Degradation of *Escherichia coli* RecN Aggregates by ClpXP Protease and Its Implications for DNA Damage Tolerance*

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Protein degradation in bacteria plays a dynamic and critical role in the cellular response to environmental stimuli such as heat shock and DNA damage and in removing damaged proteins or protein aggregates. *Escherichia coli* recN is a member of the structural maintenance of chromosomes family and is required for DNA double strand break (DSB) repair. This study shows that RecN protein has a short half-life and its degradation is dependent on the cytoplasmic protease ClpXP and a degradational signal at the C terminus of RecN. In cells with DNA DSBs, green fluorescent protein-RecN localized in discrete foci on nucleoids and formed visible aggregates in the cytoplasm, both of which disappeared rapidly in wild-type cells when DSBs were repaired. In contrast, in ΔclpX cells, RecN aggregates persisted in the cytoplasm after release from DNA damage. Furthermore, analysis of cells experiencing chronic DNA damage revealed that proteolytic removal of RecN aggregates by ClpXP was important for cell viability. These data demonstrate that ClpXP is a critical factor in the cellular clearance of cytoplasmic RecN aggregates from the cell and therefore plays an important role in DNA damage tolerance.

DNA double strand breaks (DSBs) are major threats to the genomic integrity of cells. DSBs can be caused by exogenous and endogenous agents such as ionizing radiation and chemical mutagens and by endogenously produced radicals or as a result of replication arrest and/or collapse of the replication fork (1, 2). DSBs are lethal if unrepaired and if repaired improperly may result in genome instability such as mutations, genomic rearrangements, and chromosome loss. Therefore, the repair of DSBs is crucial for cell survival and for maintaining the integrity of the genome.

In *Escherichia coli*, the RecBCD pathway of homologous recombination is responsible for the repair of DSBs. RecBCD initially processes broken ends into 3′ single-stranded DNA tails by its helicase nuclease activities (2). These single-stranded DNA tails then invade homologous duplex DNA via a RecA-mediated mechanism. However, the RecF pathway, which is another RecA-dependent recombination pathway, can also promote DSB repair when the RecBCD pathway is inactivated by mutations (e.g. recBC sbcBC). *E. coli* recN has two SOS boxes in the promoter region of recN that confer inducibility on recN in response to SOS signaling (3, 4). Mutation of recN reduces conjugational recombination in *recBC sbcBC* strains (3, 5), suggesting that RecN is part of the RecF pathway of recombination. However, in contrast to the other genes of the RecF pathway, mutations in recN do not restore resistance to thymineless death (6), and there is evidence suggesting that the RecN protein is also required for RecBCD-dependent repair of DSBs (6, 7). Recently, it was shown that RecN is required to repair chromosomal breaks at a specific location by *I-SceI* endonuclease (8). Furthermore, recN mutants are sensitive to ionizing radiation and mitomycin C but not to UV irradiation (5). Thus, RecN does not fall clearly into either pathway of recombination, but rather it may be involved in the repair of DNA DSBs by homologous recombination.

RecN contains an extensive centrally located coiled-coil domain and globular N- and C-terminal domains containing nucleotide binding Walker A and Walker B motifs, respectively, which are characteristic of the SMC (structural maintenance of chromosomes) family (9, 10). SMC proteins play fundamental roles in DNA replication and/or chromosomal segregation by maintaining and modulating chromosome structure in prokaryotic and eukaryotic cells. Recent studies show that SMC proteins also play roles in global gene regulation, cell cycle checkpoints, and DNA repair and that the functions of SMC proteins and their non-SMC subunits are regulated by post-translational modification (11–15). In this study, we show that RecN protein is degraded by the cytoplasmic energy-dependent protease ClpXP in a manner dependent upon the signal residues at the C terminus of RecN. Furthermore, we also show that in ΔclpX cells, DNA damage-inducible RecN formed visible aggregates in the cytoplasm that persisted after release from DNA damage and were deleterious to cell survival. These data demonstrate that proteolytic removal of toxic RecN aggregates plays an important role in efficient recovery after release from DNA damage and is therefore crucial for cell homeostasis.

**EXPERIMENTAL PROCEDURES**

**Media and General Methods**—Standard methods for *E. coli* genetics and recombinant DNA techniques were as described...
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by Miller (16) and Sambrook et al. (17). Ampicillin (50 μg/ml), tetracycline (10 μg/ml), chloramphenicol (100 μg/ml), and kanamycin (30 μg/ml) were used where indicated. The source of ionizing radiation was 60Co with an output of 20 grays/min.

