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Profiling of the assembly of RecA nucleofilaments implies a potential target for environmental factors to disturb DNA repair

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ABSTRACT

Double-strand breaks (DSBs), one class of the most harmful DNA damage forms that bring elevated health risks, need to be repaired timely and effectively. However, an increasing number of environmental pollutants have been identified to impair DSB repair from various mechanisms. Our previous work indicated that the formation of unsaturated RecA nucleofilaments plays an essential role in homology recombination (HR) pathway which can accurately repair DSBs. In this study, by developing a benzonase cutting protection assay and combining it with traditional electrophoretic mobility shift assay (EMSA) analysis, we further investigated the assembly patterns of four RecA mutants that display differential DSB repair ability and ATPase activity. We observed that the mutants (G204S and S69G) possessing both ATP hydrolysis and DSB repair activities form unsaturated nucleofilaments similar to that formed by the wild type RecA, whereas the other two ATP hydrolysis-deficient mutants (K72R and E96D) that fail to mediate HR form more compacted nucleofilaments in the presence of ATP. These results establish a coupling of ATPase activity and effective DSB repair ability via the assembly status of RecA nucleofilaments. This linkage provides a potential target for environmental factors to disturb the essential HR pathway for DSB repair by suppressing the ATPase activity and altering the assembly pattern of nucleofilaments.

Introduction

Environmental exposures of toxic pollutants and dysregulation of endogenous metabolism can lead to various types of DNA damage including DNA adducts, base mismatches, strand breaks and others (Chatterjee and Walker, 2017; Hou et al., 2012; Kucab et al., 2019; Wang et al., 2002; Xu et al., 2018). DNA double-strand breaks (DSBs) are one class of the most severe DNA damage that may cause genomic instability and bring elevated health risks. Numerous environmental factors such as ultraviolet radiation, ionizing radiation and toxic pollutants are able to induce DSBs directly by attacking DNA molecules or indirectly by stimulating oxidative stresses arising from the reactive oxygen species (ROS) (Franco et al., 2009; Greinert et al., 2012; Rolfsmeier et al., 2010; Srinivas et al., 2019). On the other hand, an increasing number of studies

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have focused on the effects of exogenous agents on the DSB repair pathways (Lan et al., 2018; Liu et al., 2020; Robles and Adami, 1998). The exposure to Bisphenol A (Allard and Colaiácovo, 2010) or diethylhexyl phthalate (Cuenca et al., 2020) would impair the DSB repair machinery in the germline of *Caenorhabditis elegans* and thereby leads to genomic instability. (Arsenite can serve as a general inhibitor for E3 ubiquitin ligases, RNF20 and RNF40, by binding to the RING finger domain and subsequently inhibit the ubiquitination of histone H2B (Zhang et al., 2014), preventing the formation of biochemically accessible chromatin structure that is conducive for recruiting of repair proteins to the DSB sites. A similar effect was recently observed for cadmium which can directly bind to the E3 ubiquitin ligase RNF168 and induce its degradation, thereby blocking downstream recruitment of DSB repair proteins (Zhang et al., 2019). demonstrated that Aldehydes at environmentally exposed concentrations can induce degradation of BRCA2, an essential tumor suppressor, and stall DNA replication, triggering DSBs in dividing cells (Tan et al., 2017).

Homologous recombination (HR) is a conserved pathway that can accurately repair DSBs by resynthesizing the lost sequence information in a homology template-dependent manner. To initiate this error-free repair procedure, recombinases, a family of proteins conserved from bacteria to humans, accompanying with the cofactor ATP, are loaded on single-stranded (ss) DNA produced by nucleases' resection after DSBs take place, forming an active presynaptic nucleofilament which is capable of searching and invading homology region routinely located on sister chromatin. Successful strand invasion results in a displacement loop structure that can serve as a primer to initiate subsequent synthesis of the lost sequence arising from DSBs (Mehta and Haber, 2014; Piazza and Heyer, 2019; Piazza et al., 2019). Notably, the assembly of recombinase proteins on ssDNA is a vital upstream molecular event that determines followed HR processes. Therefore, regulation of this assembly process is much of importance and has been widely investigated (Tavares et al., 2019; Zhao et al., 2017, Zhao et al., 2017). We previously revealed that RecA, a representative recombinase from *E. coli*, is able to employ its intrinsic DNA binding-dependent ATPase activity to generate an unsaturated nucleofilament that has been identified as the main active form responsible for HR *in vivo* (Zhao et al., 2017).

