THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 51, Issue of December 17, pp. 52991–52997, 2004
Printed in U.S.A.

Inhibition of RecA Protein by the *Escherichia coli* RecX Protein

MODULATION BY THE RecA C TERMINUS AND FILAMENT FUNCTIONAL STATE*

Received for publication, August 6, 2004, and in revised form, October 4, 2004
Published, JBC Papers in Press, October 4, 2004, DOI 10.1074/jbc.M409050200

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The RecX protein is a potent inhibitor of RecA activities. We identified several factors that affect RecX-RecA interaction. The interaction is enhanced by the RecA C terminus and by significant concentrations of free Mg^{2+} ion. The interaction is also enhanced by an N-terminal His_6 tag on the RecX protein. We conclude that RecX protein interacts most effectively with a RecA functional state designated A_o and that the RecA C terminus has a role in modulating the interaction. We further identified a C-terminal point mutation in RecA protein (E343K) that significantly alters the interaction between RecA and RecX proteins.

Homologous recombinational repair of damaged DNA mediated by the bacterial RecA protein is crucial in maintaining the integrity of the genome (1–6). *In vitro*, the RecA protein promotes a series of DNA pairing and strand exchange reactions that are thought to mimic the function of this protein *in vivo* (6, 7). RecA functions as a nucleoprotein filament. Filaments are assembled most readily on single-stranded DNA (ssDNA).[1] However, filament assembly on duplex DNA (dsDNA) can also be achieved under some conditions, especially if a suitable nucleation site is available. RecA is a DNA-dependent ATPase, promoting ATP hydrolysis with a k_{cat} of about 30 or 20 min^{-1} when bound to ssDNA or dsDNA, respectively. RecA filaments assemble and disassemble in the 5′ to 3′ direction on ssDNA, with protomers added to one end and subtracted from the other under appropriate conditions (6, 7).

The activities of RecA protein must be regulated in the cell to target RecA to locations where it is needed and to avoid aberrant DNA transactions. The same must be true of all RecA homologs. In humans, aberrant recombination reactions could include the gross chromosomal rearrangements that lead to many human diseases, including cancer (8). Therefore, understanding the mechanisms by which RecA family recombinases are regulated is of utmost importance.

RecA itself provides one level of control. This involves autoregulation mediated by the RecA C terminus (9). In *Escherichia coli,* many additional proteins are known to regulate RecA function. The RecF, RecO, and RecR proteins have been implicated in the modulation of RecA filament assembly and disassembly (10–13). The DinI protein has been studied as a modulator of RecA function during the SOS response (14–18). The RecX protein is an inhibitor of RecA function both *in vivo* and *in vitro* (19). The RecX protein is among the least understood of these RecA modulator proteins. Recently, evidence has been obtained that RecX protein acts to cap the growing end of a RecA filament (20). This activity would serve to limit the length of RecA filaments formed *in vivo*, and we have proposed that this is the major mode of action of RecX (20).

The RecX protein (19.3 kDa) is encoded by a widespread bacterial gene, often but not always found as it is in *E. coli*, just downstream of the *recA* gene (21–26). A palindromic sequence separates the two *E. coli* genes (27). The resulting hairpin interrupts transcriptional read-through and limits the amount of *recA-recX* transcript to about 5–10% of the levels of the *recA* transcript alone. Protein levels of RecX were estimated to be 500 times less than those of the RecA protein (27). RecX interacts directly with the RecA protein (19), and direct binding to ssDNA is limited (19, 20).

Overexpression of RecA proteins in the absence of *recX* is toxic in *Pseudomonas aeruginosa, Streptomyces lividans, Mycobacterium smegmatis,* and *Xanthomonas oryzae* (23, 24, 28, 29). This provided early evidence that RecX is a negative modulator of RecA expression or function. Overexpression of the *recX* gene can reduce the induction of the SOS response (19), but there are no other clear phenotypes (27). When purified, RecX protein from both the *Mycobacterium* (30) and *E. coli* (19, 20) inhibit RecA functions *in vitro*. The RecX protein binds in the major helical groove of an AMPPNP-stabilized RecA filament (31). The effects of RecX are not identical in all bacteria, as RecX seems to enhance RecA activity in *Neisseria gonorrhoeae* (26).

Seifert and co-workers (19) found that *E. coli* RecX protein inhibits RecA protein ATPase activity *in vitro*, coprotease activity *in vivo*, and recombinase activity both *in vivo* and *in vitro*. The *in vitro* assays demonstrated inhibition at levels of RecX far below the concentration of RecA protein (19, 20), while the *in vivo* experiments employed a plasmid that overexpressed RecX protein. These same workers also demonstrated that RecA and RecX proteins physically interact using yeast two-hybrid analysis in *E. coli* (19). Electron Microscopy (EM) and the RecA crystal structure (32, 33) have been used to build three-dimensional reconstructions of RecA nucleoprotein filaments (stabilized by binding to AMPPNP) with bound *E. coli* RecX protein (31). Their data show RecX protein bound to the RecA filament, spanning the monomer-monomer interface from the C-terminal domain of one RecA monomer to the core domain of the second (31). These data suggest that the effects of RecX protein might involve an interaction with the C terminus of RecA.

