RecA Protein Recruits Structural Maintenance of Chromosomes (SMC)-like RecN Protein to DNA Double-strand Breaks*§

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Background: RecN is an SMC (structural maintenance of chromosomes) family protein that is required for DNA double-strand break (DSB) repair. Previous studies show that GFP-RecN forms nucleoid-associated foci in response to DNA damage, but the mechanism by which RecN is recruited to the nucleoid is unknown. Here, we show that the assembly of GFP-RecN foci on the nucleoid in response to DNA damage involves a functional interaction between RecN and RecA. A novel RecA allele identified in this work, recAQ300R, is proficient in SOS induction and repair of UV-induced DNA damage, but is deficient in repair of mitomycin C (MMC)-induced DNA damage. Cells carrying recAQ300R fail to recruit RecN to DSBs and accumulate fragmented chromosomes after exposure to MMC. The ATPase-deficient RecNK35A binds and forms foci at MMC-induced DSBs, but is not released from the MMC-induced DNA lesions, resulting in a defect in homologous recombination-dependent DSB repair. These data suggest that RecN plays a crucial role in homologous recombination-dependent DSB repair and that it is required upstream of RecA-mediated strand exchange.

DNA double-strand breaks (DSBs)3 are serious genomic lesions that are potentially lethal at the cellular level. DSBs are caused by exogenous agents such as ionizing radiation, chemical mutagens, reactive oxygen species, and replicative stress (i.e. collapsed replication forks) (1, 2). In bacteria, homologous recombination (HR) plays a major role in repairing DSBs. HR enzymes and pathways have been extensively characterized in Escherichia coli, and E. coli HR is a paradigm for understanding HR-related processes in all organisms (3–5).

In E. coli, the repair of DSBs is initiated by RecBCD, which generates 3’ single-stranded DNA (ssDNA) tails at DSB sites via its helicase and nuclease activities; the ssDNA tails are then substrates for homologous pairing by RecA protein (4, 6). The RecF pathway is involved in the daughter strand gap repair in wild-type cells (3). RecF also provides an alternative pathway for HR-dependent DSB repair in recBC mutants when two additional nucleases, ExoI and SbcCD, are inactivated (7, 8).

Significance: RecN is critical for protecting the structural integrity of chromosomes during DSBs repair.

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3 The abbreviations used are: DSB, DNA double-strand break; HR, homologous recombination, MMC, mitomycin C, SMC, structural maintenance of chromosomes; ECFP, enhanced cyan fluorescent protein.
dependent repair of DSBs (31–33). Thus, RecN plays a specific role in the repair of DNA DSBs, and its role is not limited to a single branch or subpathway of HR.

In this study, we examine the mechanism by which RecN is recruited to the nucleoid in response to DNA damage. We show that a functional interaction between RecN and RecA is required for assembly of RecN foci at MMC-induced DSBs; conversely, conditions that abrogate or disrupt a stable RecN-RecA interaction lead to chromosome fragmentation and loss of cell viability in cells exposed to MMC. The RecN ATPase is not required for formation of RecN-DSB foci, but is required for release of RecN from DSBs and completion of RecA/HR-dependent DSB repair. These data demonstrate that the SMC-like protein RecN plays a crucial role in promoting RecA-dependent DSB repair.

EXPERIMENTAL PROCEDURES

Media and General Methods—Standard methods for E. coli genetics and recombinant DNA techniques were as described by Miller (34) and Sambrook et al. (35). Ampicillin (50 µg/ml), tetracycline (10 µg/ml), chloramphenicol (100 µg/ml), and kanamycin (30 µg/ml) were used where indicated. Mitomycin C (2.5 mg/ml) was dissolved in 10 mM Tris-HCl (pH 8.5) buffer. Sensitivity to UV damage was measured as described previously (36). To measure sensitivity to MMC, cultures were grown in LB broth to an A560 of ~0.4, serially diluted, spotted onto LB medium containing the indicated concentration of MMC, and incubated at 37 °C.

