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ATP Binding, ATP Hydrolysis, and Protein Dimerization Are Required for RecF to Catalyze an Early Step in the Processing and Recovery of Replication Forks Disrupted by DNA Damage

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In *Escherichia coli*, the recovery of replication following disruption by UV-induced DNA damage requires the RecF protein and occurs through a process that involves stabilization of replication fork DNA, resection of nascent DNA to allow the offending lesion to be repaired, and reestablishment of a productive replisome on the DNA. RecF forms a homodimer and contains an ATP binding cassette ATPase domain that is conserved among eukaryotic SMC (*s*tructural *m*aintenance of *c*hromosome) proteins, including cohesin, condensin, and Rad50. Here, we investigated the functions of RecF dimerization, ATP binding, and ATP hydrolysis in the progressive steps involved in recovering DNA synthesis following disruption by DNA damage. RecF point mutations with altered biochemical properties were constructed in the chromosome. We observed that protein dimerization, ATP binding, and ATP hydrolysis were essential for maintaining and processing the arrested replication fork, as well as for restoring DNA synthesis. In contrast, stabilization of the RecF protein dimer partially protected the DNA at the arrested fork from degradation, although overall processing and recovery remained severely impaired.

Keywords: RecF; DNA binding; DNA replication; structural maintenance of chromosome (SMC) proteins; ATP binding cassette (ABC) ATPases

Edited by J. Karn

Introduction

RecF is part of a ubiquitous family of recombination mediator proteins that includes Rad52, BRCA2, BLM, and WRN. These proteins are required to maintain genomic stability, but their precise cellular functions remain poorly understood.[1–7] The structure of the RecF protein of *Escherichia coli* reveals a strong similarity to the globular head domain of human Rad50, a protein involved in the detection and repair of double-strand DNA breaks.[8] Both RecF and Rad50 contain a conserved ATP binding cassette ABC-type ATPase, which is conserved among many structural maintenance of chromosome (SMC) proteins, DNA repair enzymes, and membrane transporters.[9–13]

In *E. coli*, RecF function, together with RecO and RecR functions, is required for replication to resume following disruption by DNA damage.[14–17] In the absence of any one of these genes, replication forks are not maintained following arrest, nascent DNA at the arrested fork is extensively degraded, and DNA synthesis fails to resume.[15,16,18] Either coordinately with or subsequent to RecF–RecO–RecR binding, the nascent lagging strand of the arrested fork is partially degraded by the combined action of RecQ, a 3′–5′ helicase, and RecJ, a 5′–3′ nuclease.[16,18] Nascent DNA degradation is thought to restore the lesion-containing region to a double-strand form that can be repaired by nucleotide excision repair, and it is essential for the rapid recovery of DNA synthesis.[19,20] In the absence of either processing or repair, the recovery of replication remains dependent on the RecF pathway, but occurs through the action of translesion synthesis polymerases.[19,20]

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Abbreviations used: ABC, ATP binding cassette; SSB, single-strand DNA binding protein.

Once the lesion is removed or bypassed, an active and functional replisome must be restored to allow replication to resume.[19,20] The precise role of RecF in processing the disrupted replication forks remains unclear and could occur at any of several steps during the recovery process, including the initial binding and recognition of the disrupted forks, the processing or regression of the fork structure away from the offending lesion, or the reestablishment of an active replisome once the lesion has been removed or bypassed (Fig. 1).

The crystal structure and solution studies of the RecF protein revealed that the protein forms a dimeric clam-like structure and contains an ABC ATPase domain.[8] Similar to the ABC ATPase domains found in other SMC-like proteins, RecF contains three conserved motifs termed the Walker A motif, Walker B motif, and signature motif. On RecF, the Walker A and Walker B motifs are located in ATPase domain 1 at the N-terminus and the C-terminus, respectively, while the signature motif resides in domain 2 on the protein.[8] The signature motifs of ABC ATPases mediate ATP-dependent dimerization, with ATP bound at the interface of two opposing molecules.[25–28] RecF lacks the striking coiled-coil region between the N-terminus and the C-terminus of the globular head domain seen in other SMC-like proteins and Rad50, but is otherwise structurally similar to Rad50 and exhibits both ATP-dependent DNA binding and DNA-dependent ATP hydrolysis characteristic of SMC proteins.[8,29–31]

The precise catalytic function(s) of the conserved RecF motifs in processing and restoring arrested replication forks remains uncharacterized, although a number of biochemical characterizations are consistent with the idea that they could participate in any of the several progressive steps associated with the recovery process. Purified RecF, RecO, and RecR are able to displace single-strand DNA binding protein (SSB) and to enhance the nucleation of a RecA filament on DNA.[32–35] Although RecO and RecR proteins are sufficient to perform this reaction *in vitro*, RecF greatly stimulates the process in the presence of double-strand DNA fragments.[5,21,22] Furthermore, RecF appears to play an important role in targeting this nucleation to regions that contain a single-strand–double-strand DNA junction and have a 5′ DNA end.[5,21,29] These observations are consistent with the idea that RecF may act to recognize the arrested replication forks and to catalyze the loading of a RecA filament.