In addition, RecA protein has several defined functional states that could affect the RecX-RecA interaction. When

* This work was supported by Grant GM 52725 from the National Institutes of Health (to M. M. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: ssDNA, single-stranded DNA; DTT, dithiothreitol; AMPPNP, adenosine 5′-(β,γ-imino)triphosphate; PEP, phosphoenolpyruvate; dsDNA, double-stranded DNA.

bound to ssDNA, RecA is in a functional state designated A (active). There are two forms of the A state (9, 34). One of these, present at low Mg^{2+} concentrations, is relatively restricted in terms of allowed DNA pairing reactions (called A_c, with the c reflecting a "closed" state). The other is present at higher Mg^{2+} concentrations and is unrestricted in DNA pairing (the open or A_o form). Addition of another strand of bound DNA to the RecA filament, either when RecA is bound to dsDNA or during DNA strand exchange, causes a transition to a state designated P (pairing) (7). In this report, we set out to determine how the RecA C terminus and the RecA functional state affected the interaction of RecA filaments with RecX protein.

MATERIALS AND METHODS

Enzymes and Biochemicals—The *E. coli* wild-type RecA, RecA E343K, and RecAΔC17 proteins were purified as described (9, 35). The RecA E343A mutant protein was purified identically to the RecAΔC17 protein (35), except that the final fraction was passed through a BioRex 70 column to remove a persistent nuclease activity. The concentration of the purified proteins were determined from the absorbance at 280 nm using the extinction coefficient 2.23×10^4 M^{-1} cm^{-1} (36). The *E. coli* SSB protein was purified as described (37). The concentration of the purified protein was determined from the absorbance at 280 nm using the extinction coefficient 2.83×10^4 M^{-1} cm^{-1} (38). Unless otherwise noted, all reagents were purchased from Fisher and were of the highest grade available. XhoI restriction endonuclease was purchased from MBI Fermentas. DTT was obtained from Research Organics. Lysozyme, phosphoenolpyruvate (PEP), pyruvate kinase, lactate dehydrogenase, ATP, polyethyleneimine, bromphenol blue, and NADH were purchased from Sigma. Hydroxyapatite and BioRex 70 resins were purchased from Bio-Rad. Isopropyl-1-thio-β-D-galactopyranoside was obtained from Gold BioTechnology, Inc. Ficoll was from Amersham Biosciences.

Buffers and Media—Phosphate buffer contained the indicated concentration of potassium phosphate (pH 6.8), 1 mM DTT, 0.1 mM EDTA, and 10% (w/v) glycerol. R buffer contained 20 mM Tris-HCl (80% cation, pH 7.5), 1 mM DTT, 0.1 mM EDTA, and 10% (w/v) glycerol. TAE buffer contained 40 mM Tris-OAc (80% cation) and 1 mM EDTA. Luria-Bertani medium (LB broth) is 10 g/liter tryptone, 5 g/liter yeast extract, and 10 g/liter NaCl, with the pH adjusted to 7.0.

DNA Substrates—Bacteriophage φX174 circular single-stranded DNA (virion) was purchased from New England Biolabs. φX174 RF I supercoiled circular duplex DNA was purchased from Invitrogen. Full-length linear duplex DNA was generated by the digestion of φX174 RF I DNA (5386 bp) with the XhoI restriction endonuclease, using conditions suggested by the enzyme supplier. The digested DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1), followed by ethanol precipitation. Circular single-stranded DNA from bacteriophage M13mp8 (7229 nucleotides) was prepared using previously described methods (39). The concentrations of ssDNA and dsDNA were determined by absorbance at 260 nm, using 36 and 50 μg ml^{-1} $A_{260}{}^{-1}$, respectively, as conversion factors. All DNA concentrations are given in micromolar nucleotides.

Cloning and Overexpressing the RecX Protein—The *recX* gene was isolated from the *E. coli* genome (strain MG1655, a gift from George Weinstock, Ref. 40) by polymerase chain reaction. One DNA primer corresponded to the first 19 bases of the *recX* gene with the sequence 5′-CACCGTTC added to the 5′-end to provide a BspHI restriction site. The second DNA primer corresponded to the last 8 bases of the *recX* gene with the sequence 5′-CGGGATCCCCGTATGCGT added to the 5′-end to provide a BamHI restriction site. These primers were used to amplify a DNA fragment of the appropriate size (529 bp) by the polymerase chain reaction. The *recX* gene fragment was digested with BspHI and BamHI and then ligated into the cloning vector pET21d (Novagen), which was digested with NcoI and BamHI (the NcoI and BspHI restriction sites have compatible cohesive ends). This plasmid containing the native *recX* gene under the control of the T7 RNA polymerase promoter was designated pEAW224. The integrity of the entire *recX* gene in this construct was verified by direct sequencing.

Competent cells of *E. coli* strain STL327/pT7pol26 (35, 41) were transformed with plasmid pEAW224. Ten liters of culture were grown in LB broth to an OD_{600} of 0.7. RecX protein expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to 0.4 mM. Following a 3-h incubation at 37 °C, ~19 g of cells were harvested by centrifugation, flash-frozen in liquid N_2, and stored at −80 °C. The protein expressed is the native polypeptide, with no protein tags or other additions present.

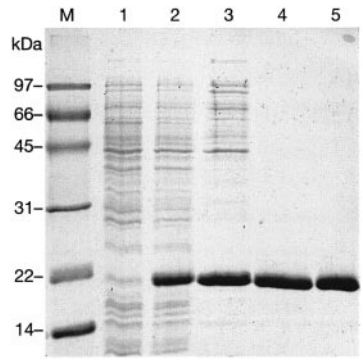

FIG. 1. **Purification of the native RecX protein.** SDS-polyacrylamide gel of purified RecX. *Lane 1*, uninduced STL327 cells. *Lane 2*, crude cell extract after induction of RecX protein. *Lane 3*, protein fraction after ammonium sulfate precipitation. *Lane 4*, purified RecX after the first hydroxyapatite column. *Lane 5*, final pure RecX protein after the second hydroxyapatite column (over 99% pure). Approximately 7.5 μg of protein was loaded in each of *lanes 3–5*.

