromatography (SEC) yielded the monomeric form of E. coli RecF in the presence of ATP (Webb et al., 1999). The caveat of such experiment is in low protein solubility, when only solution with limited protein concentration can be run through column, and in a non-equilibrium nature of SEC, which may lead to dissociation of weak dimers. Later, it was shown that DrRecF nonspecifically interacts with the column resin even in a 1M KCl buffer (Koroleva et al., 2007). Therefore, a combination of SEC with static light scattering was utilized to determine the true molecular weight of eluted fractions. DrRecF does form an ATP-dependent dimer, though relatively unstable, which could dissociate on the column under non-equilibrium conditions at low protein concentration. The dimerization of wild type protein and specific mutants under equilibrium conditions was tested with a *d*ynamic *l*ight *s*cattering (DLS). DrRecF dimerizes only in the presence of ATP but not with ADP. Mutation of signature motif S276R resulted in lack of dimerization, as well as mutation of Walker motif A K39M, which prevents ATP binding. Walker A motif mutant K39R which binds, but does not hydrolyses ATP, forms dimer as well as mutants of Walker B motif D300N. Surprisingly, non-hydrolizable ATP analogs did not support dimerization in initial experiments, suggesting that RecF dimerization is highly sensitive to specific ATP-bound conformation. While DLS method is not suitable for quantitative analysis, it is highly sensitive

to the presence of high molecular weight protein aggregates, and it was utilized to optimize RecF solution conditions for other experiments.

4. Functional significance of ABC-type ATPase properties of RecF

4.1 ATP-dependent dimerization is required for DNA binding

The DNA binding properties of RecF and their role in recombination initiation remain poorly understood and controversial. Different publications presented contradicting results of DNA junction recognition by RecF (Hegde et al., 1996; Webb et al., 1999). RecR was shown to stabilize ATP-dependent interaction of RecF with DNA. However, RecR also stimulated ATP hydrolysis, which theoretically should lead to destabilizing of RecF complex with DNA (Webb et al., 1995). Therefore, multiple complimentary equilibrium binding techniques were utilized to comprehensively address the relationship between dimerization, DNA binding and ATP binding and hydrolysis (Makharashvili et al., 2009). Quantitative characterization of RecF dimerization was performed using Föster (or Fluorescence) Resonance Energy Transfer (FRET) technique with a mixture of Cy3- and FAM(fluorescein)-labeled DrRecF (Fig. 4). The cysteine substitutions were introduced either at a topical part of domain II arm or at the C-terminal tail to crosslink DrRecF with fluorophores. The labeling of domain II interfered with DNA-binding (Makharashvili, 2009), indirectly confirming the dimer model presented in Fig. 3, where apical parts of domain II arms are situated close to each other in the dimer and the presence of bulky polar fluorophores may interfere with DNA binding. C-terminally labeled protein (A355C) was fully functional. Apparent dimerization constant of L_d = 0.15 ± 0.02 μM was calculated from the plot of FRET signal versus DrRecF concentration (Fig. 4C). Alternatively, multiple data sets (Fig. 4B) were globally fitted into a two-step reaction model consisting of the ATP-binding and dimerization processes resulting in a dimerization constant of L_d = 0.13 ± 0.02 μM and an ATP-binding constant of K_d^{ATP} = 13 ± 2 μM.