Given that the formation of this HR-favorable unsaturated RecA-ssDNA nucleofilament is tightly regulated by ATP hydrolysis, it is reasonable to speculate that endogenous and environmental factors may alter the RecA nucleofilament's status by suppressing its ATPase activity and consequently impair HR repair of DSBs. Previous results showed that RecA mutants (G204S, S69G, K72R, and E96D) displaying differential ATP hydrolytic activity possess much differential HR activity (Britt et al., 2011; Bryant, 1999; Cox et al., 2006; Zhao et al., 2017). In this study, to further figure out and establish the relationship of the nucleofilament's ATPase and HR activities, we explored the assembly patterns of these RecA mutants on ssDNA. By combining a benzonase-mediated nuclease protection assay and the electrophoretic mobility shift assay (EMSA), we observed that the mutants (G204S and S69G) in favor of effective *in vivo* HR form similar unsaturated nucleofilaments as wild type (WT) RecA forms under physiologically relevant ATP conditions, whereas the other two mutants (K72R and E96D) that fail to catalyze homology-directed DSB repair form more compacted nucleofilaments. Together with previous data, the presented results suggest an ATP hydrolysis-mediated coupling of the lightly assembled RecA nucleofilaments to the effective *in vivo* HR consequence. Notably, this linkage provides a potential target for exogenous chemicals to indirectly disturb the essential HR pathway.

1. Materials and methods

1.1. Chemicals and DNA probes

The benzonase was purchased from Sino Biological Inc. (Beijing, China) and the powder was dissolved in 1 × TH buffer (20 mmol/L Tris–HCl, pH 7.5) prior to use. The wild type RecA protein and Proteinase K (molecular biology grade) were purchased from New England Biolabs Inc. (Ipswich, MA, USA). All oligodeoxynucleotides were synthesized and purified by Sangon Biological Engineering Technology and Services (Shanghai, China), and the related sequences and usages are listed in Table 1. ATP and AMP-PNP (adenylyl-imidodiphosphate) were supplied by Sigma-Aldrich (St Louis, MO, USA). The 40% acrylamide (mass ratio of acrylamide to bis-acrylamide: 29:1) was purchased from Solarbio (Beijing, China). Glycerol and tris-(hydroxymethyl)aminomethane (Tris) were biotechnology grade and supplied by Ameresco (Tully, NY, USA). N,N,N,N-Tetramethylethylenediamine (TEMED, ≥ 99%) and ammonium persulfate (APS, 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Sangon, respectively. Other chemical reagents were purchased from National Pharmaceutical Group Chemical Reagent Company (Beijing, China) unless otherwise stated. All solutions were prepared using ultrapure water from a Purelab Ultra Elga Labwater system (VWS Ltd., UK) with an electrical resistivity of 18.2 MΩ•cm and filtered through a 0.45 μm filter.

1.2. Protein expression and purification

RecA K72R gene was a kind gift from Dr. Michael M. Cox at the University of Wisconsin-Madison. Other RecA mutants' genes (G204S, E96D, S69G) were generated by using the Fast site-directed mutagenesis kit (Tiangen Biotech (Beijing) Co, Ltd., Beijing, China) according to the manufacturer's instructions. All RecA mutants were expressed and purified in our lab as previously described (Shan et al., 1996; Zhao et al., 2017).

1.3. RecA assembly reactions

E. coli wild type or mutant RecA protein (0–3.0 μmol/L as indicated) was mixed with a single-stranded oligonucleotide (20 nmol/L, estimated as whole oligomer molecule) and one nucleotide cofactor (1.0 mmol/L ATP or 0.1 mmol/L AMP-PNP) unless otherwise stated in 1 × TH-Mg^{2+} buffer (20 mmol/L Tris–HCl, pH 7.4, 10 mmol/L Mg^{2+}). After a 10-min incubation at 37 °C maintained by A K30 dry bath incubator (Allsheng, Hangzhou, China), the solutions were immediately subjected to benzonase cutting assay or PAGE analysis.

Table 1 – The sequences of oligodeoxynucleotides used in this work.