Purification of the RecX Protein—All steps were carried out at 4 °C. Cell paste (19 g) was thawed and fully resuspended in 95 ml of a solution of 25% (w/v) sucrose and 250 mM Tris-HCl (80% cation, pH 7.5). Cells were lysed by a 60-min incubation with 37 ml of 5 mg/ml solution of lysozyme in 250 mM Tris-HCl (80% cation, pH 7.5), followed by the addition of 52 ml of 25 mM EDTA, sonication, and centrifugation. The lysate was precipitated with 16 ml of 5% (w/v) polyethyleneimine, pH 7.5, and centrifuged. The pellet was extracted two times with R buffer plus 150 mM ammonium sulfate. The extracted protein solution was precipitated by the addition of 0.144 g of solid ammonium sulfate per ml of solution. The RecX protein was precipitated from the supernatant fraction after centrifugation with an additional 0.227 g of solid ammonium sulfate per ml of solution. The resulting pellet was washed two times with R buffer plus 2.9 M ammonium sulfate and resuspended in 200 ml of 500 mM phosphate buffer. The protein solution was dialyzed against 2 liters of 320 mM phosphate buffer, then two times against 2 liters of 220 mM phosphate buffer. The protein solution was then loaded onto a hydroxyapatite column, washed with two column volumes of 220 mM phosphate buffer, and eluted with a linear gradient from 220 to 700 mM phosphate buffer over 8 column volumes. Peak fractions were identified by SDS-PAGE analysis, pooled, and dialyzed two times against 220 mM phosphate buffer. The protein was then loaded over the same hydroxyapatite column equilibrated with 220 mM phosphate buffer, washed with two column volumes of 220 mM phosphate buffer, and eluted with a wash of 270 mM phosphate buffer. Peak fractions were identified by SDS-PAGE analysis, pooled, and concentrated with a Centricon Plus-20, 10,000 molecular mass cut-off concentrator (Amicon). The concentrated protein was dialyzed against R buffer plus 300 mM potassium chloride or potassium glutamate, 30% (w/v) glycerol, then twice against RecX storage buffer (R buffer, 100 mM KCl or KGlu, 50% (w/v) glycerol). The protein was flash frozen in liquid N_2 and stored at −80 °C.

The concentration of the RecX protein was determined from the absorbance at 280 nm using the extinction coefficient 2.57×10^4 M^{-1} cm^{-1}. The RecX extinction coefficient ($\epsilon_{nat,\ 280\ nm} = 2.57 \times 10^4 \pm 0.027$ M^{-1} cm^{-1}) was determined during the course of the present work, using procedures described elsewhere (42, 43). The A_{280}/A_{260} ratio for the native RecX is 1.59 ± 0.031. The error in both numbers is 1 S.D. The protein was free of detectable nuclease activities and was determined to be greater than 99% pure by SDS-PAGE (Fig. 1). The identity of the purified protein was confirmed by matrix-assisted laser desorption/ionization-time of flight ass spectrometry. The measured mass of the protein was 19,274, in good agreement with the calculated mass of RecX protein of 19,294 (with the initiator Met residue removed).

The RecX protein with a His_6 N-terminal tag, used in one experiment, was generously provided by Elizabeth Stohl and Hank Seifert (Northwestern University). The purification of this protein has been described (19, 31).

In the course of these studies we noted that the native RecX protein was relatively unstable. RecX protein preparations stored at −80 °C exhibit a measurable loss of activity after four or more months of storage (data not shown). When thawed aliquots of the purified native RecX protein were kept at 4 °C for several days, its inhibitory effects declined (Fig. 2). For this reason, all of the experiments in this study made use of RecX aliquots thawed on the day of the experiment. Fur-

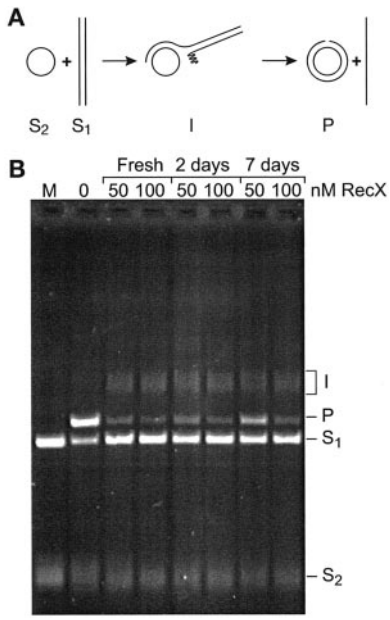

FIG. 2. **Limited stability of native RecX protein.** *A*, reaction diagram. In the first step, RecA forms a filament on circular single-stranded DNA (labeled S_2). RecA then recruits complementary linear double-stranded DNA (labeled S_1) and pairs the two molecules, aligning homology. A joint molecule intermediate (*I*) is formed while the identical strand is displaced. Finally, a nicked circular product (*P*) and a linear single-stranded molecule result. Substrates, products, and intermediates are distinguishable by agarose gel electrophoresis. *B*, reactions were carried out as described under "Experimental Procedures," and contained 6.7 μM RecA protein, 20 μM ϕX174 circular ssDNA, 20 μM linear ϕX174 dsDNA, 10 μM $Mg(OAc)_2$, 3 mM ATP, and the indicated concentration of RecX protein. Reactions were incubated for 90 min. *M* is a marker of unreacted linear double-stranded DNA and circular ssDNA. A thawed aliquot of RecX protein was stored at 4 °C for the indicated time prior to use in these trials. The fresh aliquot was used 4 h after thawing.

ther, all native RecX protein, stored at −80 °C, was used within 12 weeks of its purification.