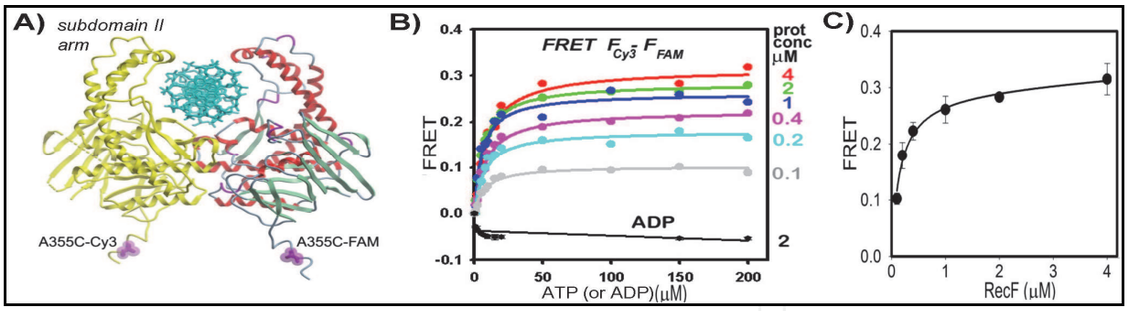

Fig. 4. ATP-dependent dimerization of DrRecF. **A**) Location of cysteines (A355C) are shown by magenta spheres on the model of DrRecF dimer with one monomer is colored in yellow and the other color-coded accordingly to its secondary structure elements with α-helixes in red and β-strands in green. The DNA is shown in cyan. **B**) Titration of labeled DrRecF by ATP. Different isotherms represent different concentration of DrRecF in solution (values are shown on the right). The black isotherm corresponds to titration of 2 μM DrRecF by ADP. **C**) A plot of maximal FRET signal versus DrRecF concentrations.

The DNA binding was first assayed using short FAM-labeled oligonucleotides with the fluorescent polarization anisotropy method (Fig. 5). To address initial DNA binding rate,

reactions were performed for a relatively short time (10-15 min) and with the excess of ATP, taking an advantage of RecF being a slow ATPase (Fig. 6C, below). Alternatively, the rate of ATP hydrolysis was measured over 1 or 2 hours time upon titration of RecF by different DNA oligonucleotides (Fig. 6B). The binding of all DNA substrates was relatively weak with the apparent dissociation constants greater than 15 μM (Fig. 5). Neither a wild type DrRecF in the presence of ADP nor a signature motif mutant S279R in the presence of ATP were able to bind DNA (Fig. 5), suggesting that the ATP-dependent dimerization is essential for RecF interaction with all DNA substrates.

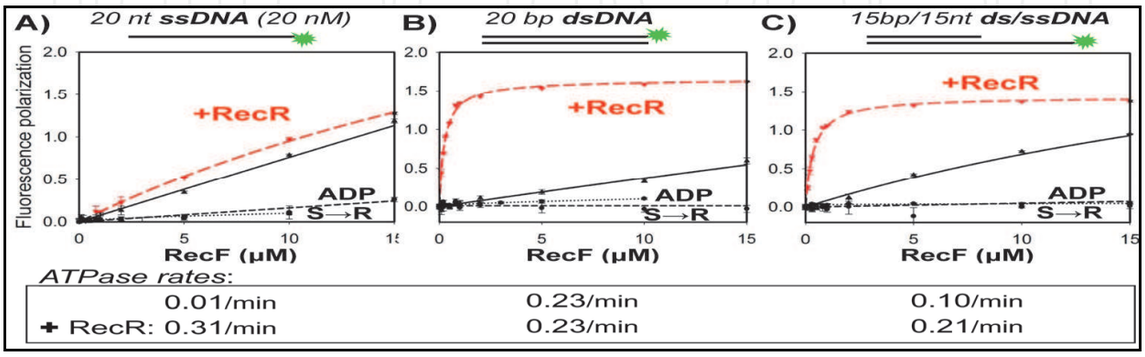

Fig. 5. ATP-dependent binding of DrRecF to different DNA substrates (top) and DNA-dependent ATP hydrolysis rates (bottom). DNA substrates are schematically represented above each plot with **A**) ssDNA, **B**) dsDNA and **C**) ds/ssDNA junction. Solid isotherms correspond to binding in the presence of ATP, dashed black – in the presence of ADP, dotted – to the binding of signature motif mutant S279R in the presence of ATP. Red isotherms correspond to DrRecF binding in the presence of ATP and 50 μM DrRecR. The maximum estimated ATP hydrolysis rates of DrRecF (Fig. 6A) are shown at the bottom with the top lane corresponding to reactions without DrRecR and the bottom – with RecR. DrRecF concentration is 10 μM, DNA- 20 nM, ATP – 2 mM.