Other studies have suggested that RecF modulates both the ability of RecA filaments to form on single-strand regions and the RecA-mediated strand exchange reaction in a way that would enhance fork regression.[34,36,37] Consistent with this idea, *in vitro*, RecF cycles through a complex pathway that involves ATP-dependent dimerization, DNA binding, and repeated interactions with RecR in a DNA-substrate-dependent manner.[21,31,34]

Still other studies support the idea that RecF–RecO–RecR, along with RecA, may functionally interact with the replication machinery and may have a direct role in removing polymerase from its arrest site or in reestablishing the replisome after the

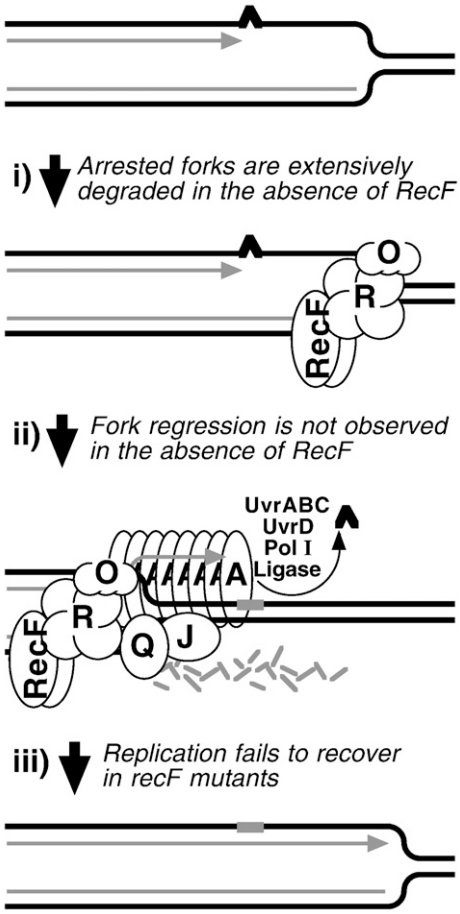

Fig. 1. Processing events involved in the recovery of replication following disruption by DNA damage and the phenotypes observed in the absence of RecF. (i) Replication is arrested following UV-induced DNA damage. *In vitro*, RecF is proposed to direct RecR and RecO to DNA junctions and to initiate the formation of a RecA filament at these sites, an activity that has been proposed to help target RecA to the arrested replication fork *in vivo*.[21,22] (ii) Either subsequent to or concurrent with this activity, the RecQ helicase and RecJ nuclease partially degrade the nascent lagging strand of the arrested DNA fork. The partial degradation is required for the rapid recovery of replication and is thought to restore the region containing the lesion to a double-strand form that allows nucleotide excision repair to remove the obstructing lesion.[18,20] In the absence of RecF, this degradation is much more extensive and eventually leads to the loss of the replication fork's integrity.[23] (iii) Once the lesion has been removed, an active replisome must be reestablished. It is not clear which components of the replisome are disrupted upon encountering a UV-induced lesion, although some evidence suggests that the replicative helicase remains bound,[24] suggesting that the replisome may remain at least partially intact. It is possible that RecF, along with RecO–RecR and RecA, may stabilize the nascent leading strand on the template to allow the replisome to resume from this structure. In the diagram, RecA, RecO, RecR, RecQ, and RecJ are denoted as A, O, R, Q, and J, respectively.

Table 1. Targeted mutations in RecF

Amino acid change	Location in protein	Functional prediction based on other SMC proteins	Confirmed for RecF *in vitro*
K36>M	Walker A motif	Prevents ATP binding	Yes
K36>R	Walker A motif	Prevents ATP hydrolysis	Yes
D303>N	Walker B motif	Stabilizes protein dimerization	Yes
S270>R	Signature motif	Prevents protein dimerization	Yes
Q273>A	Signature motif	Prevents protein dimerization	No

lesion has been repaired. In reconstituted replication assays, RecF, RecO, and RecR, along with RecA, are able to displace a stably bound polymerase from SSB-bound DNA to expose the arresting lesion.[38] Also, suggestively, both *recF* and *recR* are coregulated and transcribed on the same operon with the replisome's *dnaN* and *dnaX* genes, respectively.[39,40] These observations are consistent with the possibility that the conserved RecF motifs may function together with RecA to catalyze the disassembly or reestablishment of the replisome at the site of replication disruption.

To characterize the role of the conserved motifs in the process of replication recovery following UV-induced arrest, we constructed five *recF* point mutations in the *E. coli* chromosome. Equivalent mutations using the highly conserved RecF from *Deinococcus radiodurans* have been shown biochemically to generate proteins that either disrupt or stabilize RecF dimerization, prevent ATP binding, or prevent ATP hydrolysis (Table 1). We then characterized the molecular events that occur during the progressive steps of restoring replication following disruption in strains containing these altered RecF proteins.

Results

The crystal structure of RecF revealed a strong structural similarity to that of human Rad50, a protein required to recognize and catalyze the repair of double-strand breaks in humans.[8] Biochemical analysis demonstrated that RecF contains a conserved ABC ATPase domain and forms a dimeric clam-like structure that is capable of accommodating single-strand or double-strand DNA. At the time, we hypothesized that upon dimerization, the protein could function as a clamp loader that targets a tetrameric RecR ring to regions of single-strand–double-strand DNA junctions and, subsequently, RecO monomers.[8,21] However, RecF could participate in any or several of the progressive steps associated with the recovery process.

To characterize the functional role of the ABC ATPase domain in the recovery process, we constructed several point mutants that were predicted to have altered RecF functions based on their homology to Rad50 and other SMC-like proteins (Table 1). In previous work, many of these altered proteins have been characterized biochemically using the RecF protein from *D. radiodurans* and have been confirmed to produce their predicted effect.[8]

We initially cloned these *recF* mutants into expression plasmids pQE9 and pMalp2. When these overexpression plasmids had been transformed into *E. coli* deleted for *recF*, the altered proteins rendered the cells hypersensitive to UV irradiation to varying extents (Koroleva *et al.*[8]; data not shown). However, previous studies have shown that altering the intracellular ratio of RecF, RecO, and RecR can affect their normal functions *in vitro* and *in vivo*, and that an excess of RecF can have an inhibitory effect on presynaptic complex formation.[36,41] Therefore, we chose to reconstruct the altered *recF* genes directly into the chromosome using gene replacements, rather than characterizing their effects when expressed from plasmids. This avoids the possibility of an observed effect being due to abnormal expression levels and ensures that all regulatory elements controlling its endogenous expression are functional.