Probe Name	Sequence (5' to 3')	Usage
10 mer	TTGTCCCCAG	marker
20 mer	TTGTCCCCAGCATTTAAAAC	marker
40 mer	TTGTCCCCAGCATTTAAAACCTCTGCCGTAAGGGATGTCC	marker
60 mer	TTGTCCCCAGCATTTAAAACCTCTGCCGTAAGGATGTCCTGGCCCCTCCTCAGCACCTT	marker
5'-Cy5-ss90	TTTCCTACCTTAAGATCGTTCCAGTCTCCGCCGGCCAGTGTTATTTTTAGAGCTCATACCATTCGCCAATTTCTTCGCACGTTAGTCTTT	marker, experimental ssDNA
mid-Cy5-ss90	TTTCCTACCTTAAGATCCTTCCAGTCTCCGCCGGCCAGTGTTATTTTTAGAGCTCATACCATTCGCCAATTTCTTCGCACGTTAGTCTTT	experimental ssDNA
3'-Cy5-ss90	TTTCCTACCTTAAGATCCTTCCAGTCTCCGCCGGCCAGTGTTATTTTTAGAGCTCATACCATTCGCCAATTTCTTCGCACGTTAGTCTTT	experimental ssDNA
com-ss90	AAAGACTAACGTGCGAAGAAATTGGCGAATGGTATGAGCTCTAAAAATAACACTGGCCGCCGGAGACTGGAAGGATCTTAAGGTAGGAAA	preparing dsDNA probe

The marked red T indicates a Cy5 labeling; com-ss90 is utilized to hybridize with 3'-Cy5-ss90 to prepare the Cy5-ds90 probe.

1.4. *Benzonase cutting protection assay*

To perform benzonase cutting assay, 5.0 U benzonase was added to the prepared samples of 20 μL. Following incubation at 37 °C for a specific period of time as indicated, 50 mmol/L EDTA was added to terminate the benzonase cutting reaction. After the addition of 1.6 U proteinase K to remove proteins including RecA and benzonase, the solutions were successively incubated at 55 °C for 40 min, 95 °C for 10 min and 25 °C for 10 min. Then the final samples were mixed with 10% (final concentration) glycerol and resolved on a 12% native polyacrylamide gel at ~20 V/cm (200 V) for 80 min.

1.5. *EMSA assay*

After RecA assembly on ssDNA was finished, the samples were mixed with 10% (final concentration) glycerol and loaded on a gradient gel consisting of two different concentrations (4% and 12% for upper and lower PAGE, respectively) of regions. Then the samples were resolved at ~20 V/cm (200 V) for 90 min.

1.6. *PAGE gel imaging and analysis*

PAGE gels were scanned using an Odyssey CLX infrared imaging system (LI-COR Biotechnology, Lincoln, NE, USA) with a 700 nm excitation through which the Cy5 signals labeled on DNA probes can be observed. The fluorescent intensities were analyzed using the Image Studio Software.

2. Results and discussion

2.1. *Benzonase cutting protection assay for assessing RecA assembly*

Benzonase, a kind of supernuclease which is able to efficiently cut DNA molecules into very short oligonucleotides of 2–5 nucleotides (nt) in length (Nestle and Roberts, 1969), has become a described option to perform nuclease protection assay for exploring DNA-protein interactions. In this study, we developed a benzonase cutting protection assay to investigate the assembly pattern of RecA protein on ssDNA (Fig. 1). RecA proteins are loaded on a fluorescently labeled ssDNA probe to form the nucleofilament. Then the RecA-unbound segments will be cut by benzonase whereas the RecA-covered segments will be protected from benzonase cutting. Following a protein digestion by Proteinase K, the protected segments are measured using PAGE analysis. Of note, the cutting time of benzonase cutting protection assay was shortened to just 30 - 60 sec compared with the supernuclease-mediated DNA protection assay (Zhao et al., 2017) to offset the cutting time effect as much as possible.

