RecX protein abrogates ATP hydrolysis and strand exchange promoted by RecA: Insights into negative regulation of homologous recombination

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In many eubacteria, coexpression of *recX* with *recA* is essential for attenuation of the deleterious effects of *recA* overexpression; however, the molecular mechanism has remained enigmatic. Here, we show that *Mycobacterium tuberculosis* RecX binds directly to *M. tuberculosis* RecA as well as *M. smegmatis* and *E. coli* RecA proteins *in vivo* and *in vitro*, but not single-stranded DNA binding protein. The direct association of RecX with RecA failed to regulate the specificity or extent of binding of RecA either to DNA or ATP, ligands that are central to activation of its functions. Significantly, RecX severely impeded ATP hydrolysis and the generation of heteroduplex DNA promoted by homologous, as well as heterologous, RecA proteins. These findings reveal a mode of negative regulation of RecA, and imply that RecX might act as an antirecombinase to quell inappropriate recombinational repair during normal DNA metabolism.

The past several years have seen a considerable progress in our understanding of the central role of *Escherichia coli* RecA protein in homologous recombination, DNA repair, restoration of stalled replication forks, induction of SOS response, and mutagenesis (1, 2). The much-studied homologous recombination process *in vitro* is the three-strand exchange reaction between circular single- and linear double-stranded DNA (1–3). In this model, the reaction proceeds in three sequential phases: (*i*) The *presynaptic* polymerization of RecA protein on single-stranded DNA; (*ii*) *synapsis*, the homologous alignment of nucleoprotein filament with linear double-stranded DNA; and (*iii*) unidirectional *strand exchange* (1–3). The mechanistic aspects of homologous recombination promoted by the prototype *E. coli* RecA protein may arguably be the best understood, but understanding its counterparts from other organisms will be essential to establish the generality of the phenomenon. To this end, we have described the biochemical characterization and x-ray structure of *M. tuberculosis* RecA (4–6).

In some eubacteria, *recX* is located on the same coding strand downstream of *recA* (7). In *Streptomyces lividans*, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, or *Thiobacillus ferrooxidans*, the ORFs of *recA* and *recX* overlap and the two genes are cotranscribed (7–12). It is known that overexpression of *recA* in *recX* mutants of *S. lividans*, *M. smegmatis*, or *P. aeruginosa*, but not mutant RecA, lead to induction of deleterious effects (8, 10, 13). However, the molecular mechanisms by which *recX* attenuates the deleterious effects induced by *recA* overexpression has remained unknown. Using *M. tuberculosis* as a model, we explored the mechanism by which RecA is regulated by RecX. Here, we show that RecX interacts directly with RecA *in vitro* and *in vivo* resulting in suppression of ATPase and strand exchange, processes that are central to homologous recombination. The negative regulation of RecA by RecX implies that RecX might act as an antirecombinase to quell inappropriate recombinational repair during normal DNA metabolism.

Materials and Methods

DNA and Proteins. RecA from *E. coli* (EcRecA), *M. tuberculosis* (MtRecA), *M. smegmatis* (MsRecA) (ref. 4; N. Ganesh and K.M., unpublished observations), and single-stranded DNA binding protein (SSB) from *E. coli* (EcSSB), *M. tuberculosis* (MtSSB), and *M. smegmatis* (MsSSB) were purified (14) and their concentrations determined as described (15). Negatively supercoiled (form I) and circular single-stranded M13 DNA (ssDNA) was prepared as described (16). The concentrations are expressed in moles of nucleotide residues.

Purification of RecX. *E. coli* BL21(DE3)[pLysS] strain harboring *M. tuberculosis* recX gene on plasmid pET15b was cultured in 1 liter of LB medium containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37°C. At mid-exponential phase ($A_{600} = 0.4$), *recX* expression was induced by adding isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.5 mM and incubated for 4 h. All subsequent steps were performed at 4°C unless indicated otherwise. Cell paste (8 g) was suspended in 30 ml of buffer containing 50 mM Tris·HCl (pH 8) and 10% (wt/vol) sucrose. Cells were disrupted by sonication. The cell lysate was centrifuged at 25,000 rpm for 60 min. Protein was precipitated by the addition of $(NH_4)_2SO_4$ (0.3 g/ml) over 1 h, and collected by centrifugation at 10,000 rpm for 20 min. The precipitate was resuspended in 12 ml of 20 mM Tris·HCl (pH 7.5) buffer containing 20% glycerol, 5 mM 2-mercaptoethanol, and 0.4 M NaCl (buffer A), and dialyzed against 500 ml of buffer A with four changes over 20 h. The dialysate was loaded onto a Ni^{2+}-agarose (Qiagen) column (3 ml), which had been equilibrated with buffer A. The column was washed with 20 ml of buffer A containing 20 mM imidazole. The bound proteins were eluted with a 20-ml linear gradient of 20–200 mM imidazole in buffer A. The fractions containing RecX were combined and dialyzed against 20 mM Tris·HCl (pH 7.5) buffer containing 20% glycerol, 1 mM DTT, and 0.4 M NaCl. Aliquots of RecX were stored at −70°C. Protein purity was assessed by SDS/10% PAGE/Coomassie blue staining (17). Protein concentration was determined using BSA as the standard (18).