We have developed a number of molecular assays to monitor the effect of RecF on maintaining replication fork, regressing replication fork, limiting fork processing by RecJ and RecQ, and restoring DNA synthesis *in vivo*. An advantage of this type of cellular approach is that it allows us to directly observe RecF function in an environment that contains all of RecF's natural endogenous substrates and multiple protein partners, which would otherwise not be possible to reconstruct biochemically.

The ABC ATPase activity of RecF is required to recognize and protect nascent DNA ends at arrested replication forks

Following disruption by UV-induced damage in wild-type cells, the nascent DNA at the replication forks is partially degraded by the combined action of the RecQ 3′–5′ helicase and the RecJ 5′–3′ nuclease.[16,18] RecF is required to maintain the replication fork DNA and to limit the nascent DNA processing that occurs following the disruption of replication.[15,16]

To examine whether the altered RecF proteins retain the ability to carry out this step in the recovery process, we pulse labeled cultures grown in the presence of [^{14}C]thymine with [^{3}H]thymidine for 5 s and then immediately transferred them to prewarmed nonradioactive media and irradiated them with 30 J/m^2 UV irradiation. At 20-min intervals, aliquots of the culture were lysed, and radioactivity in the DNA was quantified to determine whether the nascent DNA remained protected and how much degradation occurred in both the overall genomic DNA (^{14}C) and the nascent DNA synthesized just prior to the arrest of replication (^{3}H).

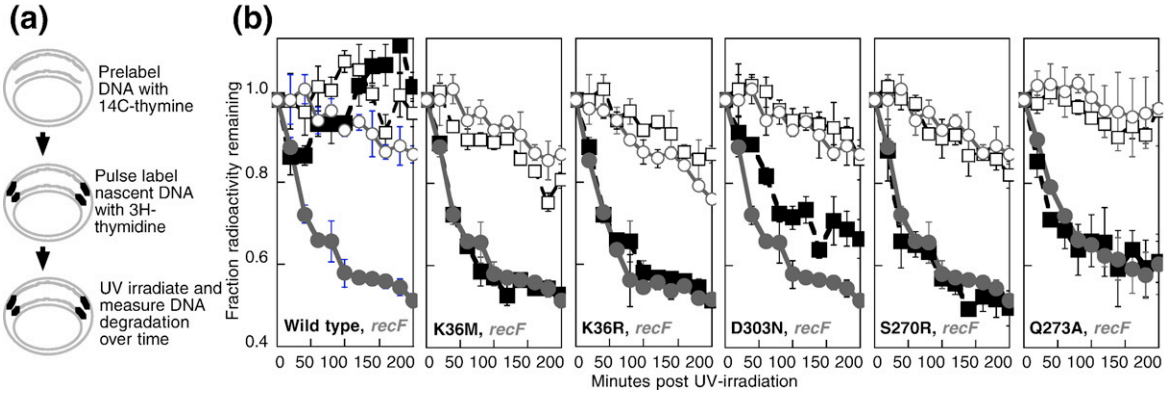

Fig. 2. A *recF* D303N mutation that stabilizes RecF in its dimeric form partially retains the ability to protect the nascent DNA from degradation following disruption by UV-induced damage. However, *recF* mutations that prevent dimerization (S270R and Q273A), ATP binding (K36M), or ATP hydrolysis (K36R) are as defective as a null mutation. (a) Schematic diagram of the approach used to monitor degradation occurring at the nascent DNA and in the overall genome. Cultures prelabeled with [^{14}C]thymine were pulse labeled for 5 s with [^{3}H]thymidine before being resuspended in nonradioactive medium and UV irradiated with 30 J/m^2. Aliquots of the culture were taken at various times following UV irradiation, and the fraction of radioactivity remaining in the DNA was plotted over time. (b) The fraction of radioactive nucleotides that remain in the total DNA (^{14}C; open squares) and newly synthesized DNA (^{3}H; filled squares) in wild type and of five *recF* point mutants following disruption by UV-induced damage is plotted. The amount of degradation occurring in the total DNA (^{14}C; open circles) and newly synthesized DNA (^{3}H; filled circles) of the *recF* deletion mutant is plotted in gray in each panel for comparison. Initial counts per minute were between 5000 and 6000 for ^{3}H and between 1000 and 2000 for ^{14}C in each experiment. Each graph represents at least two independent experiments. Error bars represent the standard error of the mean.

Similar to previous studies, we observed a limited amount of degradation of the nascent DNA at early times after UV irradiation in wild-type cells (Fig. 2). The amount of ^{3}H-labeled DNA decreased by approximately 10% initially, but began to increase at later times, even exceeding 100% of the initial DNA labeled. In principle, the amount of ^{3}H-labeled DNA should only be able to decrease over time. In previous work, we have shown that the increase at later times is likely due to reincorporation of intracellular pools once replication has resumed.[15,18] tures lacking the *recF* gene, we observe that the degradation of the nascent DNA was more extensive and continued for the first 100 min until approximately 50% of the pulse label in the nascent DNA had been degraded (Fig. 2). This observation has previously been interpreted to indicate that RecF is required to recognize and protect the replication fork DNA following disruption.

When we examined the altered *recF* mutants in this assay, we observed that the pattern of nascent DNA degradation was nearly identical with that of the *recF* deletion mutant for the *recF* K36M, K36R, S270R, and Q273A mutations. The results indicate that ATP binding and ATP hydrolysis, as well as RecF dimerization, are essential to the ability of RecF to protect the nascent DNA ends at disrupted replication forks from extensive degradation. In contrast, the *recF* D303N point mutant, which has been shown biochemically to trap the ATP-bound RecF protein in its dimeric form, partially retained the ability to protect the nascent DNA from degradation, relative to the *recF* deletion mutant. However, the amount of nascent DNA degradation that occurred in the *recF* D303N point mutant still exceeded that occurring in wild-type cells. Taken together, these results could be consistent with the idea that RecF dimer formation, but not ATP-hydrolysis-dependent dissociation, is required to initiate the recruitment of protein partners to protect the disrupted replication fork. The inability of the *recF* D303N mutant to restore nascent DNA protection to wild-type levels suggests that the ATP-bound dimeric form of the protein is loaded onto the DNA less efficiently. This type of function would be consistent with the idea that RecF plays an initiating role in the recruitment of factors to protect the nascent DNA.