We first tested the feasibility of the benzonase cutting protection assay. For this purpose, two types of DNA probes, double-stranded (ds) DNA probe (Cy5-ds90) and ssDNA probe (Cy5-ss90) were employed as the substrates of benzonase cutting assay. As shown in Fig. 2, the addition of RecA protein (3.0 μmol/L) successfully protected the ssDNA probe from benzonase cutting whereas it failed to protect the dsDNA probe which is unable to be bound by RecA protein, suggesting that

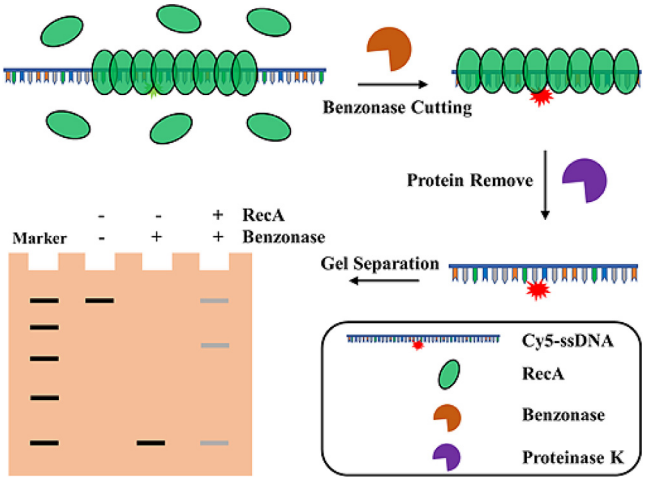

Fig. 1 – Schematic illustration of the benzonase cutting protection assay for assessing the assembly of RecA protein on ssDNA.

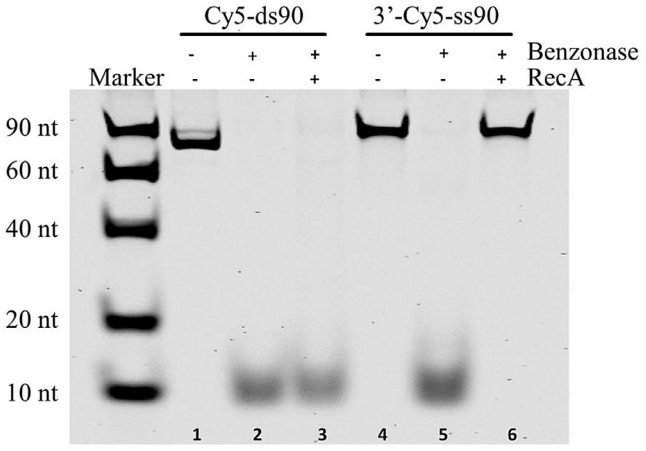

Fig. 2 – RecA specifically protects ssDNA but not dsDNA from benzonase cutting. No ATP and ATP analog was used. Benzonase cutting time: 0.5 min.

RecA itself does not directly affect the cutting efficiency of benzonase. Therefore benzonase can be used to perform the nuclease protection assay for assessing RecA assembly on ssDNA.

2.2. *ATP hydrolysis promotes the formation of unsaturated RecA nucleofilaments*

To further verify this method, we applied it to investigate a well-known assembly manner by incubating varying concentrations (0–3.0 μmol/L) of RecA with 20 nmol/L Cy5-ss90. As shown in Fig. 3, more ssDNA molecules were protected as the concentration of RecA protein increased. Notably, RecA assembles on ssDNA with a stoichiometry of 3 nt per RecA monomer (Bell and Kowalczykowski, 2016). Thus complete protection of 20 nmol/L ssDNA of 90 nt theoretically requires at least 600 nmol/L RecA monomers. Indeed, when >600 nmol/L RecA was added, we observed complete protection of the whole ssDNA (Fig. 3a–c). In addition, 600 nmol/L RecA was able to provide complete protection for over 90% of ssDNA probes (Fig. 3d), suggesting a very high affinity of RecA to this ssDNA probe (K_d < 30 nmol/L) in the absence of ATP. More importantly, when <600 nmol/L RecA was added, almost two bands, completely protected or digested, were exclusively observed for all three 90-nt ssDNA probes which were labeled with Cy5 at different sites (Fig. 3a–c), suggesting that RecA assembles on ssDNA in a cooperative manner under the present condition lacking ATP. These data are highly consistent with previous assembly model of wild type RecA protein, indicating the benzonase cutting protection assay is feasible.

Interestingly, RecA nucleofilaments possess ATPase activity and our previous work indicated that ATP hydrolysis, in turn, stimulates RecA to dissociate from ssDNA and consequently form unsaturated RecA nucleofilaments. We thus utilized the developed benzonase cutting assay to confirm the unsaturated RecA nucleofilaments under ATP hydrolysis. As shown in Fig. 4, more bands between the completely protected and digested segments were observed when ATP was added as a cofactor to form the RecA nucelofilaments, suggesting that these ssDNA probes were partially covered by RecA proteins in the presence of ATP. Evidently, shortening benzonase cutting allows us to characterize the assembly pattern for the binding of RecA to single stranded DNA. In addition, this assembly pattern is not affected by the tested RecA concentration (0.2 - 3.0 μmol/L), indicating that the distribution pattern of RecA protein on this unsaturated nucleofilament is well conserved.