4.2 RecR-dependent DNA specificity of RecF

DNA binding of DrRecF is drastically alters in the presence of DrRecR (red isotherms in Fig. 5). DrRecR significantly increases the affinity of DrRecF to dsDNA (Fig. 5B) with the estimated association binding constant at least two orders of magnitude stronger than without DrRecR. DrRecR does not alter DrRecF ssDNA binding according to the DNA binding assay. However, the ATPase assay clearly demonstrated interaction of DrRecR with DrRecF in the presence of ssDNA. ssDNA does not stimulate ATP hydrolysis by DrRecF, while the presence of both DrRecR and ssDNA results in strongest ATPase rate. This suggests that DrRecR stimulates the ATPase rate of DrRecF bound to ssDNA, potentially destabilizing dimerization and ssDNA binding. In case of dsDNA, maximum ATPase rates were similar with and without DrRecR. Therefore, DrRecR stabilizes DrRecF complex with dsDNA without increasing its ATPase rate. Due to this stabilization effect of RecR, we are able to measure DNA binding and dimerization of DrRecF in the presence of ATP analogs (Fig. 6B). Curiously, a weak dimerization is observed at highest DrRecF concentration even in the presence of ADP. Therefore, DrRecR selectively stimulates binding of DrRecF dimer to dsDNA, while potentially destabilizing DrRecF complex with ssDNA. Both dimerization and DNA binding reactions were also measured as a function of time to verify that under

these conditions ATP hydrolysis does not significantly alter either interaction within first 10 minutes (Fig. 6C).

DrRecR is characterized by a weak DNA binding affinity in a millimolar range, while binding of *E. coli* RecR to DNA was not detected. DrRecR forms a tetrameric DNA clamp-like structure (Lee et al., 2004). This conformation is likely to be conserved for other RecR homologs since *E. coli* RecR is either a dimer or tetramer in solution (Umezu et al., 1993), and *H. influenzae* RecR also was crystallized in a similar tetrameric conformation (Koroleva, O., Baranova, E., Korolev, S. unpublished data). One way to explain the DNA-dependent interaction of RecR with RecF is through the binding of both proteins to a shared DNA substrate, as beads on a string. Moreover, since dimer to tetramer transition was proposed as a clamp loading mechanism (although not confirmed), the ATP-dependent dimerization of RecF may stimulate such loading of RecR clamp on DNA. To test the hypothesis of shared DNA substrate requirement for RecF interaction with RecR, the RecR-stimulated DNA binding of RecF and the ATPase rate were tested in the presence of different length dsDNA substrates. Surprisingly, 10 bp short oligonucleotide stimulates DrRecF interaction with DrRecR. Structural modeling suggests that RecF dimer can bind 12-15 bp long DNA, while RecR clamp may cover up to 8-12 bp. These results rule out the beads-on-a-string model of RecFR binding to dsDNA. Alternatively, RecR may interact with the domain II arms encircling RecF bound DNA in a model similar to that of Rad50/Mre11 complex (Hopfner et al., 2001; Lammens et al., 2011; Williams et al., 2011).

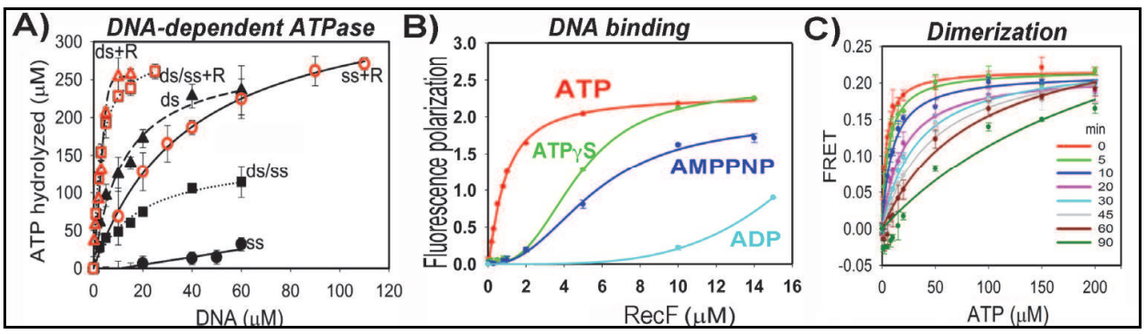

Fig. 6. **A**) ATP hydrolysis by DrRecF over 120 min was measured upon titration by different DNA substrates, with circles corresponding to ssDNA, triangles to dsDNA, and squares to ds/ssDNA. Red symbols correspond to titrations in the presence of 50 μM RecR. Concentration of RecF is 10 μM, and ATP 2 mM. **B**) dsDNA binding by RecFR in the presence of ATP analogs measured with the fluorescence polarization assay performed similarly to that in Fig. 5 with the following nucleotides: ATP (red), ATPγS (green), AMPPNP (blue), and ADP (cyan). **C**) Time dependence of RecF dimerization upon titration with ATP as measured by FRET of labeled RecF. Isotherms of different colors correspond to the FRET value at different time points shown on the right.