Alternatively, partial restoration of nascent DNA protection could also be consistent with the idea that RecF must repeatedly dimerize and disassociate on the DNA during the recovery process. Mutations equivalent to the D303N change in other SMC proteins retain some residual ATP hydrolysis activities.[26] Although the *recF* D303N mutant did not retain any ATP hydrolysis *in vitro*,[8] we cannot rule out the possibility that *in vivo*, in the presence of RecR or other protein partners, residual ATP hydrolysis that allows the stabilized dimer to cycle, albeit with greatly reduced efficiency, may occur. This type of role would be more consistent with the idea that RecF plays a more active role in the processing and regression of the replication fork DNA during the recovery process.

The ABC ATPase activity of the RecF protein is required to process replication forks disrupted by DNA damage

In vivo, the processing of replication forks following disruption by DNA lesions involves a transient

regression of the fork structure that persists until a time correlating with when lesions are repaired and replication resumes. The processing and intermediate structures that occur at the replication fork during the recovery process can be visualized on plasmids such as pBR322 *in vivo*. In previous work, the formation of these processing intermediates has been shown to depend on the RecF protein.[16]

To examine whether replication fork processing and replication fork intermediates occurred in strains expressing the altered RecF proteins, we examined the structural intermediates that occurred on replicating plasmids of pBR322 in each strain using two-dimensional agarose gel analysis. Strains containing the plasmid were irradiated with 50 J/m^2 UV irradiation and then sampled immediately following irradiation and at 15 and 30 min post-UV irradiation. This dose and this recovery period produce an average of one lesion per plasmid strand.[16] At the times indicated, genomic DNA was purified from each sample and digested with PvuII, which linearizes the plasmid just downstream of its origin of replication. The intermediate structures were then visualized by Southern blot analysis following separation in two-dimensional agarose gels. In this technique, nonreplicating plasmids migrate through the gel as a linear 4.4-kb fragment, forming a prominent large spot. Replicating plasmids are observed as Y-shaped structures that migrate more slowly through the gel (due to their larger size and nonlinear shape) and form an arc that extends out from the linear fragments (Fig. 3). Following UV irradiation, transient replication intermediates that have a double-Y or X-shaped structure are observed. The nonlinearity of these structures causes the intermediates to migrate even more slowly and to be observed as a cone region above the arc of replicating Y-structures.

In the absence of UV irradiation, only Y-shaped replication intermediates are observed. Following UV irradiation in wild-type cultures, however, both Y-shaped replication intermediates and cone region intermediates are observed to accumulate. Previous work has shown that a portion of the cone region intermediates is associated with a RecF-dependent processing of the replication fork prior to the time that replication resumes.[16] In cells without a functional copy of *recF*, the processing intermediates in the cone region are not observed, and replicating intermediates remain and accumulate as simple Y-shaped structures. When we examined each of the strains containing an altered *recF* gene, we observed that, in each case, the cone region intermediates were absent or greatly reduced (Fig. 3). This observation indicates that the ATP hydrolysis and dimerization dynamics of RecF are required for the accumulation of replication fork intermediates and for the replication fork processing that occurs following disruption. Unlike the previous assay where the stabilized RecF dimer (*recF* D303N) mutant retained some modest protective functions with respect to degradation at the replication fork, no increase in replication fork intermediates was observed relative to the other *recF* mutants. However, it is possible that a modest retention of activity, such as that seen with the *recF* D303N mutant's ability to protect the nascent DNA in the previous assay, would be below the level that could be detected by this type of assay.

The ABC ATPase activity of the RecF protein is required to reestablish the replication machinery and to resume DNA synthesis following DNA damage that blocks replication

Following the processing and restoration of the damaged region, survival requires the reestablishment of a functional replisome at, or proximal to, the site of disruption. This latter step fails to occur in the absence of RecF and can be monitored by adding radioactive nucleotide precursors to the media and following their rate of incorporation into the DNA.[15,19]

To examine DNA synthesis in *recF* point mutants, we monitored the overall DNA accumulation and the rate at which synthesis recovered following UV-induced DNA damage. To this end, duplicate aliquots of cultures grown in the presence of [^{14}C] thymine were pulse labeled with [^{3}H]thymidine for 2 min at various times following 30 J/m^2 UV irradiation or mock irradiation. In this way, the rate of DNA synthesis (^{3}H incorporation/2 min) and the overall DNA accumulation (^{14}C incorporation) at various times during the recovery period could be determined relative to those occurring in unirradiated cultures.

By this assay, the rate of DNA synthesis of irradiated wild-type cultures initially decreased by approximately 90%, but had recovered to a rate near preirradiation levels within 100 min postirradiation. Similarly, the overall accumulation of DNA in the irradiated cultures increased within this time to approach that of the unirradiated culture. By comparison, although the rate of synthesis in irradiated *recF* deletion strain cultures was reduced to a similar extent, the rate did not recover, and little further DNA was observed to accumulate within the time course examined (Fig. 4). When we examined the recovery of DNA synthesis in cultures containing the altered forms of *recF*, all mutants were severely impaired in their ability to resume DNA synthesis. Similar to the initial assay, the possible exception to this was observed for the *recF* D303N mutant, which stabilizes RecF in its dimeric form. In this mutant, we observed a very modest increase in the rate of synthesis over the time course of recovery. However, although the trend was repeatedly observed in individual assays, the overall amount of synthesis was not significant when compared to other *recF* point mutants.

