Dissociation of RecA filaments from duplex DNA by the RuvA and RuvB DNA repair proteins

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Communicated by Nicholas R. Cozzarelli, June 15, 1994

ABSTRACT The RuvA and RuvB proteins of Escherichia coli act late in recombination and DNA repair to catalyze the branch migration of Holliday junctions made by RecA. In this paper, we show that addition of RuvAB to supercoiled DNA that is bound by RecA leads to the rapid dissociation of the RecA nucleoprotein filament, as determined by a topological assay that measures DNA underwinding and a restriction endonuclease protection assay. Disruption of the RecA filament requires RuvA, RuvB, and hydrolysis of ATP. These findings suggest several important roles for the RuvAB helicase during genetic recombination and DNA repair: (i) displacement of RecA filaments from double-stranded DNA, (ii) interruption of RecA-mediated strand exchange, (iii) RuvAB-catalyzed branch migration, and (iv) recycling of RecA protein.

In Escherichia coli, the RecA protein plays a central role in recombination by initiating homologous pairing and strand exchange to produce intermediates in which duplex molecules are linked by Holliday junctions (1). As a first step, RecA binds to single-stranded or gapped duplex DNA to form a nucleoprotein filament in which the DNA is extensively underwound to 1.5 times the length of B-form DNA. This underwinding can be detected by topological methods (2, 3) and may help to promote the formation of multistranded DNA helices that act as intermediates in strand exchange, and to create torsional stress that can unwind blocks of DNA heteroduplex during recombinational repair (4).

Cells carrying mutations in any of the three ruv genes (ruvA, ruvB, ruvC) are sensitive to UV light and ionizing radiation and exhibit reduced recombination frequencies (5). Biochemical studies have shown that the RuvA and RuvB proteins interact with Holliday junctions to promote their movement along DNA, by a process known as branch migration (6-8). The RuvC protein regulates Holliday junctions by endonucleolytic cleavage (9, 10). RuvA (22 kDa) and RuvB (37 kDa) each play a defined role in branch migration, with RuvA targeting RuvB to the Holliday junction (11, 12). The selective binding of RuvA to junctions (7, 11), the stimulation of RuvB's ATPase by RuvA/DNA (8), and observations which show that saturating concentrations of RuvB alone can promote branch migration (13) suggest that RuvB is the motor that drives strand exchange during branch migration, with RuvA playing an ancillary role (12). Further biochemical studies of RuvAB have revealed a DNA helicase activity that unwinds DNA with a 5'-to-3' polarity (14). This finding may be significant, since DNA helicases could promote strand exchange through regions of chromatin, blocks of DNA heteroduplex, and DNA lesions during genetic exchange and recombinational repair.

Several groups have investigated how DNA polymerases and helicases cope with the problem of translocating through regions of tightly bound protein or compacted chromatin (15, 16). A similar problem may occur during the late steps of recombination in E. coli in which branch migration proteins such as RuvAB or RecG (17) act upon DNA intermediates bound by RecA. Since the RuvAB complex enhances the rate of DNA strand exchange when added to ongoing RecA reactions (6), it is possible that RuvAB actively displaces RecA from DNA. To determine whether RuvAB can dissociate RecA filaments from duplex DNA we conducted the experiments described in this paper. We found that RuvAB promotes the dissociation of RecA filaments from duplex DNA, and causes transient unwinding of the DNA double helix. These data are consistent with a model in which RuvAB displaces RecA from double-stranded DNA to promote branch migration during recombinational repair.

MATERIALS AND METHODS

Proteins. E. coli RecA, RuvA, and RuvB and wheat germ DNA topoisomerase (topo I) were purified as described (18, 19). Restriction enzymes and bovine serum albumin (BSA) were bought from New England Biolabs and BRL, creatine kinase was from Sigma, and proteinase K was from Boehringer Mannheim. Protein concentrations were determined by the Bradford and Lowry methods using BSA as standard and were confirmed by spectroscopy. Amounts of protein are expressed in moles of monomeric protein.