Bacterial Strains and Plasmids—The strains that we used in this study were isogenic with BW25141 (18) except forftsH null mutants. Wild-type and deletion mutants were provided by National BioResource Project (39). TheftsH sfhC mutant was a gift from T. Ogura (19). A fragment containing the SOS promoter and open reading frame of gift from T. Ogura (19). A fragment containing the SOS pro-

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Regulatory gene was inserted into pTF271 (21), giving pRecN. RecN was tagged with an enhanced green fluorescent protein cassette at its N terminus, giving pSG101. Arabinose-inducible recN (pTF270) or GFP-recN (pTF271) was constructed by cloning the recN or GFP-recN gene into pTF200 (21). All recN mutants were generated by PCR and replaced with the wild-type recN gene of pRecN. The structure of recombinant plasmids was confirmed by DNA sequencing.

Sensitivity to Mitomycin C (MMC)—For quantitative assays, cells were grown to early log phase in Luria Bertani (LB) (or LB supplemented with 0.5% glucose at 28°C) and post-translational regulation of RecN in stressed and unstressed cells. Cells were transiently exposed to γ-irradiation to generate DNA DSBs, and RecN stability was monitored using anti-RecN antibody. In wild-type cells exposed to ionizing radiation, RecN protein increased rapidly for 60 min in vivo (Fig. 1A). The fact that RecN is transiently induced in irradiated cells suggests that RecN is unstable or that its stability is regulated in stressed cells. To directly examine the stability of RecN in vivo, we quantified its level per cell using anti-RecN antibody and compared it to MMC followed by protein synthesis inhibitor chloramphenicol. The level of RecN protein increased rapidly for 60 min in the presence of 0.5 μg/ml MMC at 37°C; however, after addition of chloramphenicol, RecN was rapidly degraded with a half-life of ~8 min (Fig. 1B). Thus, RecN is unstable and has a short half-life under these conditions.

RecN Is Unstable in Vivo—We examined the translational and post-translational regulation of RecN in stressed and unstressed cells. Cells were transiently exposed to γ-irradiation to generate DNA DSBs, and RecN stability was monitored using anti-RecN antibody. In wild-type cells exposed to ionizing radiation, RecN protein was dramatically induced within 20 min (Fig. 1A), which is consistent with the fact that E. coli recN is a member of the SOS regulon (3, 4). Notably, RecN rapidly declined to near basal levels within 160 min after exposure to ionizing radiation (Fig. 1A). The fact that RecN is transiently induced in irradiated cells suggests that RecN is unstable or that its stability is regulated in stressed cells. To directly examine the stability of RecN in vivo, we quantified its level per cell using anti-RecN antibody and compared it to MMC followed by protein synthesis inhibitor chloramphenicol. The level of RecN protein increased rapidly for 60 min in the presence of 0.5 μg/ml MMC at 37°C; however, after addition of chloramphenicol, RecN was rapidly degraded with a half-life of ~8 min (Fig. 1B). Thus, RecN is unstable and has a short half-life under these conditions.

RecN Is a Substrate for ClpXP Protease—In E. coli, targeted intracellular proteolysis is largely carried out by energy-dependent proteases encoded bylon, hslV, clpP, and ftsH (24, 25). Because RecN might be a substrate for one or more of these proteases, RecN stability was examined inlon, hslV, clpP, and ftsH mutant strains. FtsH is essential for viability (26), so theftsH null strain also carried the suppressor mutationsfhC. The results indicated that the stability of RecN was significantly greater in theclpP deletion strain, but not in other protease-
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deficient strains (Fig. 1C), suggesting that RecN is a substrate for a ClpP-dependent protease. ClpP protein associates with either of two ATPases, ClpA or ClpX, to form two distinct proteolytic complexes, ClpAP and ClpXP, that are remarkably similar to the 26S proteasome in eukaryotic cells (27–29). To determine whether RecN is degraded by ClpAP and ClpXP, or both, the stability of RecN was measured in strains expressing truncated forms of RecN composed of N-terminal (DI or DI–II), C-terminal (DII–III), or central (DII) fragments of RecN. Cells were treated with MMC to induce the truncated forms of RecN and then treated with chloramphenicol, and the level of RecN was measured by Western blotting. The C-terminal fragment (DII–III) was rapidly degraded after addition of chloramphenicol (Fig. 1D). In contrast, the N-terminal or central fragments (DI, DII, or DI–II) were much more stable (Fig. 1D). These results indicate that the C-terminal fragment of RecN is essential for ClpXP-mediated degradation of RecN.