Bacterial Strains and Plasmids—Strains used in this study were isogenic with BW25141 (37) except for strains with PβBAD-1-SceI. Wild-type strains and deletion mutants were provided by the National BioResource Project (NBRP) (38). The strains carrying the inducible fluorescent repressor gene araC PBAD-lacI-ecfp (40, 42, 43). Cells were grown at 37 °C for 90 min in LB medium containing 0.2% arabinose and 1 µg/ml MMC and then analyzed by fluorescence microscopy.

RESULTS

RecA Is Required for the Formation of Nucleoid-associated GFP-RecN Foci in MMC-treated Cells—When DNA is damaged or replication is inhibited, ssDNA-bound RecA becomes conformationally active and promotes cleavage of the LexA repressor, which results in the induction of SOS genes including recN (21, 44). Previously, we showed that GFP-RecN formed foci on nucleoids after DNA damage (27). This implied that RecN could be recruited to the nucleoid at a step after RecA is loaded onto damaged DNA. To specifically examine this sequence of events, we measured GFP-RecN foci in a ΔrecA strain. Wild-type recN is a part of the SOS regulon, and its expression is completely dependent on activated RecA. Therefore, this experiment was performed in cells that expressed GFP-recN under control of the inducible PBAD promoter. Fig. 1A shows that arabinose-inducible GFP-recN fully complements the MMC sensitivity of ΔrecN, when cells are grown in the presence of arabinose, but not when cells are grown in the presence of glucose. Furthermore, GFP-RecN foci form in the cytoplasm of both MMC-treated and untreated wild-type cells and ΔrecA cells, whereas nucleoid-associated GFP-RecN foci form in wild-type MMC-treated cells but are absent in ΔrecA.
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**FIGURE 1. RecN foci in wild-type and ΔrecA cells with or without DNA damage.** A, ΔrecN cells carrying either an arabinose-inducible GFP-recN gene (pTF271) or a pBAD vector (pTF200) were diluted and spotted onto LB plates with or without MMC (0.5 μg/ml) in the presence of either glucose or arabinose. B, the subcellular localization of GFP-RecN foci in response to MMC-induced damage. Wild-type or ΔrecA cells carrying pTF271 were exposed to MMC (0.5 μg/ml) followed by the addition of arabinose (0.05%, w/v) to induce GFP-RecN. The panels show GFP/DAPI-merged images of cells 30 min after the addition of arabinose. Scale bar indicates 2.5 μm. C, quantitative analysis of GFP-RecN foci. For cells incubated with or without MMC, ~150 cells were examined. The results represent the average of at least three independent measurements. Error bars indicate S.D.

**FIGURE 2. Effect of recA<sup>Q300R</sup> on DNA repair and SOS response.** A, sensitivity of cells to MMC and UV irradiation. 10-fold serial dilutions of the indicated strains were spotted on LB plates. DNA damage was induced by either MMC or UV irradiation. B, Q300 is conserved in bacterial RecA orthologs. A map of the E. coli RecA region between amino acids 290 and 305 is shown. Ec, E. coli; Hi, H. influenzae; Bs, B. subtilis; Tt, Thermus thermophilus; Dr, D. radiodurans. C, sensitivity of recA<sup>Q300R</sup> cells to MMC and UV irradiation was examined as in A. D, recA<sup>Q300R</sup> is proficient in SOS induction. Protein extracts from cells treated with MMC for the indicated times were prepared and analyzed by Western blot using anti-RecA, anti-RecN, or anti-LexA antibodies. For the LexA degradation assay, chloramphenicol (100 μg/ml) was added at time 0 to inhibit resynthesis of LexA protein.

**Isolation of a recA Mutant That Mimics the Phenotype of ΔrecN**—Although RecA plays the central role in recombinational repair and is the master inducer of the SOS pathway, it is unclear what functions of RecA are required to recruit RecN to the nucleoid in MMC-treated cells. The phenotype of ΔrecA or SOS-deficient lexA3 cells (45) differs from the phenotype of ΔrecN cells; the former are hypersensitive to MMC and UV, whereas the latter are hypersensitive to MMC but insensitive to UV (Fig. 2A). Therefore, a library of recA mutants was generated and screened for mutants that provide resistance to UV but confer sensitivity to MMC. Three candidate mutants were isolated from ~2,000 clones. All three mutants carry an arginine substitution at the highly conserved C-terminal Gln-300 of RecA (Fig. 2B). Fig. 2C shows that this allele, recA<sup>Q300R</sup>, fully complements the UV sensitivity of ΔrecA cells, but does not complement their MMC sensitivity. In the wild-type strain, MMC-induced DNA damage leads to proteolytic cleavage of LexA, the repressor of the SOS regulon, and induces the SOS response (Fig. 2D). Similarly, recA<sup>Q300R</sup> is capable of inducing expression of the SOS regulon and specific proteolytic cleavage of LexA in MMC-treated cells (Fig. 2D). Thus, recA<sup>Q300R</sup> is proficient in the DNA damage-induced SOS response, and its phenotype is similar to the phenotype of ΔrecN.