4.3 The lack of ss/dsDNA junction specificity

The steps of RecF interaction with DNA and RecR are schematically represented in Fig. 7. ATP binding stimulates RecF dimerization, essential for binding of all DNA substrates. The DNA-bound RecF dimer interacts with RecR, which either stabilizes the complex with dsDNA or destabilizes with ssDNA. Importantly, neither of the performed assays revealed any specificity of RecF and RecFR complex for ss/dsDNA junction. Both DNA binding and

ATPase rates had an average between ss- and dsDNA substrates values. Although all data were obtained with *D. radiodurans* proteins, RecF and RecR are highly homologous proteins. Moreover, *E. coli* RecR stimulates DNA binding of *D. radiodurans* RecF similarly to that of *D. radiodurans* RecR suggesting that DrRecF binds both Dr- and *E. coli* RecR proteins with similar affinities (Makharashvili, 2009). Therefore, the described above properties of *D. radiodurans* proteins are likely to be conserved for *E. coli* homologs. While DNA binding and ATPase assays did not reveal specificity of RecF towards DNA junction, functional studies clearly evidence the role of RecF at ss/dsDNA junction (Chow and Courcelle, 2004; Handa et al., 2009; McInerney and O'Donnell, 2007; Morimatsu and Kowalczykowski, 2003; Webb et al., 1997). The potential specificity of RecF to ds/ssDNA junction is likely to require additional protein partners of recombination initiation reaction including SSB, RecO and RecA. For example, RecR can be recruited to SSB-bound ssDNA while in complex with RecO (Ryzhikov et al., 2011). The increased local concentration of RecR on SSB-coated ssDNA may subsequently stimulate RecF interaction with the adjacent dsDNA region.

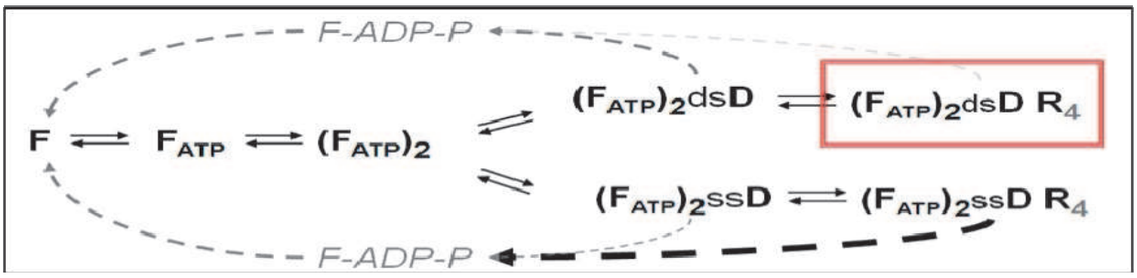

Fig. 7. Schematic representation of RecF interaction with ATP, ATP-dependent dimerization, DNA binding, and the effect of RecR on DNA binding and ATP hydrolysis. The complex formed on dsDNA in the presence of RecR (red box) is the most stable intermediate. In case of *D. radiodurans* homologs, RecF dimer interacts with RecR tetramer.

4.4 In vivo function of RecF conserved motifs

The role of RecF SMC motifs *in vivo* was initially addressed with *E. coli RecF* mutant cells transformed with RecF-containing vector (Koroleva et al., 2007). Only wild type RecF complemented the UV sensitivity of a *recF* cells. Mutations of Walker A, -B and signature motifs did not restore the UV resistance. Since the overexpression of RecF can potentially affect its function, similar mutants of *RecF* were constructed in chromosome (Michel-Marks et al., 2010). Importantly, different steps of RecFOR function were tested with each mutant. Those include the rate of DNA synthesis, degradation of nascent DNA, the presence of DNA intermediates, and cell survival upon UV irradiation. Mutants included Walker A motif K36M, deficient in ATP binding, a Walker A motif K36R and a Walker B D303N, which both retain ATP binding but are deficient in ATP hydrolysis, and two signature motif mutants S270R and Q273A, which prevent an ATP-dependent dimerization.