2.3. *Profiling of the assembly patterns of RecA mutants on ssDNA*

Given that the assembly manner of RecA on ssDNA is tightly regulated by ATP hydrolysis, we next explored the assembly patterns of four RecA mutants (G204S, S69G, K72R, and E96D) that possess similar ATP binding but differential ATP hydrolytic ability. For this purpose, the assembly of these RecA mutants were performed under three different conditions: without a cofactor (Fig. 5a), with 0.1 mmol/L AMP-PNP (a non-hydrolyzable ATP analog, Fig. 5b) or 1.0 mmol/L ATP (Fig. 5c). Interestingly, for the two RecA mutants, G204S and S69G, which are able to hydrolyze ATP, the addition of ATP but not AMP-PNP significantly change their assembly patterns, leading to less fraction of the completely protected probes, which is similar to the unsaturated nucleofilaments formed by the wild type RecA. The other two mutants, K72R and E96D, however, displayed negligible ATP hydrolysis activity (Zhao et al., 2017). Interestingly, we observed that the addition of either AMP-PNP or ATP weakens the assembly of K72R but obviously enhances the assembly of E96D, suggesting that ATP binding is adverse to K72R but helpful to E96D for nucleofilaments' assembly. Without the addition of ATP or ATP analog, the E96D cannot protect ssDNA from the 30-min benzonase cutting (Fig. 5a), indicating that ATP binding stimulates the enhanced binding and assembly of E96D on ssDNA.

To further confirm the altered assembly patterns of RecA mutants, we performed a traditional EMSA analysis which is routinely used to investigate protein-nucleic acids interactions. As shown in Fig. 6, AMP-PNP did not change the ssDNA binding properties of the wild type RecA, G204S, and S69G pro-