Immunoprecipitation and Immunoblotting. Polyclonal antibodies against EcRecA and *M. tuberculosis* RecX were generated in rabbits. Anti-EcRecA antibodies cross-reacted with MtRecA (4), as well as MsRecA (14). Anti-RecX antibodies cross-reacted with purified RecX, as well as a 19-kDa protein in the cell lysates of *M. tuberculosis* and *M. smegmatis*.

Cell lysates from *E. coli* BL21 bearing *M. tuberculosis* recX

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Abbreviations: ssDNA, single-stranded DNA; SSB, single-stranded DNA binding protein.

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gene, *M. tuberculosis* H37 Rv, *M. smegmatis* mc^{2}155, and its isogenic Δ*recA* strain, HS42 (13), were prepared as described (19). Where specified, cell lysates (200–300 μg) were incubated with 10 μg of DNase I at 37°C for 15 min, before immunoprecipitation reactions. Under these conditions, 10 ng of [^{3}H]ssDNA incubated with the lysates was completely degraded to acid-soluble nucleotide residues by 10 μg of DNaseI (data not shown). Immunoprecipitation and immunoblotting experiments were performed as described (14). An aliquot of cell lysate (0.5–0.75 mg protein) in 20 mM Tris·HCl (pH 7.5)/0.2 M NaCl/1 mM ATP (buffer TNA) was incubated with anti-RecA or anti-RecX antibodies at 4°C for 6 h, and then with 50 μl of slurry of protein A-Sepharose for an additional 2 h. Immunoprecipitates were collected by centrifugation, washed six times with TNA buffer containing 0.1% Triton X-100, stained with appropriate primary and secondary antibody, and visualized by chemiluminescence (14, 20).

Interaction of RecX with RecA Proteins *in Vitro*. Approximately, 370 μg of RecX was immobilized onto Ni^{2+}-agarose (1.5 ml) via $(His)_6$-tag at the N-terminal end with gentle agitation at 4°C. The amount of protein bound to the resin was determined from A_{280} measurements of unbound protein in the supernatant. An equal amount of specified RecA or SSB was incubated with the resin. Protein-bound matrices were poured into individual columns and washed extensively with loading buffer containing 0.1 M NaCl until the eluate contained no material that absorbed light at 280 nm. These columns were then eluted with buffer A containing 0.3 M imidazole, and fractions of 0.5 ml were collected at a flow rate of 0.5 ml/4 min. An aliquot from each fraction was analyzed by SDS/PAGE and visualized by staining with Coomassie blue. Surface plasmon resonance (SPR) measurements were performed with biosensor chips containing immobilized RecA or 60-mer immobilized ssDNA according to the manufacturer's suggestions (Pharmacia Biosciences).

Mobility Shift Assay. Reaction mixture (20 μl) containing 30 mM Tris·HCl (pH 7.5), 12 mM $MgCl_2$, 0.1 mM ATPγS, or 1 mM ATP (Fig. 4*C*) with 3 μM ^{32}P-labeled 40-mer, single- or double-stranded DNA, 1 μM RecA, and increasing concentrations of RecX were incubated at 37°C for 10 min. In competition reactions, RecX was incubated with RecA for 10 min before the addition of DNA. The reactions were terminated by the addition of 3 μl of loading buffer [20% glycerol containing 0.12% (wt/vol) each of bromophenol blue and xylene cyanol] to each sample. The samples were separated on an 8% polyacrylamide gel by electrophoresis in 13.2 mM Tris/acetate buffer (pH 7.4) at 12 V/cm for 4 h at 4°C. The gel was dried on to a Whatman 3 MM filter paper and DNA–protein complexes were visualized by autoradiography.