Following the UV-induced arrest of replication, the nascent DNA is partially degraded at the replication fork by RecQ helicase and RecJ nuclease and RecF limits such degradation (Courcelle and Hanawalt, 1999). The degree of nascent DNA degradation was measured with pulse labeling of growing cell culture with [^{14}C]thymine and [^{3}H]thymidine. Similarly to a null mutant(Courcelle and Hanawalt, 1999), approximately 50% of nascent DNA was degraded with all mutants with the exception of D303N, where degradation was less severe.

Therefore, all steps of the dynamic interactions of RecF with ATP and DNA are important for the very first step of RecFOR function in replication repair. The weak functionality of D303N can be explained by a potential residual ATPase activity of this mutant, as shown for other SMC proteins (Lammens et al., 2004). Experiments with ATP analogs (Fig. 6B) demonstrated that even minor conformational changes significantly affect RecF properties. Therefore, an alternative explanation may be that D303N mutant introduces the least conformational distortion at the ATP-binding site and may retain conformation of a wild type wild type dimer and DNA-binding activities better than K36R mutant.

The rate of DNA synthesis is reduced by approximately 90% immediately after UV irradiation, but is recovered to nearly initial rate within 100 min in wild-type cells. The overall accumulation of DNA is increased at that time approaching the level of unirradiated cells. In *recF* cells the initial reduction of DNA synthesis rate is similar, but there is no recovery. Like in the previous assay, all mutants with exception of D303N were similar to the null mutant. D303N mutant did support slight recovery of DNA replication rate, yet it was significantly weaker than that of a wild type. RecF is associated with appearance of specific replication intermediates during DNA damage, as visualized on two-dimensional agarose gel (Courcelle et al., 2003). In this assay, all mutants were equally deficient in accumulation of such intermediates similarly to the null mutant, although the detection level of this assay may not be sufficient to reveal weak activity of D303N mutant. Finally, the survival rate of cell culture after UV irradiation was assayed. D303N mutant was partially resistant, while all other mutants were as hypersensitive to UV irradiation as deletion of *recF*. These studies demonstrate that all steps of ATP binding, dimerization and hydrolysis by RecF are important to maintain stalled replication and to restart cell growth after DNA damage.

5. Conclusions

RecFOR proteins regulate RecA binding to ssDNA under DNA damage conditions. This reaction initiates a variety of DNA repair pathways including maintenance and restart of stalled replication. Correspondingly, recombinational repair is tightly regulated in cell. While the exact role and mechanism of RecF in these pathways remain controversial, the majority of known data suggest a regulatory function of RecF during initiation and subsequent steps of recombinational DNA repair. Intricate properties of the ATP-dependent interaction of RecF with DNA and of the DNA-dependent ATP hydrolysis as well as the dependence of these interactions on RecR strongly supports this hypothesis.

Regulatory function is further reinforced by the sequence and structural homology with the head domain of Rad50 and SMC proteins. Rad50 is involved in multiple steps of DNA damage response including initial detection of DSBs, triggering of cell signaling cascades, and in resection of dsDNA to create 3' ssDNA tail for recombinase binding (Nicolette et al., 2010). In bacteria, RecF is likely to be involved in multiple steps of replication restart as well, including initial detection of replication arrest. Neither Rad50 nor RecF specifically recognizes functionally relevant DNA substrates, blunt-end DNA and ss/dsDNA junction, correspondingly (de Jager et al., 2002). Rad50 functions in complex with other DNA binding proteins, including Mre11 nuclease, and protein-protein interactions regulate DNA binding and ATPase activities (Lammens et al., 2011; Lim et al., 2011; Williams et al., 2011). By analogy, we can speculate that ATP binding and hydrolysis may not simply control DNA binding and dissociation of RecF, but also regulate binding of RecF dimer to different

protein partners. For example, the ability of short DNA fragments to promote RecR binding suggests that the DNA-dependent conformational changes of RecF are important for protein-protein recognition rather than simple binding to the shared DNA substrate.