Finally, the survival of each strain expressing an altered RecF protein was compared to wild-type cultures and strains deleted for *recF*. Fresh overnight cultures were spotted on LB agar plates at appropriate dilutions, exposed to UV at the indicated doses, and incubated at 37 °C overnight. Cells that survived

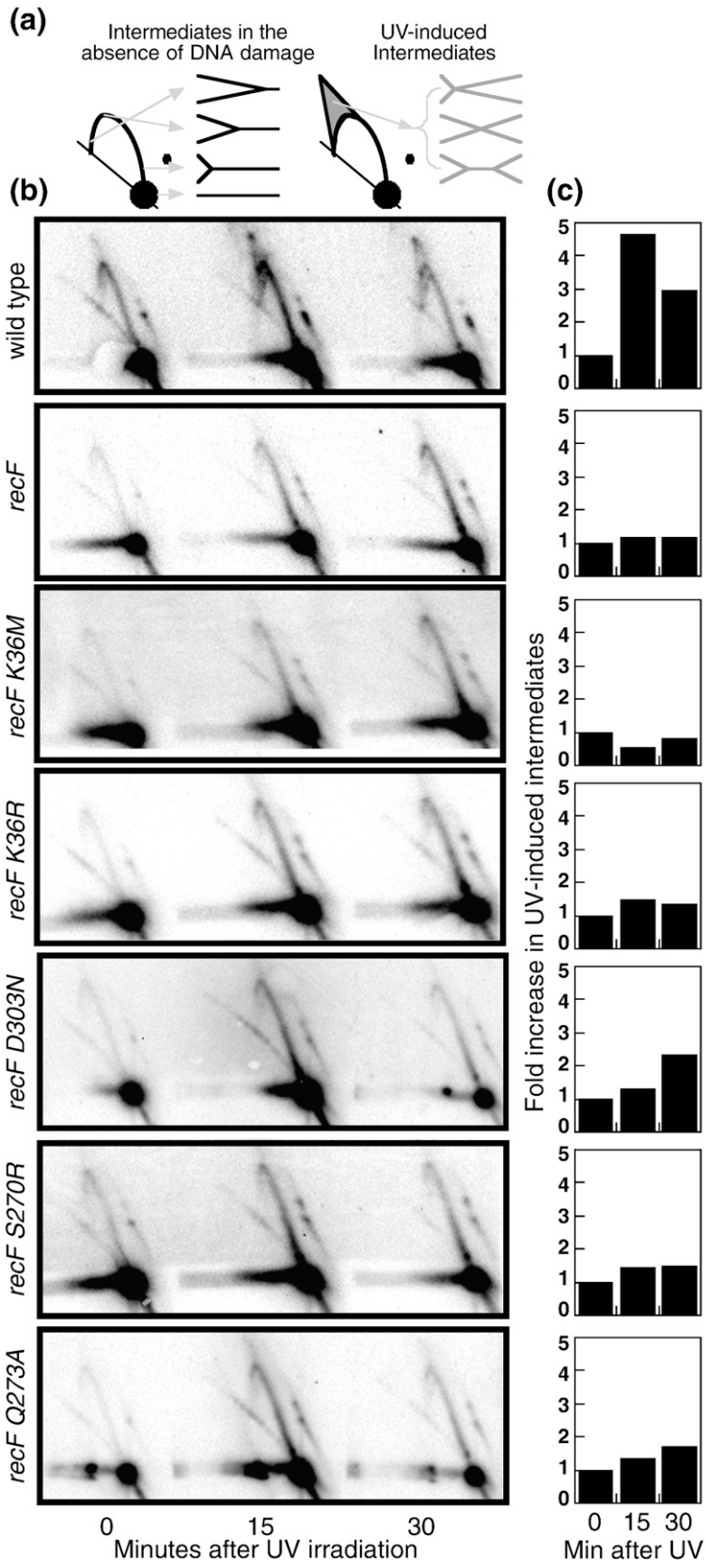

Fig. 3. Mutations that affect RecF protein dimerization (S270R, Q273A, and D303N), ATP binding (K36M), and ATP hydrolysis (K36R) are unable to process replication forks following disruption by UV-induced DNA damage. (a) Diagram depicting the replication intermediates observed in the absence or in the presence of UV-induced DNA damage. (b) Cells containing the plasmid pBR322 were exposed to 50 J/m^2 UV irradiation. At the indicated times, the genomic DNA was purified and digested with PvuII, and the structural intermediates for each strain were observed using a two-dimensional agarose gel. (c) The increase in UV-induced intermediates at each time point is plotted relative to time 0. UV-induced intermediates were quantified by determining the amount of radioactivity migrating in the cone region of UV-induced intermediates, normalized against the total amount of non-replicating linear DNA.

to form colonies were counted, and the fraction of surviving colonies was determined (Fig. 5).