ATPase Assay—A coupled spectrophotometric enzyme assay (44, 45) was used to measure the DNA-dependent ATPase activities of the wild-type RecA and RecAΔC17 proteins. The regeneration of ATP from PEP and ADP was coupled to the oxidation of NADH and followed by the decrease in absorbance of NADH at 380 nm (380-nm wavelength was used so that the signal remained within the linear range of the spectrophotometer for the duration of the experiment). The assays were carried out on a Varian Cary 300 dual beam spectrophotometer equipped with a temperature controller and a 12-position cell changer. The cell path length and band pass were 0.5 cm and 2 nm, respectively. The NADH extinction coefficient at 380 nm of 1.21 mM^{-1} cm^{-1} was used to calculate the rate of ATP hydrolysis.

The reactions were carried out at 37 °C in 25 mM Tris-OAc (80% cation, pH 7.4), 1 mM DTT, 3 mM potassium glutamate, the indicated concentration of $Mg(OAc)_2$, 5% (w/v) glycerol, an ATP regeneration system (10 units/ml pyruvate kinase and 3.5 mM phosphoenolpyruvate), a coupling system (3 mM NADH and 10 units/ml lactate dehydrogenase), and 5 μM M13mp8 circular single-stranded DNA. The aforementioned components were incubated for 10 min. The figure legends note the time of addition of wild-type RecA or RecA mutant proteins (3 μM), the RecX protein (concentration indicated in the figure legends), the SSB protein (to 0.5 μM), and ATP (to 3 mM).

DNA Three Strand Exchange Reactions Promoted by the Wild-type RecA and RecAΔC17 Proteins—Three strand exchange reactions were carried out in 25 mM Tris-OAc (80% cation, pH 7.4), 1 mM DTT, 5% (w/v) glycerol, 3 mM potassium glutamate, and the concentration of $Mg(OAc)_2$ indicated in the figure legend. An ATP regeneration system (10 units/ml pyruvate kinase and 2 mM phosphoenolpyruvate) was also included. All incubations were carried out at 37 °C. The following are final concentrations. The wild-type RecA or RecA mutant protein (6.7 μM) and the indicated concentration of RecX protein were preincubated with 20 μM ϕX174 circular ssDNA for 10 min. SSB protein (2 μM) and ATP (3 mM) were then added, followed by another 10-min of incubation. The reactions were initiated by the addition of ϕX174 linear dsDNA to 20 μM. The reactions were incubated for 90 min. To stop the reaction, a 10-μl aliquot was removed and added to 5 μl of a solution containing 15% Ficoll, 0.25% bromphenol blue, 0.25% xylene cyanol, 72 mM EDTA, and 4% SDS. Samples were subjected to electrophoresis in 0.8% agarose gels with TAE buffer, stained with ethidium bromide, and exposed to ultraviolet light. Gel images were captured with a digital CCD camera utilizing GelExpert software (Nucleotech). When indicated, the intensity of DNA bands was quantitated with the software package TotalLab version 1.10 from Phoretix.

RESULTS

The RecX Protein Inhibits the Circular ssDNA-dependent ATPase Activity of the RecA Protein—The RecA protein hydrolyzes ATP when bound to DNA and the rate of ATP hydrolysis generally correlates well to the amount of RecA bound to the DNA (46). We previously showed that when RecX protein was added to RecA filaments assembled on circular ssDNA, a slow decline in ATP hydrolysis was observed that reflected a net end-dependent disassembly of the filaments (20) and occurred over a time span of 15–20 min. Analysis of this effect led to the conclusion that RecX regulated the extension of RecA filaments, capping the growing end (the 3′-proximal end) (20).

Here, we wished to examine the effects of Mg^{2+} and the RecA C terminus on the inhibitory effects of RecX. These factors are of interest since both of them modulate the functional state of RecA filaments *in vitro* (9). We therefore determined the effects of the RecX protein on the rate of the circular ssDNA-dependent ATP hydrolysis activity of the wild-type RecA and RecAΔC17 proteins, at two different Mg^{2+} concentrations. The RecA and RecX proteins were added at the same time in all experiments in this study, preincubated with the DNA for 10 min prior to the addition of ATP and SSB. Fig. 3 shows the amount of ATP hydrolyzed by the wild-type (Fig. 3*A*) or the RecAΔC17 (Fig. 3*B*) protein over time as a function of RecX concentration. At lower concentrations of RecX protein, the wild-type RecA-mediated ATP hydrolysis reaction is quite robust initially, suggesting that RecA filaments have been able to form. In the presence of 20–60 nM RecX protein, there is then a slow decline in the rate of ATP hydrolysis, indicating a relatively slow effect of RecX on the activity of the RecA filament. A significant inhibitory effect is obtained by the addition of 10 nM RecX protein. The addition of 80 nM RecX brings about almost complete inhibition in which the assembly of filaments on the DNA appears to be limited. As demonstrated elsewhere (20), the slow change reflects the slow and end-dependent dissociation of assembled RecA protein filaments from the circular ssDNA after the growing end of the filaments have been capped.

In contrast, the RecAΔC17 protein is less inhibited by RecX protein. There is only a slight decrease in the rate of ATP hydrolysis at 10 nM RecX, and a comparable level of inhibition of the RecAΔC17 protein requires about a 3-fold higher concentration of RecX protein. Complete inhibition of ATP hydrolysis occurs at RecX concentrations above 200 nM.