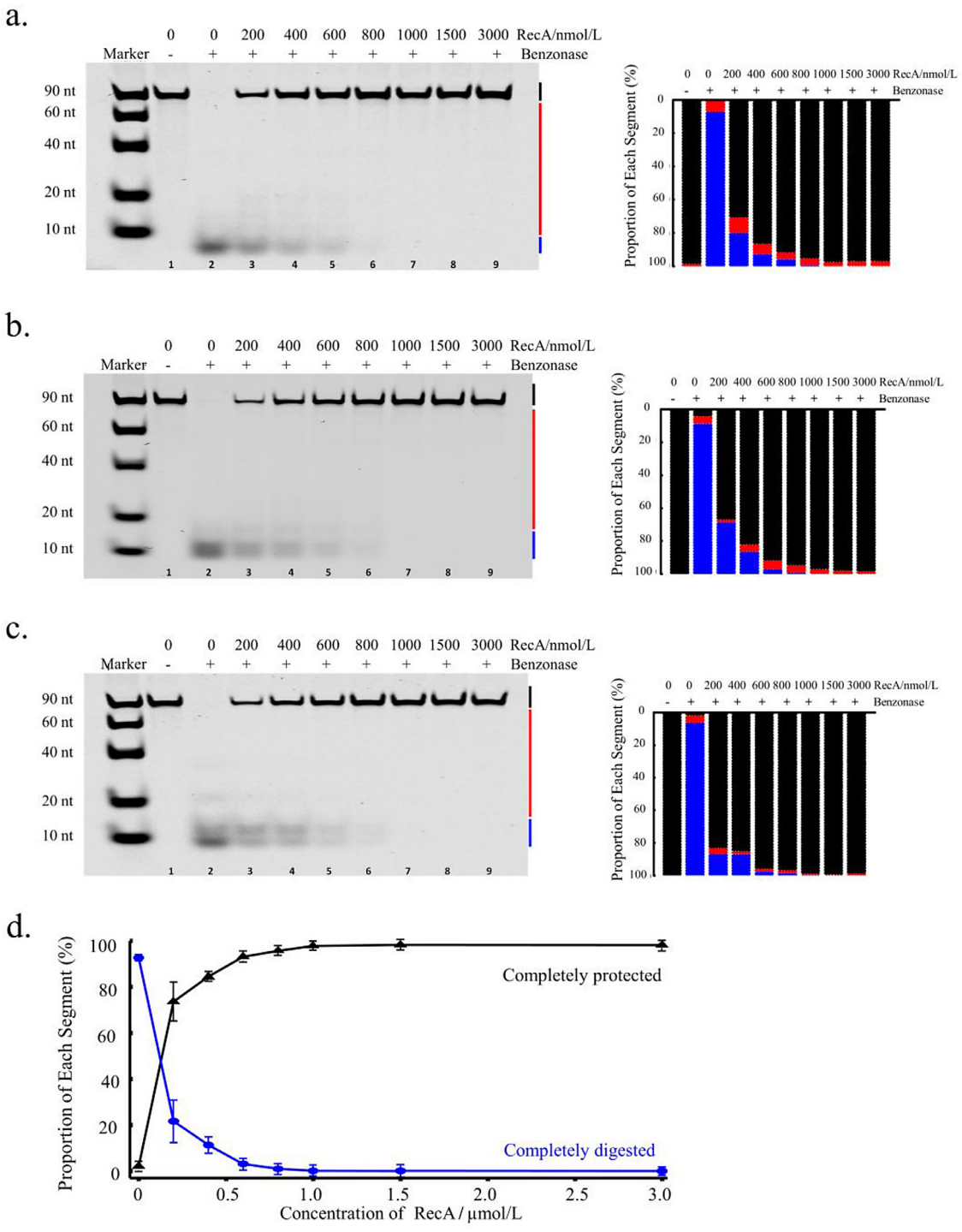

Fig. 3 – Benzonase cutting protection assay to confirm the assembly manner of wild type RecA proteins on ssDNA in the absence of ATP and ATP analog. (a-c) PAGE analysis of the RecA-protected DNA segments (left) and their corresponding proportions counted from normalized intensities (right). The ssDNA probes were labeled with Cy5 at 5'-end (a), 46th dT (b), and 3'-end (c), respectively. The completely protected, partially protected and completely digested bands are indicted in black, red and blue, respectively. (d) Statistic analysis of the proportion of the completely protected or digested segments versus RecA concentration. Benzonase cutting: 1.0 min. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

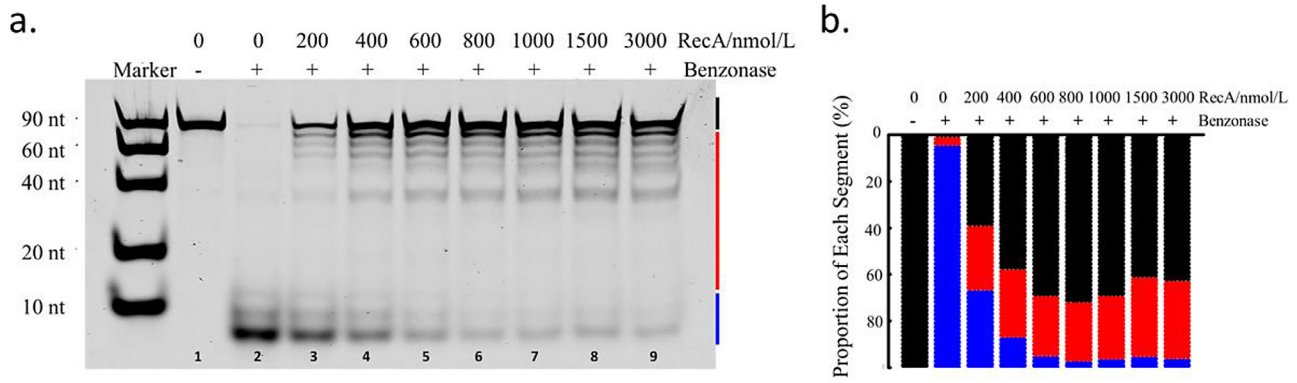

Fig. 4 – Benzonase cutting protection assay to investigate the assembly manner of wild type RecA proteins on ssDNA in the presence of ATP. (a) PAGE analysis of the RecA-protected DNA segments. (b) Proportion of each segment counted from normalized intensities. The completely protected, partially protected and completely digested bands are indicted in black, red and blue, respectively. Benzonase cutting: 1.0 min. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