DNAs. The plasmid pDEA1 (4.44 kb) was derived from the plasmids pBR322, pACYC184, and pGEM-7Zf(+) (Promega). It contains the ColE1 and phage fl replication origins and was used for the production of both double-stranded and single-stranded DNA. 32P-labeled supercoiled DNA (scDNA) was made by growth of pDEA1 in E. coli JM109 cells in the presence of [32P]orthophosphate (370 MBq/ml, 10 μCi/ml; Amersham) and purified with a Qiagen column. Linear duplex DNA was produced by cleavage at the unique Pst I restriction site. DNA concentrations are expressed in moles of nucleotide residues.

Topological Assay for Displacement of RecA. To produce nucleoprotein filaments on duplex DNA, supercoiled 32P-labeled pDEA1 DNA (20 μM) was incubated with RecA (20 μM) in 1 ml of buffer A (27 mM Tris-HCl, pH 7.5, 1 mM MgCl2/2 mM dithiothreitol/1.3% (vol/vol) glycerol/0.01% (wt/vol) BSA/27 mM phosphocreatine with creatine kinase at 4 units/ml) containing 2.7 mM ATP. Mixtures were prepared on ice and then transferred to 37°C. Following this initial RecA binding (typically 45 min), MgCl2 was increased to 12 mM for RuvAB-DNA binding. The higher MgCl2 concentration did not affect the stability of the RecA filaments but inhibited rebinding of RecA to scDNA (20). To do
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RESULTS

RuvA and RuvB Proteins Displace RecA from scDNA. To determine whether RuvA and RuvB displace RecA protein from duplex DNA, we took advantage of the ability of RecA to bind and underwind negatively supercoiled plasmid DNA (Fig. 1A). The assay we used was a modification of that described by Iwabuchi et al. (22), in which RecA-mediated DNA underwinding produces positive superhelical turns which can be relaxed by eukaryotic topo I (indicated schematically in Fig. 1 B and C). Upon deproteinization, the DNA is seen as a highly negatively supercoiled product, defined as form X DNA (Fig. 1D). Addition of RuvAB and consequent dissociation of the RecA nucleoprotein filament would be expected to lead to the formation of relaxed DNA (rather than form X DNA) after topo I treatment (Fig. 1 F-H).

To bind RecA to supercoiled plasmid 32P-labeled DNA, we used low concentrations of MgCl2 followed by a shift up to 12 mM (22). At the higher Mg2+ concentration, the RecA filament is stable for at least 90 min. Addition of wheat germ topo I led to the formation of highly supercoiled form X DNA (Fig. 2, lane d), whose agarose gel electrophoretic mobility (after deproteinization) was greater than that of native scDNA (lane a). Under these conditions, >90% of the DNA was converted to form X, indicating the formation of stable nucleoprotein filaments. As expected, in the absence of RecA, the DNA was completely relaxed by topo I, resulting in a profile of topoisomers whose gel electrophoretic mobility was slightly positively supercoiled in the gel (lane c).

When RuvA and RuvB were added to the nucleoprotein filament following the shift to 12 mM MgCl2, the DNA was relaxed by topo treatment, indicating that RecA had been dissociated (Fig. 2, lane e). The profile of final topoisomer products was the same as that obtained in control reactions containing RuvAB but without RecA (Fig. 2, lane h). Neither RuvA nor RuvB alone was able to dissociate RecA from the DNA (Fig. 2, lanes f and g). In these incomplete reactions, form X DNA was observed. Similar results were obtained in experiments in which single-stranded DNA fragments were