The concentration of Mg^{2+} was 10 mM in the experiments of Fig. 3, *A* and *B*. This is the optimum Mg^{2+} concentration for the DNA strand exchange mediated by the wild-type RecA protein, but the RecAΔC17 protein promotes DNA strand exchange better at lower Mg^{2+} concentrations (9). Mg^{2+} affects the functional state of RecA filaments (9). To complete the data set, we therefore carried out the same set of experiments under conditions that entail Mg^{2+} concentrations stoichiometric with the available ATP (9). At 3 mM Mg^{2+} ion, the wild-type RecA protein filaments appeared to be somewhat unstable even in the absence of the RecX protein. However, the effect of RecX protein was reduced relative to the results obtained at higher Mg^{2+} concentrations (Fig. 3*C*). The inhibition of RecA-medi-

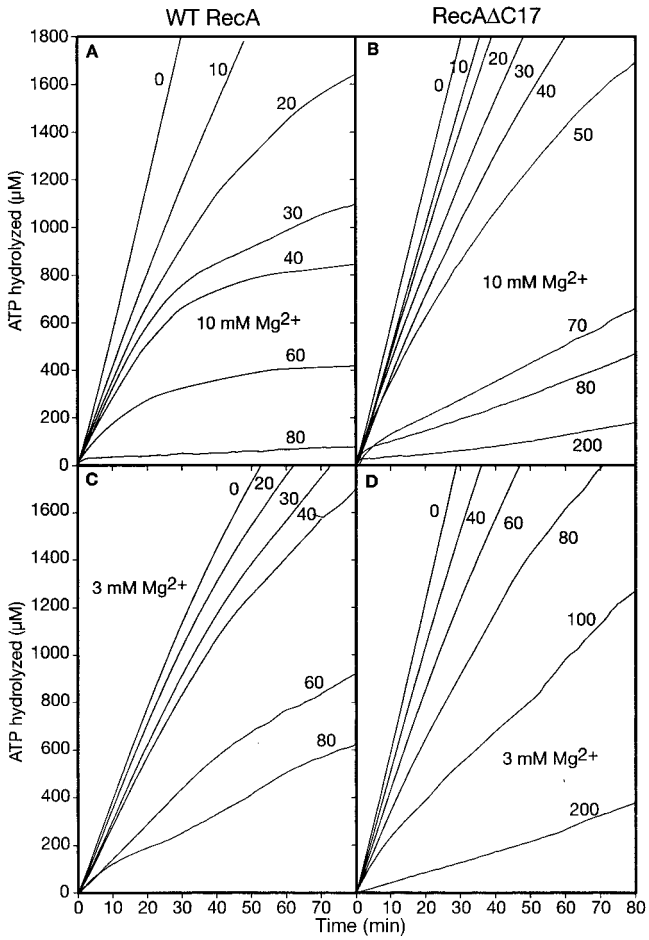

FIG. 3. **The effect of the RecX protein on the circular ssDNA-dependent ATPase activity of the wild-type RecA and RecAΔC17 proteins.** Reactions were carried out as described under "Experimental Procedures" and contained 3 mM ATP, 5 μM M13mp8 circular ssDNA, 3 μM wild-type RecA (*A* and *C*) or RecAΔC17 (*B* and *D*) protein and the indicated concentration of RecX protein (in nanomolar concentration). The concentrations of magnesium acetate used in each set of experiments are also indicated in the panels. In these trials, RecX and RecA were incubated together with the ssDNA for 10 min at 37 °C prior to the addition of the SSB and ATP to start the reaction.

ated ATP hydrolysis was alleviated to an even greater extent for the mutant RecAΔC17 protein (Fig. 3*D*). Thus, there are two factors that enhance the inhibition of RecA-mediated ATP hydrolysis by RecX protein, the presence of the RecA C terminus and significant concentrations of free Mg^{2+} ion. We assume that both of these factors enhance the RecA filament end-capping function of RecX protein, which involves an interaction with the growing end of a RecA filament (20).

Alterations of the RecA C terminus Render RecA Less Sensitive to Inhibition of RecA-mediated DNA Strand Exchange by the RecX Protein—Central to the recombinase activities of the RecA protein is the exchange of homologous DNA strands. The assay generally used to assess this activity *in vitro* is the DNA three strand exchange reaction diagrammed in Fig. 2*A*. The effect of RecX on the wild-type RecA-promoted DNA strand exchange reaction as a function of RecX concentration is shown in Fig. 4*A*. After 90 min, the final product of DNA strand exchange, nicked circular duplex, is reduced by 83% when the RecX protein concentration is 40 nM (a RecX:RecA ratio of 1:167.5) and is completely abolished at 10-fold higher RecX concentrations. As in the other experiments, the RecX protein was added to the reaction with the RecA protein. It is clear that RecX is able to inhibit the wild-type RecA protein at substoichiometric levels.