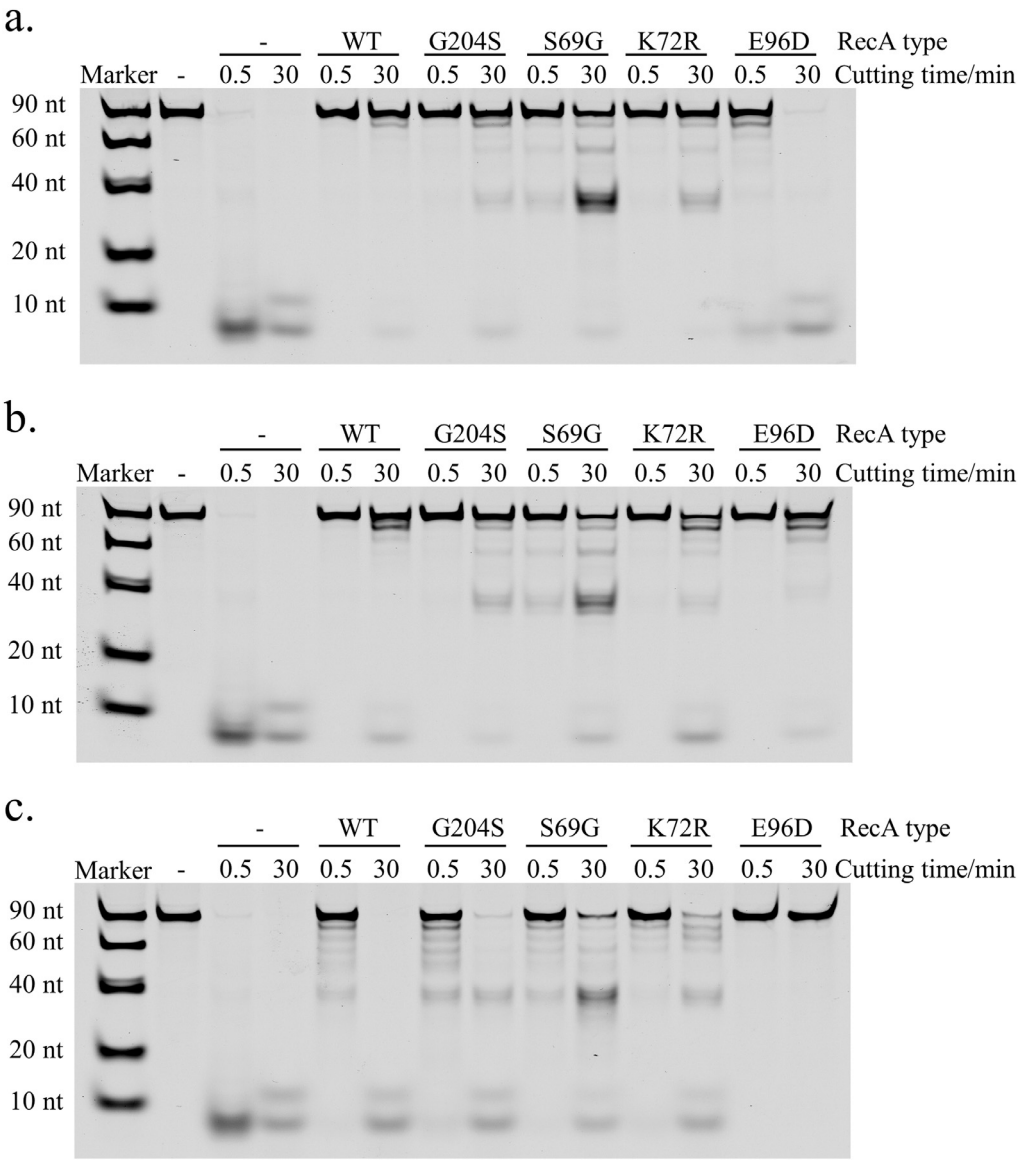

Fig. 5 – Benzonase cutting protection assay to explore the assembly patterns of RecA mutants without a cofactor (a) or with AMP-PNP (b) and ATP (c).