Cells containing the *recF* K36M, K36R, S270R, or Q273A point mutations were as hypersensitive to UV irradiation as a mutant that was deleted for *recF*. By comparison, the *recF* D303N point mutant was approximately 1 order of magnitude more resistant to UV at each of the examined doses. Taken together

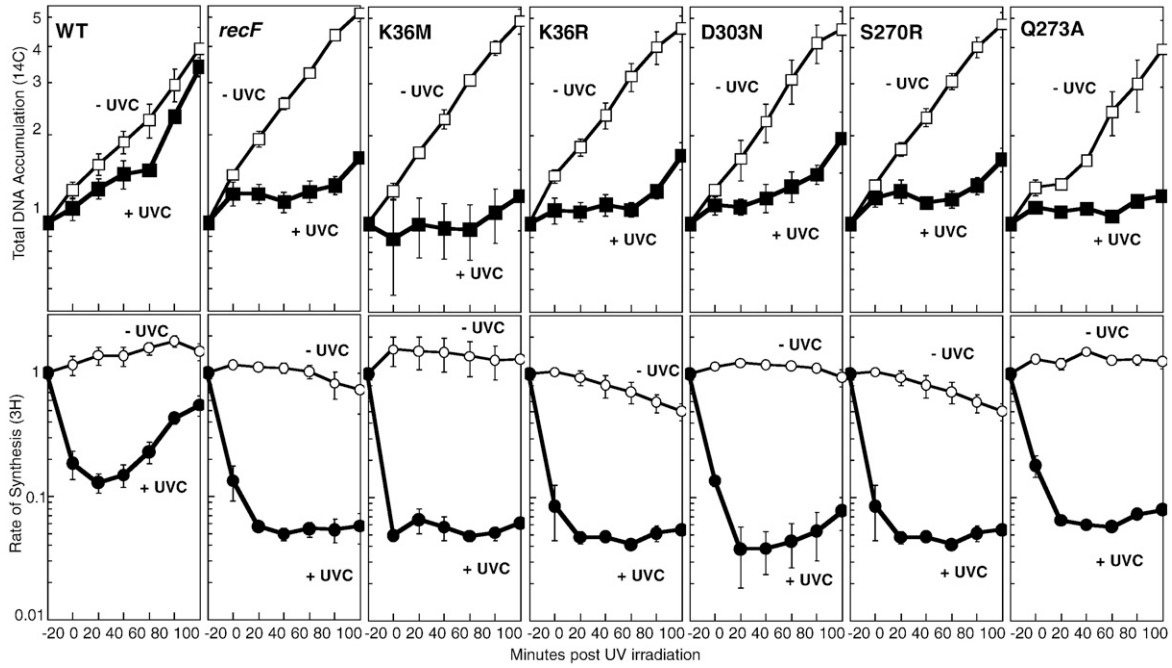

Fig. 4. RecF protein dimerization, ATP binding, and ATP hydrolysis are required to resume DNA synthesis following disruption by UV-induced DNA damage. [^{14}C]Thymine-labeled cultures were pulse labeled with [^{3}H]thymidine for 2 min at the indicated times following either 30 J/m^2 UV irradiation (filled symbols) or mock irradiation (open symbols) at time 0. The relative DNA accumulation (^{14}C; squares, top) and the rate of DNA synthesis (^{3}H; circles, bottom) are plotted. Each graph represents at least three independent experiments. Error bars represent the standard error of the mean.

with the previous assays, these results indicate that ATP binding and ATP hydrolysis, as well as the ability of RecF to form protein dimers, are essential for RecF function in processing and restoring replication after disruption by UV-induced damage. By contrast, although the effect was modest, the *recF* D303N mutant, which forms stable RecF protein dimers, appeared to retain some minimal activity relative to a *recF* deletion mutant with respect to protection of the nascent DNA of the disrupted fork, allowing DNA synthesis to resume and, ultimately, allowing cells to survive. The result would suggest that in this mutant, an initial round of RecF loading and interactions can occur; in a small fraction of cases, this may be sufficient to allow survival and recovery to occur. However, it also suggests that RecF dimerization and ATP hydrolysis involve repeated cycling *in vivo*, as has been observed to occur *in vitro* upon interaction with RecR.[21,31] This cycling appears to be important for full resistance and function *in vivo*.

Discussion

The RecF protein is required for survival following disruption of replication by DNA damage in a process that includes maintaining the structure of the replication fork DNA, protecting and processing the DNA ends at arrested replication forks to allow repair enzymes to access and repair the damage, and, finally, reestablishing an active replisome at the site of disruption.[15,16,18,42]

Here, we investigated how altering the ATP binding, ATP hydrolysis, and protein dimerization activities of RecF affect each of the progressive steps involved in restoring replication following the arrest of the replication fork. We initially hypothesized that one or more of these altered RecF proteins could retain the ability to bind and protect the replication fork, but fail to proceed further into the recovery process. Instead, we observed that ATP binding, ATP hydrolysis, protein dimerization, and, to a lesser extent, the ability of the protein dimers to dissociate upon ATP hydrolysis each rendered cells deficient in every step associated with the recovery of replication. The inability of these mutants to maintain the replication fork DNA or to limit nascent DNA processing argues that RecF is involved in catalyzing an early step in the recovery process, although it does not preclude the possibility that RecF could also participate in later steps of the recovery process.

Two possible initiating roles for the RecFOR complex in the recovery process are consistent with biochemical studies. *In vitro*, RecFOR is able to displace SSB from single-strand DNA to allow RecA to bind the filament and to initiate filament formation.[5,33,43,44] *In vivo*, the failure of *recF* deletion strains to maintain the replication fork DNA and to limit nascent DNA degradation supports this type of role, since DNA ends are rapidly degraded in the absence of RecA binding.[15,16,18] Similarly, *recF* deletion mutants exhibit a delayed induction of the SOS response, which requires loading RecA filaments onto single-strand DNA before activation can occur.[45–48]