**Nucleoid Fragmentation in MMC-treated ΔrecN and recA<sup>Q300R</sup> Cells**—To explore these results further, ΔrecA, recA<sup>Q300R</sup>, ΔrecN, and ΔruvB cells were stained with DAPI and examined by fluorescence and phase-contrast microscopy for genome integrity and cell morphology. Under conditions of exponential growth in the absence of MMC, all cells had a normal morphology, with two centrally located nucleoids per cell (Fig. 3A). Wild-type cells treated with MMC for 90 min became highly filamented with elongated, evenly spaced nucleoids (Fig. 3A). This morphology is typical of SOS-activated cells (46). By contrast, a large fraction of MMC-treated SOS-defective ΔrecA were anucleate, and filamentous cells were hardly detected (Fig. 3A). RuvABC resolvasome branch-migrates and resolves Holliday junctions, and inactivation of any of the three Ruv functions blocks resolution of recombinational repair intermediates (3). Therefore, RuvB plays a role in the later steps of HR. MMC-treated ruvB mutants formed both filamentous and anucleate cells. As reported previously (47), the nucleoids of filamentous ruvB cells were centrally located and little to no DNA migrated to cell poles, which is in contrast to the morphology of filamentous wild-type cells (i.e. well partitioned nucleoids). This indicates that the accumulation of intermediates of HR-mediated DSB repair results in chromosome nondisjunction and the production of anucleate cells. MMC-treated ΔrecN and recA<sup>Q300R</sup>...
cells were as filamentous as wild-type cells, but had an abnormal morphology characterized by multiple, short, diffuse nucleoids (Fig. 3A). In wild-type cells, the number of nucleoids per cell was largely unaffected by exposure to MMC, whereas the number of nucleoids per cell increased when \( \Delta recN \) and \( \Delta recA^{Q300R} \) cells were exposed to MMC (Fig. 3B). Furthermore, abnormal nucleoid morphology was not generally observed in UV-irradiated \( \Delta recN \) and \( \Delta recA^{Q300R} \) cells (supplemental Fig. S1). These results support the conclusion that \( \Delta recA^{Q300R} \) is a phenocopy of \( \Delta recN \). Our interpretation of this result is that RecN is dysfunctional in the \( \Delta recA^{Q300R} \) mutant.

We hypothesized that the abnormal nucleoid morphology of MMC-treated \( \Delta recN \) cells might reflect the presence of unrepairred DSBs and chromosome fragmentation. Therefore, a fluorescence-based method was used to visualize chromosome fragments. For this purpose, oriC was labeled indirectly, via LacI-ECP (enhanced cyan fluorescent protein) bound to an ectopic tandem array of Lac repressor-binding sites (240\( \times \) lacO) at ori1 (15 kb counterclockwise of oriC). The panels show merged images of LacI-ECP (light blue) and nucleoids (dark blue). White arrows indicate nucleoids that fail to bind LacI-ECP, and by implication, oriC-lacking nucleoids. D, quantification of cells lacking ori1 foci. Wild-type and \( \Delta recN \) cells were treated with MMC for 90 min and examined by fluorescence microscopy. For cells with or without MMC-induced damage, \( \geq 200 \) cells were examined.