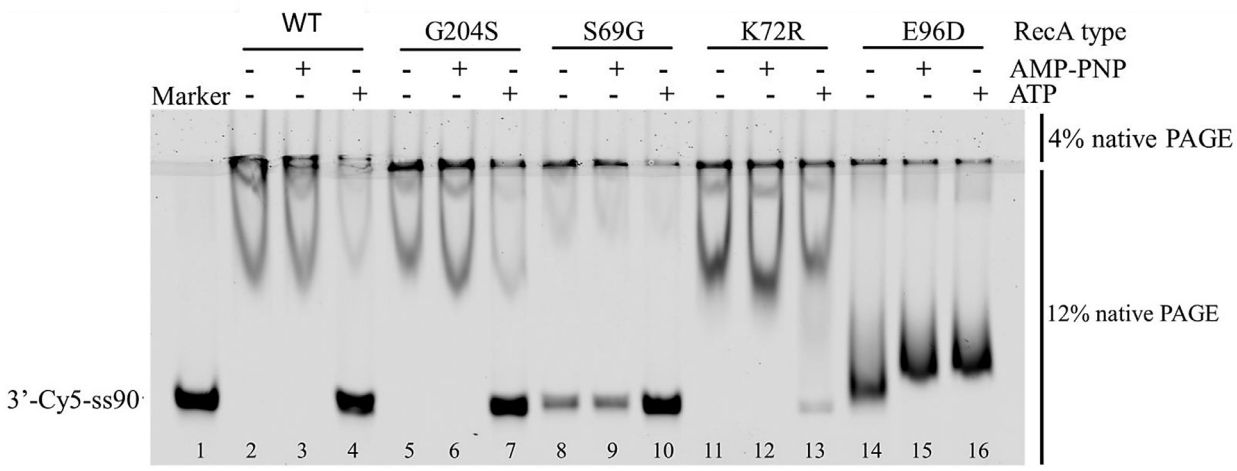

Fig. 6 – EMSA analysis of the assembly patterns of RecA mutants.

teins, while ATP significantly weakened their binding to ssDNA. In contrast, both AMP-PNP and ATP were able to change the ssDNA binding ability of the K72R and E96D mutants. Notably, the binding of K72R to ssDNA is slightly weakened in the presence of AMP-PNP or ATP. This is manifested by the presence of unbound 3'-Cy5-ss90. However, as assisted with ATP or AMP-PNP, E96D-ssDNA complex migrates slower than that in the absence of nucleotide cofactor (Fig. 6). These results are consistent with above analysis from the benzonase cutting protection assay.

It seems that the S69G and K72R displayed similar intensities for band of protected ssDNA probe (90 nt) in the three conditions. However, the underlying mechanism is different. For S69G, ATP hydrolysis may contribute the weakened protection of RecA on ssDNA in the presence of ATP, but for K72R (ATP hydrolysis-deficient), ATP binding may contribute to the lost protection in the absence of ATP.

As described above, the ATP hydrolysis-favorable mutants G204S and S69G form similar unsaturated nucleofilaments to the wild type RecA protein in the presence of ATP while the ATP hydrolysis-deficient mutants K72R and E96D form more compacted nucleofilaments under the identical conditions. Given that these two classes of RecA mutants displayed totally different DSB repair ability in previous studies (Britt et al., 2011; Davis, 1999; Zhao et al., 2017), we speculate that the assembly pattern of presynaptic nucleofilaments plays an essential role in this conserved HR pathway. Therefore, this study implies that exogenous factors such as environmental pollutants may disturb this pivotal DSB repair pathway by altering the assembly manner of recombinase proteins, resulting in genomic instability and elevated health risks.

3. Conclusions

By developing a benzonase cutting protection assay and combining traditional EMSA analysis, this study investigated the assembly patterns of four RecA mutants that displayed differential DSB repair ability and ATPase activity. We observed that the mutants (G204S and S69G) which possess ATPase activity and favor homology-directed DSB repair *in vivo* form similar unsaturated nucleofilaments to the wild type RecA protein whereas the other two ATP hydrolysis-deficient mutants (K72R and E96D) that fail to mediate HR form more compacted nucleofilaments. These results established a coupling of ATP hydrolysis activity and effective DSB repair ability via the assembly status of RecA nucleofilaments. More importantly, this linkage provides a potential target for environmental factors to regulate the essential HR pathway for DSB repair. In the future, environmental pollutants that display ATP hydrolysis suppressing effect need to be further investigated because they are probably able to disturb DSB repair and lead to genomic instability by altering the assembly of RecA nucleofilaments.

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