![Fig. 1. Diagram of the DNA-unwinding assay used to show RuvAB-mediated dissociation of RecA filaments. Underwinding of negatively supercoiled DNA (A) by RecA results in the introduction of positive supercoils (B). Underwinding is initially limited by the positive supercoiling but becomes greater upon topo I-mediated relaxation of the DNA (C). Upon deproteinization, highly underwound DNA adopts a tightly interwound configuration, known as form X DNA (D). The high degree of negative supercoiling in this product differs sharply from that (zero, on average) generated by topo I in the absence of RecA (E). If RuvAB is added to a RecA-DNA binding reaction mixture before (F) or after (G) topo I addition, RuvAB rapidly displaces RecA from the DNA which is relaxed by topo I. Transient unwinding and/or underwinding of the DNA (small bubbles) by RuvAB (F and G) results in partially relaxed negative (-) topoisomer products (H). ProK, proteinase K.](image-url)

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FIG. 2. Dissociation of RecA filament from scDNA by RuvAB. RecA protein was bound to 32P-labeled scDNA by the Mg2+-shift method as described in Materials and Methods. RuvA and RuvB were added as indicated prior to treatment with wheat germ topoisomerase I. 32P-labeled products were analyzed by agarose gel electrophoresis followed by autoradiography. The positions of supercoiled (sc) and open circular (oc) pDEA1 plasmid DNA (4.44 kb in length) and a small amount of dimeric DNA (8.88 kb) are indicated. The relaxed topoisomerase products are shown.

used to load RecA onto the scDNA at 12 mM Mg2+ (data not shown).

Unexpectedly, without RecA, the profile of topo I relaxation products obtained in the presence of RuvA and RuvB (Fig. 2, lane h) differed from those obtained with RuvA or RuvB alone (lanes i and j) or with naked DNA (lane c). Most likely, DNA binding by RuvAB leads to a transient unwinding and/or underwinding of the substrate, and this small change was detected by the topoisomerase assay.

Reaction Requirements and ATP Dependence. Dissociation of RecA by RuvAB required ATP (Fig. 3) and 7.5–30 mM Mg2+ (Fig. 4). We did not observe substantial disruption of the RecA filament when ATP was replaced with ATPγS (Fig. 3, lane i). This result indicates that ATP hydrolysis is required, although we cannot exclude the possibility that the RecA filament was irreversibly fixed by the ATPγS. In the presence of 1 mM ATP, dissociation of RecA was only slightly inhibited by the addition of 0.5 mM ATPγS (lane j). Control reactions revealed that RuvAB, in the presence of ATPγS, bound the supercoiled DNA, resulting in a wide distribution of topoisomers (lanes l–o). As noted earlier, this observation is consistent with the hypothesis that RuvAB can introduce torsional stress into the DNA.

A time course of the standard RecA displacement reaction is shown in Fig. 5. Within 2 min after RuvA and RuvB addition, >90% of the substrate DNA was fully relaxed (lanes c–g). We conclude that RuvAB acts rapidly to disrupt RecA–DNA filaments.

Requirement for RuvA and RuvB. To determine the specific requirement for RuvA and RuvB in mediating RecA dissociation, the concentration of one protein was varied while the

FIG. 3. Requirements for RuvAB-mediated dissociation of RecA: Effect of ATP, ATPγS, and low Mg2+. Reactions were performed and the products were analyzed as described in Fig. 2 legend. The final reaction mixtures contained ATP (1 mM) or ATPγS (0.5 mM) as indicated. In some reactions, the Mg2+ concentration was not increased and remained 1 mM. Topo I was omitted from the sample shown in lane a.
Materials and final binding

RuvB RecA protein stopped
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In filtration increased from 0.075 percentage reaction decreased sharply

deproteinized where topo other was held.

These results described method.

and percentage of uM (0.35 tM) (0.35 uM)

constant and percentage of RuvB held constant (2 uM) and RuvB was varied. In this case, the percentage of form X DNA decreased as the RuvB concentration increased from 0.075 to 0.6 uM RuvB (Fig. 6B). At 7.5 mM Mg2+, displacement of RecA by RuvAB was again less efficient. These results indicate that the amount of RuvA (0.35 uM) and RuvB (0.15 uM) required to displace >50% of the bound RecA is one RuvA tetramer per 85 bp and one RuvB dodecamer per 600 bp.