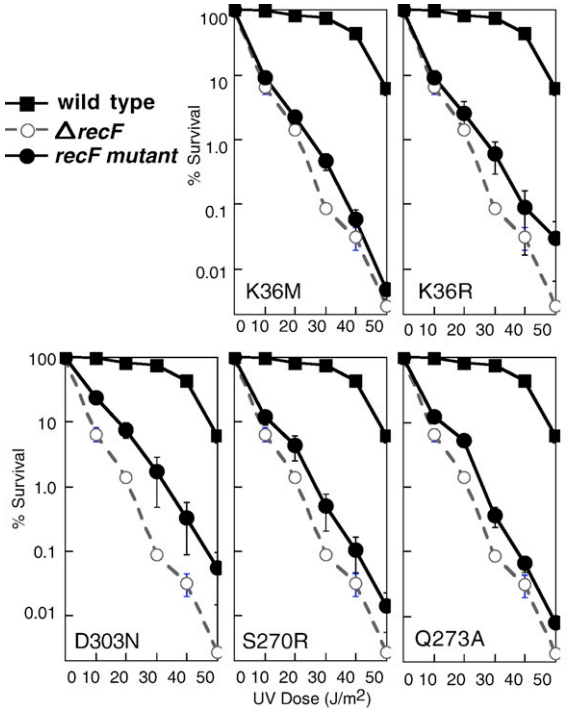

Fig. 5. RecF protein dimerization, ATP binding, and ATP hydrolysis are required for resistance to UV-induced DNA damage. The percentage of cells surviving the indicated doses of UV irradiation is plotted for the *recF* K36M, K36R, S270R, D303N, and Q273A (filled squares) point mutations, as indicated. Survival for wild type (filled squares) and survival for the *recF* deletion strain (open circles) are plotted on each graph for comparison. Survival curves represent at least three independent experiments. Error bars represent the standard error of the mean.

In addition to displacing SSB, other studies have shown that RecFOR, along with RecA, is capable of displacing a DNA polymerase from its template when it is arrested at a DNA lesion.[38] This type of activity may be critical to orienting RecA-catalyzed fork regression and allowing repair enzymes to gain access to the offending lesion. *In vivo*, after UV-induced damage, replication forks remain stalled in cells that lack the enzymes to repair or resect the nascent DNA away from the lesion.[19,20] In these cells, the recovery of DNA synthesis depends entirely on translesion synthesis by polymerase V, which is induced in active form only 50 min after SOS induction. It has also been reported that polymerase V function depends directly on RecF function, in addition to RecA function,[49] although other studies have reached different conclusions as to whether this effect is direct or indirect.[47,50,51]

In vitro, RecO and RecR interact and, at high concentrations, these proteins are able to displace SSB and to load RecA in the absence of RecF.[22,32,43,52] The presence of RecF appears to be important for reducing the protein concentrations required for this reaction to occur and for targeting the RecF/RecR complex to appropriate single-strand–double-strand DNA junctions, such as those found at arrested replication forks, although this targeting appears to occur through complex interactions with RecF protein partners.[5,21,31,34] In solution, *D. radiodurans* RecR forms a tetrameric clamp that is capable of encircling DNA and interacts with RecF in a 4:2 RecR/RecF stoichiometry.[21,53] RecF ATP-dependent dimers form a clam-like structure whose mouth is capable of accommodating single-strand or double-strand DNA.[8] Thus, the ATP-dependent interaction of RecF and RecR suggests a role for RecF in the initial steps of damage recognition. Interestingly, Makharashvili *et al.* recently demonstrated the ATPase-dependent selectivity of the RecF/RecR complex for double-strand DNA substrates.[21] Thus, RecF may provide specificity for initial placement of RecA loading at the boundaries of single-strand and double-strand DNA, and it may be important for limiting RecA filament formation within single-strand DNA regions.[5,22,31,34,54] The observation that RecF D303N mutants, stabilized in their dimeric form, partially protect nascent DNA at arrested replication forks may also be consistent with the idea that the dimeric clam-like structure is critical and could play a more dynamic role in later steps of the recovery process. Although the purified D303N RecF protein dimer did not display any residual ATPase activity, we cannot rule out the possibility that it may retain some ability to hydrolyze ATP when in the presence of other protein partners such as RecR.[8,21,26]

RecF displays a strong degree of structural similarity to the human Rad50 protein and has homology to several eukaryotic SMC proteins whose biochemical functions remain poorly understood but are critical for maintaining genome stability and resistance to DNA damage.[1–4,6,7,55] Taken together, the results presented here support the idea that RecF plays an initiating role in the process by which disrupted replication forks are processed and restored. Furthermore, they demonstrate that ATP binding and ATP hydrolysis, as well as RecF dimer formation and dissociation, are critical activities that allow the RecF protein to initiate the recovery process *in vivo*. The conservation between RecF and Rad50 provides an opportunity to use structure-guided biochemical and cellular approaches with RecF to dissect the mechanism by which this important class of proteins functions in both prokaryotes and eukaryotes.

Experimental Procedures

Bacterial strains

SR108 is a *thyA36 deoC2* derivative of W3110.[56] CL579 (*recF6206*::*tet857*) has been previously described.[16]

Gene replacements of *recF* were constructed using the recombineering strain DY329.[57] A cat-sacB cassette was PCR amplified from plasmid pEL04[58] using primers 5′ CGGCTTATGTTGTCATGCCAATGAGACTGT-AATGTCCCTCCCTGTGACGGAAGATCACTTCG3′ and 5′CATCAACGTTTCTCGCTCATTTATACTTGGG-

TTAATCCGTCTGAGGTTCTTATGGCTCTTG3′. The PCR product was then transformed into DY329 to generate CL1204 (*recF*::cat-sacB) selecting for chloramphenicol resistance. The kanamycin resistance cassette was then inserted into the region downstream of *tnaA* in this strain using primers 5′CACTTCACCGCAAAACTTAAAGAAGTTTAATTAATACTACTATGGACAGCAAGCGAACCG3′ and 5′TAGAGGAAGGCTATTTTTGTTATTGAGGATGTAGGGTAAGTCAGAAGAACTCGTCAAGAAG3′ to amplify the kan^R cassette from Tn*5*. The PCR product was transformed into CL1204 to generate CL1206 (*recF*::cat-sacB *tna*::kan).