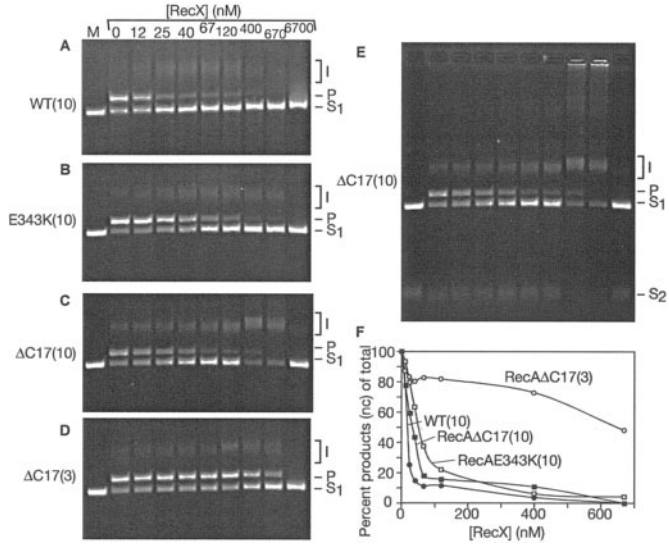

FIG. 4. **Effects of RecX protein on the RecA-promoted DNA strand exchange reaction *in vitro*.** RecX inhibition is dependent on the RecA C terminus and Mg^{2+} concentration. Reactions were carried out as described under "Experimental Procedures" and contained 6.7 μM RecA protein (wild-type, *A*; E343K, *B*; and RecAΔC17, *C–E*) and either 10 mM (*A*, *B*, *C*, *E*) or 3 mM (*D*) $Mg(OAc)_2$. *E* shows the entire gel from which *C* was derived to show the DNA complexes trapped in the wells in two of the lanes. *F*, quantitation of nicked circular products for each of the experiments *A–D*. The reaction is illustrated in Fig. 2, and the gel labels are described in Fig. 2 and its legend.

The effect of RecX protein was significantly moderated when the RecA protein had a point mutation near the C terminus (Fig. 4*B*). The RecA C terminus features seven negatively charged amino acid residues (35), and one of these is converted to a positive charge in the mutation in question (E343K). The RecA E343K mutant protein is fully competent in DNA strand exchange activities. Approximately 3–4-fold more RecX protein is required to produce a given level of inhibition of DNA strand exchange when this mutant protein is used.

Next, we assayed the effect of RecX on a C-terminal deletion mutant of RecA protein that removed all seven of the negatively charged amino acid residues. Assays were conducted under the same conditions used for the wild-type RecA protein. The RecAΔC17 protein-promoted DNA strand exchange reaction (Fig. 4*C*) was also inhibited by the RecX protein, but to a somewhat lesser extent than the wild-type RecA reaction. In this case, about twice as much RecX protein was needed to achieve a given level of inhibition.

As already noted, the optimum magnesium ion concentration for DNA strand exchange promoted by RecAΔC17 is actually much lower than the concentration optimal for wild-type RecA protein (9), approximately stoichiometric with the available ATP. We thus explored the effect of RecX at this lower (3 mM) concentration of Mg^{2+}. As indicated in *panel D*, the removal of 17 residues from the C terminus of RecA results in substantial protection of RecA from the inhibitory effects of the RecX protein, when the assay is carried out under the optimal conditions for RecAΔC17-mediated DNA strand exchange. Complete inhibition of the mutant-promoted reaction is not observed until the ratio of RecX to RecAΔC17 is 1:1 (Fig. 4*D*). Thus, both free Mg^{2+} and the RecA C terminus enhance the inhibitory effect of the RecX protein on RecA protein activity.

In Fig. 4*C*, much of the loaded DNA seems to disappear in the lanes containing 400 and 670 nM RecX protein. The missing DNA is present in what appear to be DNA aggregates that remain in the well of the gel. The full gel is shown in Fig. 4*E*. Aggregated complexes of this kind have been observed previously with the RecAΔC17 mutant protein at higher Mg^{2+} con-

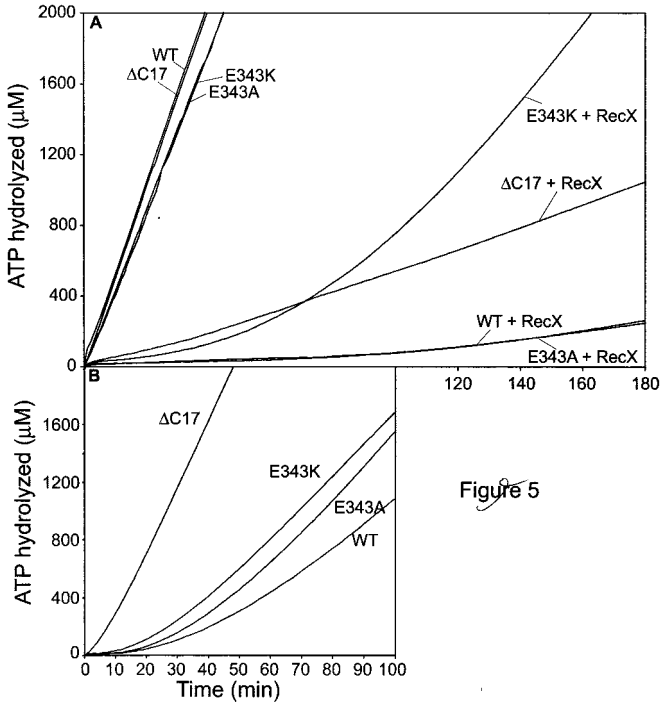

FIG. 5. **An altered interaction between RecA and RecX protein in the RecA E343K mutant.** *A*, ATPase assays were carried out as described in "Experimental Procedures" and the legend to Fig. 3, and contained 3 mM ATP, 10 mM $Mg(OAc)_2$, 5 μM M13mp8 circular ssDNA, 3 μM wild-type, or mutant RecA proteins, and 100 nM RecX where indicated. *B*, SSB displacement by the RecA mutants. Reactions were carried out as described in "Experimental Procedures" and the legend to Fig. 3 and contained 10 mM $Mg(OAc)_2$, except that ATP (3 mM) and SSB protein (0.5 μM) were preincubated with M13mp8 circular ssDNA (5 μM) for 10 min before adding the wild-type, ΔC17, E343K, or E343A RecA proteins to 3 μM to initiate the reaction.