Previous studies showed that ClpX recognizes short signals called degradation tags near the N or C terminus of its protein substrates (31–33). One of the better characterized degradation tags is SsrA, an 11-amino acid peptide (AANDENYALAA) that is attached co-translationally to the C terminus of nascent polypeptides when ribosomes stall (34). Notably, the C-terminal sequence of RecN is very similar to the SsrA degradation tag (Figs. 1E and 3). Recently, Neher et al. (30) reported that proteomic profiling of ClpXP-targeted substrates after DNA damage revealed extensive instability within the SOS regulon and identified RecN as one of the proteins targeted for ClpXP degradation. Thus, our results, taken together with those of previous studies, support the conclusion that RecN is a substrate for ClpXP protease.

The C Terminus of RecN Is Required for Its Degradation—The stability of RecN was also measured in strains expressing truncated forms of RecN composed of N-terminal (DI or DI–II), C-terminal (DII–III), or central (DII) fragments of RecN. Cells were treated with MMC to induce the truncated forms of RecN and then treated with chloramphenicol, and the level of RecN was measured by Western blotting. The C-terminal fragment (DII–III) was rapidly degraded after addition of chloramphenicol (Fig. 1D). In contrast, the N-terminal or central fragments (DI, DII, or DI–II) were much more stable (Fig. 1D). These results indicate that the C-terminal fragment of RecN is essential for ClpXP-mediated degradation of RecN.

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Intracellular Localization of RecN in Cells with DNA Damage—
The expression and stability of RecN were examined in vivo using an SOS-regulated N-terminal fusion between GFP and RecN. GFP-RecN was expressed from a low copy plasmid under its native promoter, and its localization was monitored using fluorescence microscopy. Control experiments established that GFP-RecN fully complemented the repair deficiency of a recN deletion strain (Fig. 2A) and that the level of GFP-RecN was comparable with that of RecN expressed from its native promoter on the chromosome (data not shown). Thus, wild-type RecN and GFP-RecN appear to function similarly in vivo.

During the exponential phase of growth, no GFP-RecN focus formation was observed. After exposure to MMC for 60 min, ~85% of treated cells contained GFP-RecN foci (Fig. 2B). This result is consistent with previous results indicating that Bacillus subtilis RecN-YFP forms foci in cells with DNA DSBs (35).

Notably, the merged image between the DAPI and GFP images revealed that GFP-RecN was localized not only as a discrete focus on the nucleoid but also at the cell poles in the cytoplasm (Fig. 2C). These foci are not detected in cells expressing GFP as a control (data not shown). In addition, RecN foci were also observed in anucleoid cells (Fig. 2D), suggesting that cytoplasmic GFP-RecN foci represent nonspecific RecN aggregates. This is also supported by the facts that E. coli RecN protein readily forms large protein aggregates (8) and protein aggregates are generally likely to accumulate at cell poles in the cytoplasm in bacteria.

The results described above indicate that the expression of recN is temporally restricted and tightly regulated in cells with DNA damage. The subcellular localization of RecN foci in vivo was examined in greater detail using cells expressing GFP-RecN from the inducible araC promoter. Cells were grown to mid-log phase, exposed to γ-rays, and immediately transferred to arabinose-containing medium to induce GFP-RecN. Control cells were not irradiated and/or not exposed to arabinose. The results showed that cytoplasmic (95% of total cells), but not nucleoid-associated (<5%), GFP-RecN foci formed in unirradiated cells exposed to arabinose, whereas both cytoplasmic (91%) and nucleoid-associated (83%) GFP-RecN foci formed in irradiated cells exposed to arabinose (Fig. 2, E and F). GFP foci were not observed in glucose-containing medium (Fig. 2E). Similar results were obtained in cells with MMC-induced DNA damage (data not shown). These results suggest that DNA DSBs trigger formation of nucleoid-associated GFP-RecN foci.

ClpXP-degraded Cytoplasmic RecN Aggregates after Release from MMC Treatment—GFP-RecN foci were also characterized in ΔclpX cells. Wild-type and mutant cells expressing SOS-inducible GFP-RecN were treated with MMC (0.5 μg/ml) for 60 min, transferred to medium lacking MMC, and allowed to recover for 2 h at 37 °C. After treatment with MMC for 60 min, cells were filamenteated and >80% of cells contained GFP-RecN foci (Fig. 3A, at time zero). Western analysis revealed that MMC-induced GFP-RecN is degraded after transfer to medium lacking MMC in wild-type cells, but not in ΔclpX cells (Fig. 3A). Consistently, in wild-type cells, nucleoid-associated and cytoplasmic GFP-RecN foci disappeared rapidly (Fig. 3, A and B). In contrast, in ΔclpX cells, whereas nucleoid-associated GFP-RecN foci disappeared after transfer to medium lacking MMC, cytoplasmic GFP-RecN foci persisted for 2 h and were transmitted to daughter cells when cell division resumed in MMC-free medium (Fig. 3, A and B). Furthermore, GFP-RecNDD also persisted in the form of cytoplasmic RecN aggregates after release from DNA damage (Fig. 3C). These data indicate that ClpXP degrades cytoplasmic RecN foci in cells with DNA damage.