Point mutations were initially generated using the Quick-change II site-directed mutagenesis kit (Stratagene) on plasmid pMalF6 that had the *recF* gene cloned into the BamHI site, as previously reported.[8] Gene replacements on the chromosome were then made by amplifying the *recF* point mutations from the plasmids using primers 5′ GCCAGAGCGCGGCTTATGTTGTCATGCCAATGAGACTGTAATGTCCCTCACCCGCTTGTTG and 5′ AGAATTCGACATCAACGTTTCTCGCTCATTTATACTTGGGTTAATCCGTTATTTTACCCTT. The products were then transformed into CL1206 to generate CL1375 (*recF*K36M *tna*::kan), CL1377 (*recF*K36R *tna*::kan), CL1379 (*recF*S270R *tna*::kan), CL1402 (*recF*D303N *tna*::kan), and CL1535 (*recF*Q273A *tna*::kan) by selecting for sucrose resistance. The point mutations were then moved into our parental background SR108 by P1 transduction and by selecting for kanamycin resistance to generate CL1412 (*recF*(K36M *tna*::kan), CL1414 (*recF*(K36R) *tna*::kan), CL1416 (*recF*(S270R) *tna*::kan), CL1418 (*recF*(D303N) *tna*::kan), and CL1569 (*recF*(Q273A) *tna*::kan). In each case, the presence of the *recF* mutation conferred hypersensitivity to UV irradiation.

Degradation of nascent DNA

Fresh overnight cultures were diluted 1:100 and grown in Davis medium supplemented with 0.4% glucose, 0.2% casamino acid, 10 μg/mL thymine (DGCthy), and [^{14}C] thymine (0.1 μCi/10 μg/mL) to an OD_{600} of 0.4 in a 37 °C shaking water bath. Cultures were then pulse labeled with [^{3}H]thymidine (1 μCi/10 μg/mL) for 5 s, filtered on Whatman 0.4-μm membrane filters, and washed twice with 3 mL of cold NET buffer [100 mM NaCl, 10 mM Tris (pH 8.0), and 1 mM ethylenediaminetetraacetic acid]. The filter was then resuspended in prewarmed nonradioactive DGCthy media, immediately UV irradiated with 30 J/m^2 (Sylvania 15-W germicidal lamp, 254 nm, 0.9 J/m^2/s incident dose), and incubated in a 37 °C shaking water bath. At the times indicated, duplicate 200-μL aliquots of the culture (triplicate at time 0) were taken. Cells were lysed, and the DNA was precipitated by addition of 5 mL of ice-cold 5% trichloroacetic acid and then collected on Fisherbrand 2.5-cm glass fiber filters. The amount of radioactivity in each filter was determined using a liquid scintillation counter.

Two-dimensional agarose gel electrophoresis

The two-dimensional gel electrophoresis technique for *E. coli* containing pBR322 has been described previously.[16] Briefly, fresh cultures containing the pBR322 plasmid were diluted 1:100 in 10 mL of DGCthy medium and grown to an OD_{600} of 0.4 at 37 °C. The cultures were then irradiated with 50 J/m^2 UV irradiation. Aliquots (750 μL) of the irradiated cultures were taken at 0, 15, and 30 min after UV irradiation, and placed in 750 μL of ice-cold NET buffer. The cells were then pelleted in a microcentrifuge and lysed in 140 μL of TE buffer [10 mM Tris (pH 8.0) and 1 mM ethylenediaminetetraacetic acid] containing 2 mg/mL lysozyme and 100 μg/mL RNase A and incubated at 37 °C for 30 min. Then, 10 μL each of 10 mg/mL proteinase K and 20% Sarkosyl was added to the suspension and incubated at 37 °C for another 30 min. Following extraction with 4 vol of phenol/chloroform, samples were dialyzed for 2 h in TE buffer on 47-mm Whatman 0.05-μm pore disks (VMWP04700; Whatman) floating on a 250-mL beaker of TE buffer. The samples were then digested with PvuII (Fermentas) and extracted with chloroform before being loaded onto an agarose gel. Samples were run in a 0.4% agarose gel with 1× TBE [90 mm Tris-borate, 2 mm ethylenediaminetetraacetic acid (pH 8.0)] buffer for 17 h at 25 V. For the second dimension, the gel lanes were sliced out, rotated by 90°, recast in a 1.0% agarose gel with 1× TBE buffer, and run for 7 h at 200 V. Gels were transferred to Hybond N$^+$ nylon membranes and probed with pBR322 that had been labeled with ^{32}P by nick translation according to the protocol supplied by Roche using α-[^{32}P]dCTP (MP Biomedicals). Radioactivity was visualized and quantified using a Storm 820 Phosphorimager and ImageQuant software (Molecular Dynamics).

Recovery of DNA synthesis

Fresh overnight cultures were diluted 1:100 in 20 mL of DGCthy medium supplemented with [^{14}C]thymine (0.1 μCi/10 μg/mL) and grown to an OD_{600} of 0.3 in a 37 °C shaking water bath, at which time the culture was split. Half of the culture was irradiated with 30 J/m^2 UV, and the other half was mock irradiated. At the times indicated, duplicate 0.5-mL aliquots of the culture were pulse labeled with [^{3}H]thymidine (1 μCi/10 μg/mL) for 2 min. The cells were then lysed, and the DNA was precipitated by addition of 5 mL of ice-cold 5% trichloroacetic acid. The precipitate was collected on Fisherbrand 2.5-cm glass fiber filters, and the amount of radioactivity in each sample was measured using a liquid scintillation counter.

UV survival

Fresh overnight cultures were diluted 1:100 and grown in DGCthy medium to an OD_{600} of 0.4 in a 37 °C shaking water bath. At this time, the cultures were serially diluted and plated in triplicate on LB plates supplemented with 10 μg/mL thymine and irradiated on a rotary platform at the indicated doses. Plates were incubated overnight at 37 °C, and colonies were counted on the next day to determine the surviving fraction.

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