It is important to note that RecF does not represent the exact analog of Rad50. It is a much smaller protein without long coiled-coil structures. RecF does not support DNA unwinding or resection, as well as additional adenylate kinase activity of Rad50 and SMC proteins (Bhaskara et al., 2007; Lammens and Hopfner, 2010). Instead, it is involved in the initiation of the presynaptic complex formation, the function performed by BRCA2 or Rad52 in eukaryotes (Moynahan et al., 2001; New et al., 1998; Shinohara and Ogawa, 1998; Sung, 1997; Yang et al., 2005). While Rad52 is rather unique protein (Singleton et al., 2002), structural and functional motifs of BRCA2 resemble that of RecFOR system (Yang et al., 2002). BRCA2 interacts with ssDNA through OB-fold domain, similarly to RecO, and has a putative dsDNA-binding domain. The latter function is likely to be performed by RecF, even with the lack of structural similarity.

RecF regulates RecQJ-dependent resection of nascent DNA at stalled replication fork (Courcelle and Hanawalt, 1999). This step occurs prior to RecA loading and initiation of SOS response. How RecF recognizes stalled replication remains unknown. It is tempting to speculate that RecF is a part of replisome (Kogoma, 1997) based on co-translation of RecF with replication initiation protein DnaA and polymerase subunit DnaN and on its early involvement in detection of replication arrest. However, no interactions of RecF with replication proteins have been identified so far. RecF may represent an alternative to PriA pathway of replication restart in case of arrested replication or postreplication repair (Sandler, 1996). Thus, it is important to find additional RecF-binding proteins. The detection of novel interactions is problematic due to low copy number of RecF in cells and poor solubility of purified RecF. The potential requirement of ATP- and DNA-dependent dimerization for RecF interaction with other proteins further complicates the search for interacting proteins.

The relationship of specific steps of ATP-dependent reactions with the DNA damage recognition and processing by RecF and Rad50 remains elusive. Since RecF is the smallest known DNA-biding ABC ATPase composed of the head domain only, it represents an excellent model system to address the role of allosteric regulations, governing function of this class of proteins. Importantly, both ATP binding and hydrolysis are likely to play an important mechanistic role in most of reactions (Fig. 7). For example, the first step of limiting degradation of nascent lagging DNA by RecQJ and loading of RecA may only require formation of a stable RecF dimer at DNA junction, while ATP hydrolysis and dimer dissociation may be important for the following steps. However, the involvement of all the conserved motifs to prevent degradation of nascent DNA suggests that both ATP binding and hydrolysis are important even for this initial step. Therefore, all steps of RecF function in DNA repair are likely to depend on dynamic interactions of RecF with ATP, DNA and DNA repair proteins. Delineating molecular basis and principles of these interactions is essential for understanding fundamental mechanisms of DNA repair, recombination and replication.

6. Acknowledgment

The research was supported by National Institutes of Health grant GM073837. The author is grateful for help during manuscript preparation to Mikhail Ryzhikov and Ekaterina Los. Significant portion of RecF studies were performed by graduate student Nodar Makharashivili.

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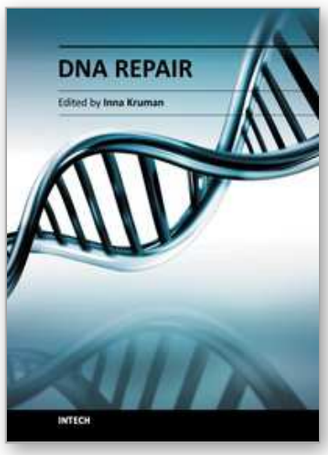

DNA Repair

Edited by Dr. Inna Kruman

ISBN 978-953-307-697-3
Hard cover, 636 pages
Publisher InTech
Published online 07, November, 2011
Published in print edition November, 2011

The book consists of 31 chapters, divided into six parts. Each chapter is written by one or several experts in the corresponding area. The scope of the book varies from the DNA damage response and DNA repair mechanisms to evolutionary aspects of DNA repair, providing a snapshot of current understanding of the DNA repair processes. A collection of articles presented by active and laboratory-based investigators provides a clear understanding of the recent advances in the field of DNA repair.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Sergey Korolev (2011). ATP-Binding Cassette Properties of Recombination Mediator Protein RecF, DNA Repair, Dr. Inna Kruman (Ed.), ISBN: 978-953-307-697-3, InTech, Available from: http://www.intechopen.com/books/dna-repair/atp-binding-cassette-properties-of-recombination-mediator-protein-recf

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