FIGURE 3. Morphological changes in MMC-treated wild-type, \( \Delta recN \), and \( \Delta recA \) cells. Exponentially growing cells were fixed and stained with DAPI and analyzed by fluorescence microscopy. A, the panels show DAPI images of cells incubated for 90 min in the presence or absence of MMC (1 \( \mu \)g/ml). Nucleoids are visualized as a light blue color. B, quantitative analysis of nucleoids. For cells with or without MMC-induced DNA damage, \( \geq 200 \) cells were examined. The results represent the average of at least three independent measurements. Error bars indicate S.D. C, localization of a LacI-ECP to the nucleoid. The wild-type and \( \Delta recN \) strains carry an ectopic tandem array of lacO at ori1 (15 kb counterclockwise of oriC). The panels show merged images of LacI-ECP (light blue) and nucleoids (dark blue). White arrows indicate nucleoids that fail to bind LacI-ECP, and by implication, oriC-lacking nucleoids. D, quantification of cells lacking ori1 foci. Wild-type and \( \Delta recN \) cells were treated with MMC for 90 min and examined by fluorescence microscopy. For cells with or without MMC-induced damage, \( \geq 200 \) cells were examined.
in wild-type cells (<1.4%) (Fig. 3D). These results demonstrate the presence of aberrant nucleoids lacking oriC in MMC-treated ΔrecN cells, which likely represent subchromosomal fragments.

RecAQ300R Is Defective in Recruiting RecN to Nucleoids in MMC-treated Cells—The results described above suggest that RecAQ300R does not recruit RecN to the nucleoid, under conditions where wild-type RecA does so (i.e. in MMC-treated wild-type cells). To explore this further, GFP-RecN foci were quantified in MMC-treated ΔrecA ΔrecN cells expressing SOS-inducible GFP-recN and either wild-type recA or recAQ300R. After exposure to MMC for 60 min, >90% of wild-type cells contained nucleoid-associated GFP-RecN foci (Fig. 4, A and B). By contrast, <5% of cells expressing recAQ300R had nucleoid-associated GFP-RecN foci, whereas the number and fraction of cells with cytoplasmic GFP-RecN foci was higher in cells expressing recAQ300R than that in cells expressing wild-type recA (Fig. 4, A and B). These results indicate that RecA is required for the formation of MMC-induced, nucleoid-associated RecN foci and that RecAQ300R has a specific defect in this function/role.

RecA Is Required to Recruit RecN to sites of DSBs—To examine the recruitment of RecN to a unique DSB site in RecA-proficient cells, I-SceI was used to introduce a site-specific DSB into a strain that carries the pBAD-I-SceI cassette on the chromosome and a single I-SceI recognition site on the F′ episome (39). Appropriately engineered cells were transformed with a plasmid expressing GFP-recN from its native SOS-inducible promoter, grown to early log phase and exposed to arabinose to induce I-SceI. Control cells were grown in medium lacking arabinose. VspI endonuclease digestion resulted in a 1.8-kb fragment containing the I-SceI cleavage site. I-SceI digestion produced two fragments, one of which with a size of 1.2 kb hybridized to the site 1 probe (Fig. 5A). The kinetics of DSB formation was monitored by Southern blot analysis of VspI-digested DNA isolated from samples taken at different times after the addition of 0.2% arabinose or glucose to the culture. A 1.2-kb fragment was not detected when cells were maintained in glucose-containing medium, whereas it was detected within 30 min in wild-type cells proficient for RecBCD after the addition of arabinose (Fig. 5, A and B). The intensity of the 1.2-kb fragments increased with time, reaching a maximum intensity ~1 h after the addition of arabinose. Similar results were obtained when the site 2 probe was used to detect the I-SceI cleavage site (Fig. 5A and supplemental Fig. S2). One possible explanation for the kinetics of DSB formation is that I-SceI digestion is not synchronous in the entire population, and the breaks may be repaired very efficiently. Thus, the only breaks generated just before samples were taken might be detected by Southern blotting. This is consistent with previous studies using chromosomally integrated I-SceI site, where DSB products are readily detected in wild-type cells even after 1 h of I-SceI induction (48).

Fig. 5C shows that nucleoid-associated GFP-RecN foci were detected in cells that expressed I-SceI and carried an F′ episome with an I-SceI cleavage site. By contrast, GFP-RecN foci were not observed when the same cells were grown in glucose-containing medium (to repress I-SceI) or if the cells did not carry an I-SceI-sensitive F′ episome (Fig. 5C). Furthermore, the number of nucleoid-associated GFP-RecN foci was much lower in recAQ300R mutant cells (<1%) than in cells expressing wild-type recA (18%) (Fig. 5D). These results indicate that, in wild-type cells, a single I-SceI-induced DSB induces an SOS response and promotes the formation of nucleoid-associated GFP-RecN foci in a RecA-dependent manner.