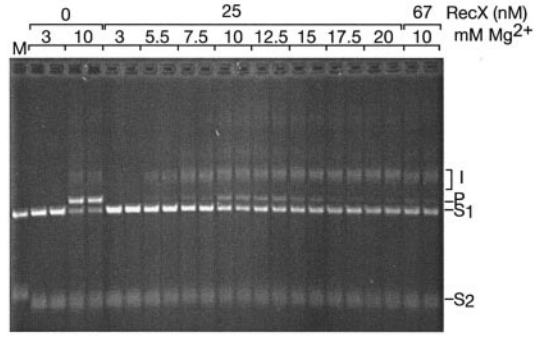

FIG. 6. **Mg^{2+} dependence of RecX inhibition of wild-type RecA promoted DNA strand exchange.** Reactions were carried out as described under "Experimental Procedures" and contained 6.7 μM wild-type RecA protein and the indicated concentrations of RecX protein (nM) and $Mg(OAc)_2$ (mM). Each experiment was done in duplicate, with the repeats presented in adjacent lanes, at each Mg^{2+} concentration. The reaction is illustrated in Fig. 2, and the gel labels are described in Fig. 2 and its legend.

centrations (9) and are thought to represent DNA networks that cannot be resolved. The added RecX protein may bring about the multiple strand invasions necessary to create these complex aggregates. Fig. 4*F* provides a quantitation of nicked circular products formed in the experiments shown in *panels A–D*.

The RecA E343K Mutation Facilitates Recovery from RecX Inhibition—Based on its moderating effect on DNA strand exchange (Fig. 4*B*), we decided to further explore the effects of RecA E343K on the RecX-RecA interaction. As shown in Fig. 5*A*, the RecA E343K mutant protein hydrolyzes ATP at rates similar to those seen for the wild-type protein, as does another mutant RecA protein with a neutral change at the same position, E343A. When the wild-type protein was preincubated with 100 nM RecX protein, the assembly of filaments was inhibited as before (Fig. 5*A*). Assembly of filaments by the RecA E343K mutant was also inhibited, but the ATPase activity of the mutant protein gradually recovered over a time course of 2 h. The wild-type RecA and the RecA E343A mutant exhibited little tendency to recover in the presence of this level of RecX protein over this same time period. The C-terminal deletion mutant RecAΔC17 was inhibited by the RecX protein (compare *panels A* and *B* in Fig. 3), but was somewhat more resistant to RecX inhibition of filament formation than was the wild-type protein. This was consistent with the greater resistance of this protein to inhibition of DNA strand exchange (Fig. 4*C*).

C-terminal deletion mutants of RecA protein have a greatly enhanced capacity to displace SSB protein (47), and the recovery of the RecA E343K mutant might be attributed to a similar capacity to nucleate enough filaments in the presence of bound SSB to circumvent the available RecX protein. We therefore examined SSB displacement by these mutants by precoating the ssDNA with the SSB before adding the RecA proteins. The RecA E343K did have a somewhat enhanced SSB displacement capacity, but it was very similar to the capacity exhibited by the RecA E343A mutant protein (Fig. 5*B*). As this latter protein did not recover in the presence of RecX we attributed the recovery of the RecA E343K mutant documented in Fig. 5*A* to an altered interaction between the RecA and RecX proteins.

RecX Inhibition of Wild-type RecA Is Highly Dependent on Magnesium Ion Concentration—Because RecX inhibition of RecAΔC17-promoted DNA strand exchange is dependent on Mg^{2+} concentration, we decided to look more closely at the Mg^{2+} dependence of RecX inhibition of wild-type RecA. As Fig. 6 shows, wild-type RecA does not promote DNA strand exchange at 3 mM Mg^{2+} either in the presence or absence of RecX. The maximum reaction in the presence of 25 nM RecX occurs at 10 mM Mg^{2+}, and the reaction is increasingly inhibited at higher Mg^{2+} concentrations. The reaction at 10 mM Mg^{2+} is inhibited in the presence of 67 nM RecX (Fig. 6). At magnesium ion concentrations above 15 mM, 25 nM RecX protein was sufficient to produce an almost complete inhibition of DNA strand exchange. Since Mg^{2+} can influence the physical state of RecA filaments, it is possible that RecX interacts preferentially with the RecA filament state present at high Mg^{2+} concentrations. Alternatively, RecX itself may be more active at high Mg^{2+} concentrations.

A RecX N-terminal His_6 Tag Increases the Inhibitory Effects of RecX Inhibition—In a previous study, a RecX protein with an N-terminal His_6 tag was utilized, and this appeared to generate more substantial levels of inhibition of RecA activities *in vitro* than we have demonstrated here (19). We obtained a sample of the His-tagged RecX protein, and carried out the experiment shown in Fig. 7. This experiment was done under the conditions used for the ATPase inhibition studies of Fig. 3. Using wild-type RecA protein and 10 mM Mg^{2+}, we show that only about half as much His-tagged RecX protein is required to bring about a given level of inhibition of RecA-mediated ATP hydrolysis. This result suggests that the His_6 tag enhances the interaction between RecX and RecA.