Restriction Cleavage Assay for Displacement of RecA. The DNA unwinding assay measures global changes in DNA topology as a result of RecA binding and topo I action. To confirm that the assay was measuring displacement of RecA,

other was held constant. When RuvA was varied from 0 to 2 uM with RuvB held constant at 1.2 uM (at 15 uM DNA, 15 uM RecA, and 15 mM MgCl2), the amount of form X DNA decreased sharply between 0.125 uM and 0.5 uM RuvA (Fig. 6A). In the presence of 7.5 mM MgCl2, the dissociation reaction was less efficient and was proportional to the RuvA concentration. Similar results were obtained when RuvA was held constant (2 uM) and RuvB was varied. In this case, the percentage of form X DNA decreased as the RuvB concentration increased from 0.075 to 0.6 uM RuvB (Fig. 6B). At 7.5 mM Mg2+, displacement of RecA by RuvAB was again less efficient. These results indicate that the amount of RuvA (0.35 uM) and RuvB (0.15 uM) required to displace >50% of the bound RecA is one RuvA tetramer per 85 bp and one RuvB dodecamer per 600 bp.

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In the experiments described here, the amounts of RuvA and RuvB required to displace >50% of the RecA filaments were significantly lower than the amount of RecA present in the reaction mixtures. The minimal stoichiometry (one RuvA tetramer per 85 bp and one RuvB dodecamer per 600 bp) as determined in Fig. 6 leads us to suggest that DNA binding, in the absence of translocation, is unlikely to be responsible for the observed degree of RecA displacement. Indeed, the requirements for RuvA and RuvB may be artificially high due to the lack of a Holliday junction which would target RuvAB to the DNA. We currently favor an active RuvAB translocation model in which RuvB rings unidirectionally translocate along duplex DNA, displacing RecA protein as the DNA passes through the center of the RuvB ring structure. The physical act of threading double-stranded DNA through the cavity of a RuvB ring, possibly coupled with DNA unwinding (14), could provide a processive mechanism of RecA displacement and branch migration.

Other possible mechanisms of RuvAB-mediated dissociation of the RecA filament, such as a direct physical interaction between RuvAB and RecA, are not ruled out by the current set of experiments. Precedent for such a direct physical interaction between a RecA-like DNA recombinase and a DNA helicase has been observed between bacteriophage T4 UvsX protein and Dda helicase (25).

The active displacement of RecA from duplex DNA by RuvAB is of particular interest given the high stability of the RecA filament in the presence of an adequate supply of ATP. Although it is known that RecA protein undergoes a transition from high- to low-affinity DNA-binding states upon ATP hydrolysis (26), there is little evidence to suggest that ATP hydrolysis and dissociation are coupled. Indeed, experiments by Pugh and Cox (27) showed that RecA protein remains associated with the heteroduplex DNA product of strand exchange well after the reaction was complete. Dissociation and recycling of RecA may therefore be consequences of subsequent RuvAB-mediated processing events that serve to dissociate the RecA filament from DNA. Removal of spent RecA protein and catalysis of branch migration would then leave the DNA accessible for Holliday-junction resolution by RuvC. Genetic data indicate an interaction among RuvA, RuvB, and RuvC in resolution (28) suggesting that the final stages of Holliday-junction processing—involving RecA removal, branch migration, and Holliday-junction resolution—may well occur via a series of coordinated events in vivo.

We thank our colleagues for suggestions, Alison Mitchell for providing RuvA protein, John Nicholson for photography, and Emily Hildebrandt and Professor Nicholas Cozzarelli (University of California, Berkeley) for their notes on purification of wheat germ topo I.