RecN ATPase Activity Is Required for Release from Growth Arrest in Cells with DNA Damage—RecN has a typical SMC family protein domain structure, including an extensive, centrally located coiled-coil domain and globular N- and C-terminal domains with Walker A and Walker B nucleotide-binding motifs, respectively (49). A previous study showed that substitution of Lys-35 with alanine in the Walker A motif resulted in a complete loss of RecN DNA repair activity in vivo (50). Biochemical characterization of Deinococcus radiodurans RecN showed that RecNK35A (an lysine-to-alanine substitution at position 67, which corresponds to E. coli RecN Lys-35) abolished ATPase activity, but did not impair ATP binding in vitro (49). Fig. 6A shows that expression of recNK35A in an ΔrecN background conferred sensitivity to MMC that was equivalent to that of ΔrecN. The overproduction of RecNK35A rendered wild-type cells sensitive to MMC (Fig. 6A), demonstrating that recNK35A is a dominant-negative allele of recN. GFP-RecNK35A formed nucleoid-associated foci in >80% of MMC-treated cells, and these foci failed to form in cells expressing recAQ300R (Fig. 6B). This result indicates that the ATPase activity of RecN is not required for formation of nucleoid-associated RecN foci. However, ΔrecN cells expressing wild-type GFP-recN resumed normal cell growth, and nucleoid-associated GFP-RecN foci dissociated after exposure to MMC was terminated (Fig. 6C). By contrast, ΔrecN cells expressing GFP-recNK35A became highly filamented, acquired fragmented nucleoid structures, and retained GFP-RecNK35A foci for 2 h after exposure to MMC was terminated (Fig. 6C). These results demonstrate that ATPase-defective RecNK35A is recruited to sites of DNA damage, but may not be properly released because of the defects in HR-mediated repair of MMC-induced DSBs.
DISCUSSION

Previous studies demonstrate that RecN protein forms both nucleoid-associated and cytoplasmic foci in cells exposed to DSB-inducing agents and that cytoplasmic RecN aggregates are degraded by the ClpXP protease (27). Here, we demonstrate that RecN is recruited to nucleoids in a RecA-dependent manner. We characterize a novel recA allele, recA\textsuperscript{Q300R}, which promotes expression of SOS-inducible genes but does not promote formation of nucleoid-associated RecN foci. RecN accumulates at a unique I-Scel-induced DSB in wild-type recA cells but not in recA\textsuperscript{Q300R} cells. Thus, we conclude that RecA plays an essential role in DNA damage-induced expression of recN and the assembly of RecN foci at the sites of DSBs. ATPase-deficient recN\textsuperscript{K35A} mutants are proficient in forming nucleoid-associated foci at DSBs, but fail to resume growth after release from MMC-induced cell-cycle arrest. This results in highly filamented cells with nucleoid-associated GFP-RecN\textsuperscript{K35A} foci and fragmented nucleoid structures. One possible explanation for the presence of persistent foci associated with damaged DNA in recN\textsuperscript{K35A} cells is that RecN\textsuperscript{K35A} is recruited to DSBs, but is not released from DSB sites because it lacks ATPase activity; under such conditions, mutant RecN\textsuperscript{K35A} DNA damage foci persist and accumulate, which interferes with RecA-mediated synaptic steps in the HR pathway.

This study also reveals that ΔrecN and recA\textsuperscript{Q300R} cells are hypersensitive to MMC but not to UV. A previous study showed that cells expressing recADΔC17 (a deletion mutant lacking residues 336–352) are hypersensitive to MMC but not to UV (51). Here, we confirm that recADΔC17 cells are sensitive to MMC, although they are less sensitive than ΔrecN and...
recAQ300R cells (supplemental Fig. S3). We found that nucleoid-associated GFP-RecN foci form normally in MMC-treated recA/H9004C17 cells (supplemental Fig. S3), indicating that the defects in the response to MMC in recAQ300R cells are not a result of a dysfunctional RecA C-terminal domain. However, it still remains possible that the C-terminal region of RecA plays a role in modulating RecN function at a later step in the repair/response to MMC-induced DSBs.