DISCUSSION

As measured by the effects on RecA-mediated ATP hydrolysis, the interaction between RecX protein and RecA protein filaments is enhanced by at least three factors: (*a*) the presence of Mg^{2+} concentrations in excess to those needed to chelate the available ATP, (*b*) the presence of the RecA C terminus or negatively charged amino acid residues therein, and (*c*) the

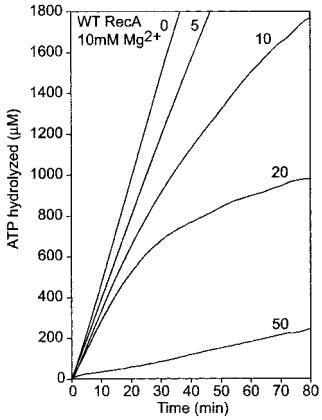

FIG. 7. **Inhibition of RecA protein-mediated ATP hydrolysis by RecX protein with an N-terminal His_6 tag.** Reactions were carried out as described under "Experimental Procedures" and the legend to Fig. 3 and contained 3 mM ATP, 10 mM $Mg(OAc)_2$, 5 μM M13mp8 circular ssDNA, 3 μM wild-type RecA protein, and the indicated concentration of RecX protein (in nanomolar).

addition of a His tag on the N terminus of RecX protein. A RecA E343K mutant protein, with an alteration in one of its C-terminal residues, also appears to moderate the RecX-RecA interaction. Together, the observations indicate that the RecA-RecX interaction is sensitive to both the RecA C terminus and to the functional state of the RecA filament. The work also suggests that the RecA-RecX interaction is mediated at least in part by the RecA C terminus. This information should help illuminate future efforts to probe the interaction between these two proteins. The work also reinforces the notion, proposed previously (20), that the RecX protein limits RecA filament extension by directly interacting with the RecA protein.

The effects of magnesium ion concentration speak to the functional state of RecA protein to which RecX protein binds most avidly. RecA filaments formed on ssDNA exist in at least two functional states, designated A_c and A_o (7, 9, 34). The two states are interconverted in response to changes in Mg^{2+} concentrations. The enhancement of the RecX inhibition by added Mg^{2+} ion in excess to that required to chelate the available ATP indicates that RecX protein interacts most effectively with a RecA A_o state (7, 9, 34).

The C terminus has a role in modulating the interchanges between the A_c and A_o states when RecA protein is bound to ssDNA (7, 9, 34). The moderating effects of a RecA C-terminal deletion and the E343K mutant (the latter point mutation being positioned in the C terminus) indicate that the C terminus plays a significant role in the RecA-RecX interaction. The last two observations, involving the His tag and the E343K mutant, further suggest that the RecX-RecA interaction involves, at least in part, ionic interactions. The helical groove in a RecA filament is a highly charged environment through which DNA strands must pass during DNA strand exchange reactions (32, 33). In an intact filament, RecX protein can bind within this groove, and interacts to a degree with the C-terminal domain of RecA (31).

When optimal conditions are used for both proteins, the RecAΔC17 mutant is not as sensitive to RecX protein as is the wild-type RecA protein. However, this compares the proteins under different conditions. We cannot compare the two RecA proteins at the lower Mg^{2+} ion concentrations that are optimal for RecAΔC17, since the wild-type protein is normally inactive in the DNA strand exchange reaction under those conditions. At the higher Mg^{2+} concentrations, the C-terminal deletion mutant is still active, but in a functional state that is apparently different than it exhibits without the excess Mg^{2+} (9). Here, we see that an increase in Mg^{2+} ion concentration changes the mutant RecA protein from a state that is relatively resistant to the effects of RecX, to one that is quite sensitive to RecX. This reinforces the previous observations indicating a Mg^{2+}-mediated change in functional state in the mutant protein (47). It again indicates that RecX protein binds most tightly to the A_o state of RecA filaments that exist at the higher Mg^{2+} concentration. RecX inhibition of wild-type RecA protein is also enhanced at higher Mg^{2+} concentrations (Fig. 6). Based on the clear effects of Mg^{2+} ion concentrations on RecA filament function (9, 34), we attribute most of the effects on changes in the functional state of RecA. However, we do not discount the possibility that the RecX protein itself is affected by Mg^{2+}, and this is an aspect of the problem that will require further investigation.

Elsewhere, we show that the effects of RecX protein on RecA activities are best explained by a RecX-mediated capping of the growing ends of RecA filaments (20). Within RecA filaments formed on circular DNAs, the capping would occur at short gaps that might exist in the filaments (20). This leads to a net dissociation of RecA from the unaffected disassembly end that can be monitored by changes in ATPase activity and by electron microscopy (20). The differences seen between the wild-type RecA and the RecAΔC17 proteins are best explained by a weaker affinity of RecX for the latter protein. It takes more RecX for a similar level of inhibition relative to the wild-type protein. In addition, there is a greater tendency for the RecA E343K protein to recover from the inhibition of RecX protein (Fig. 5) than is seen with the wild-type RecA protein. This further implicates the C terminus in the RecX-RecA interaction. However, if enough RecX protein is present, the assembly of RecAΔC17 protein filaments is strongly impeded, so that the RecX protein is clearly interacting with additional structural features of the RecA protein.

The RecX protein appears to be part of a growing suite of proteins that serves to modulate the assembly and disassembly of RecA filaments. With the RecFOR proteins (10–13), RecX protein would be present under normal growth conditions and would serve to modulate the formation and disassembly of RecA filaments for recombinational DNA repair and other functions. RecX is present at elevated levels during the SOS response. The DinI protein also appears to be a modulator, one that is specific to the SOS response (14–18). With its capacity to limit RecA filament extension, most likely by capping the filament (20), RecX would be expected to play a prominent role in limiting the length of RecA filaments *in vivo*. That role may complement the function of DinI, the RecFOR proteins, and perhaps other *E. coli* proteins.

Acknowledgments—We thank Elizabeth Wood for technical support in constructing the strains overproducing the native RecX protein. We also thank Hank Seifert (Northwestern University) and colleagues for the generous gift of His_6-tagged RecX protein.

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