FIGURE 3. ClpXP degraded cytoplasmic RecN aggregates after release from MMC treatment. A, cells containing pSG101 (SOS-inducible GFP-recN) were treated with 0.5 mg/ml MMC for 60 min, released into fresh LB medium without MMC, and further incubated at 37 °C. At the indicated times after release from MMC treatment, cells were observed by fluorescence microscopy. At the same time points, aliquots of whole cell extracts were analyzed by Western blotting with anti-RecN antibody. B, quantitative analysis of GFP-RecN after release from MMC treatment. Filled squares and filled circles indicate the percentage of cells with RecN foci and cytoplasmic RecN foci, respectively. For each of the strains, ~300 cells were examined. C, accumulation of RecNDD after release from DNA damage. Cells containing SOS-inducible GFP-recN or GFP-recNDD plasmids were treated as in panel A. The panel shows DAPI and GFP images of cells 100 min after removal of MMC.
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Degradation of Cytoplasmic RecN Aggregates by ClpXP Is Important for Cell Viability—Non-native polypeptides that tend to form protein aggregates can have toxic effects and ultimately cause human disease (36, 37). Therefore, the role of RecN aggregates in promoting survival and/or DNA repair in cells with DNA damage was examined by measuring cell survival in wild-type, ΔrecN, and ΔclpX cells treated with MMC. When cells were treated with MMC for 1 h, cell survival was lower in ΔrecN cells than in wild-type cells, but similar in ΔclpX and wild-type cells (Fig. 4A). However, when cells were treated with MMC for relatively long periods of time (≈10 h), cell survival was lower in ΔclpX cells than in wild-type cells (Fig. 4B). To determine whether excess levels of cytoplasmic RecN aggregates are responsible for low cell viability, we examined the MMC sensitivity of ΔrecN cells harboring a low copy number plasmid that expresses RecN or RecNDD. The cell survival of ΔrecN cells expressing RecNDD was similar to the survival of those expressing wild-type RecN after 1 h of exposure to MMC (Fig. 4C) but was lower than the survival of those expressing wild-type RecN after 10 h of exposure to MMC (Fig. 4D). These results suggest that cytoplasmic RecN aggregates may negatively affect cell survival in stressed cells with chronic DNA damage, thus confirming the functional roles of the targeting of RecN for degradation by ClpXP. One possible explanation for these results is that the accumulated RecN aggregates in ΔclpX or recNDD cells might lead to sequestration of de novo RecN protein and/or other DNA repair proteins and specifically interfere with DNA repair pathways. Indeed, this toxic effect caused by RecN aggregates is specific to cells with DNA damage, because wild-type and ΔclpX cells had similar viability (>90%) in the absence of DNA damage even if RecN aggregates were induced in wild-type or ΔclpX cells harboring the arabinose-inducible recN plasmid (data not shown). It should be noted that MMC-treated ΔclpX cells had lower survival than MMC-treated recNDD cells (Fig. 4, B and D). This probably reflects the fact that ClpXP carries out targeted degradation of multiple cellular proteins (22, 30, 38), and therefore the accumulation of these proteins might also affect the survival of ΔclpX cells exposed to MMC.

Because cells are frequently exposed to various environmental stimuli such as heat shock or DNA damage, adaptation to changing conditions or efficient recovery after their release is crucial for cell homeostasis. Targeted intracellular proteolysis by energy-dependent proteases is an essential component of many response pathways. This study showed that RecN protein is degraded by the ClpXP protease in a manner dependent upon the signaling residues at the C terminus of RecN. In addition, DNA damage-induced RecN forms both nucleoid-associated and cytoplasmic foci. Formation of the former is triggered specifically by DNA damage, and degradation of the latter requires ClpXP after DNA damage. In ΔclpX cells, cytoplasmic RecN aggregates persist after the release from DNA damage and are deleterious to cell survival when they accumulate to deleterious levels in stressed cells with chronic DNA damage. These data suggest that ClpXP is involved in efficient recovery after the release from DNA damage by promoting the turnover of DNA damage-inducible RecN protein and is therefore crucial for cell homeostasis in stressed cells with DNA damage. Thus, the present findings provide evidence that intracellular proteolysis is an essential component of DNA damage tolerance that allows cells to survive repeated cycles of DNA breakage and repair. Further studies will advance our understanding of the complex interplay between protein quality control and DNA damage tolerance.

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