Previous studies suggest that the SOS response plays a critical role in DSB repair in E. coli but not in Bacillus subtilis (52). Indeed, unlike in E. coli, the expression of B. subtilis RecN appears to be SOS-independent (53), and GFP-B. subtilis RecN foci associate with DSBs before RecA is recruited to the DNA lesion (10). By contrast, E. coli recN is typical of SOS-regulated genes in that its expression is tightly repressed in unstressed cells. This suggests that E. coli RecN participates in HR repair of DSBs after RecA senses DNA damage. Consistent with this, the present study indicates that RecA actively recruits RecN to DSBs. These results may reflect species-specific attributes of E. coli and B. subtilis HR pathways. The purified B. subtilis RecN (and also D. radiodurans RecN) binds to DNA and has DNA-stimulated ATPase activity in vitro (54, 55). Unfortunately, it has been difficult to purify E. coli RecN because it is relatively insoluble and highly susceptible to degradation (data not shown). A recent study showed that Haemophilus influenzae RecN can be purified to near homogeneity and is fully functional in E. coli (50). Purified Haemophilus influenzae RecN does not bind DNA, and DNA had no significant effect on Haemophilus influenzae RecN ATPase activity in vitro, which contrasts with the activity of B. subtilis RecN. This observation supports our conclusion that E. coli RecN is recruited to DSBs through its interaction with RecA. Thus, the difference in the DNA binding specificities of RecN orthologs may explain their different affinities for their respective bacterial nucleoids. How-

**FIGURE 6.** RecNK35A is deficient in HR-mediated recovery after exposure to MMC. A, sensitivity of cells to MMC. The indicated strains were grown in LB. Cells were diluted and spotted onto LB with or without MMC (0.5 μg/ml). B, subcellular localization of GFP-RecNK35A. The panels show GFP/DAPI images of ΔrecN cells containing SOS-inducible GFP-recNK35A after 30 min of incubation in the presence of MMC. Quantitative analysis of GFP-RecNK35A foci is shown to the right. The results represent the average of at least three independent measurements. C, wild-type and recNK35A cells were treated with MMC for 10 min and then transferred to MMC-free medium (t = 0). At the indicated time points, cells were analyzed for the presence of RecN foci. The right panels show the GFP/DAPI images of cells at the indicated time after transfer to MMC-free media. Scale bar indicates 2.5 μm. Error bars indicate S.D.
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ever, our data do not exclude the possibility that E. coli RecN has DNA binding activity. It is also conceivable that RecA facilitates the binding and/or retention of RecN on damaged DNA.

SMC proteins are highly conserved ATPas whose role in higher order chromosome organization and dynamics is conserved from bacteria to humans. DSBs are one of the most cytotoxic forms of DNA damage, and therefore, the repair of DSBs is crucial for cell survival and for maintaining the integrity of the genome. In this study, we provide evidence that the recruitment of RecN to DSBs requires interaction with RecA. Any defect in the interaction results in chromosomal fragmentation, such as that observed in cells exposed to the DSB-inducing agent MMC. Based on these results and implications, we propose a mechanism by which RecN promotes RecA-dependent DSB repair. The initial presynaptic step of the DNA strand exchange reaction is formation of a RecA-ssDNA nucleoprotein filament. RecA-dependent recruitment of SMC-like RecN to DSBs follows, serving a scaffolding function to facilitate subsequent search by RecA for homologous templates in the segregated sister chromatids. Lastly, RecA mediates strand exchange. This model might be compatible with the recN studies in B. subtilis; here, we allow for the fact that RecN plays a role in an early step of DSB repair and that the mechanism by which RecN is recruited to DSBs differs.

In future studies, it will be interesting to investigate how RecN SMC complexes actually promote RecA-dependent DSB repair. Therefore, novel integrated biochemical and structural approaches to examine this and other questions concerning the roles of RecN and RecA will be required. The results of such studies should advance our understanding of the mechanism of DSB repair in prokaryotic and eukaryotic cells.

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