ATPase Assay. The effect of RecX on ATP hydrolysis promoted by RecA was monitored using a coupled enzymatic assay (21). Reactions were initiated by the addition of RecA (0.8 μM). Where indicated, SSB (0.2 μM) or RecX (0.5 μM) were added 5 min after the addition of RecA. In experiments involving RecA, SSB, and RecX, after the initiation of the reaction with RecA, SSB, and RecX were added at 2.5 min and 5 min, respectively. ATP hydrolysis was monitored for 30 min at 37°C by using a Shimadzu spectrophotometer equipped with a thermostated cuvette holder and recording attachment. The rates of the reaction were calculated from the slopes of the curves and expressed as μmoles of ATP hydrolyzed min^{-1}·$liter^{-1}$.

Strand Exchange Assay. Reaction mixture (40 μl) contained 33 mM Hepes-KCl buffer (pH 7), 1 mM DTT, 1.5 mM ATP, 12 mM $MgCl_2$, 5 mM phosphocreatine, 10 units/ml phosphocreatine kinase, 10 μM ssDNA, and 5 μM of the indicated RecA protein.

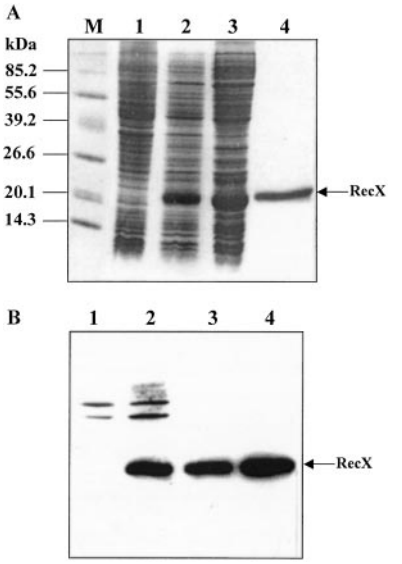

Fig. 1. SDS/PAGE analysis showing induced expression of RecX in *E. coli* and at various stages during its purification. (*A*) Five micrograms of protein was separated by SDS/PAGE and visualized by staining with Coomassie blue. Lane M, molecular mass markers; lane 1, uninduced cell lysate; lane 2, induced cell lysate; lane 3, $(NH_4)_2SO_4$ pellet fraction; lane 4, purified RecX. (*B*) Western blot analysis. Lane 1, uninduced cell lysate; lane 2, induced cell lysate; lane 3, 5 μg of purified RecX; lane 4, 10 μg of purified RecX.

Reaction mixtures were incubated at 37°C for 5 min before the addition of 0.66 μM of the indicated SSB and incubation was continued for 5 min. For *M. tuberculosis* RecA, the reaction mixture was similar except that the buffer was 33 mM Tris·HCl (pH 8.5) containing 9 mM Mg^{2+} (5). RecX was added at the indicated concentration to the reaction mixtures and incubation was continued for an additional 5 min. Reaction was then initiated by the addition of 10 μM linear duplex DNA (form I DNA cut with *Hin*cII). All reaction mixtures were incubated for 60 min. Samples were deproteinized by incubation with SDS (0.1%) and proteinase K (0.2 mg/ml) at 37°C for 15 min and electrophoresed through 0.8% agarose gel in 89 mM Tris/borate buffer (pH 8.3) at 2V/cm for 11 h. The substrates and products of the reaction were visualized by staining with ethidium bromide (0.5 μg/ml).

Results

Affinity Purification of RecX. *M. tuberculosis* recX gene was overexpressed in *E. coli* as a fusion protein bearing six histidine residues at the N-terminal end. RecX was purified by affinity chromatography to >95% homogeneity (Fig. 1*A*). The identity of His-tagged RecX in cell lysates and in fractions during purification was ascertained by Western analysis with anti-His antibodies (Fig. 1*B*). The N-terminal His-tag was removed by proteolytic digestion (22). The identity of RecX was verified by sequencing 10-aa residues at the N-terminal end. The sequence analysis revealed that the determined sequence matched with the predicted sequence (23). The His-tagged RecX behaved similar to that of RecX lacking the tag in both ATPase and strand exchange assays (data not shown).

RecX Is Associated with RecA *in Vivo*. The observation that *recX* alleviates deleterious effects of *recA* overexpression imply that their gene products might be associated with each other *in vivo*. To investigate this possibility, immunoprecipitation assays were performed with cell lysates of *M. tuberculosis*, *M. smegmatis*, and *E. coli* expressing *M. tuberculosis recX*. In wild-type cells, *recA* expression is constitutively activated by ssDNA that are tran-

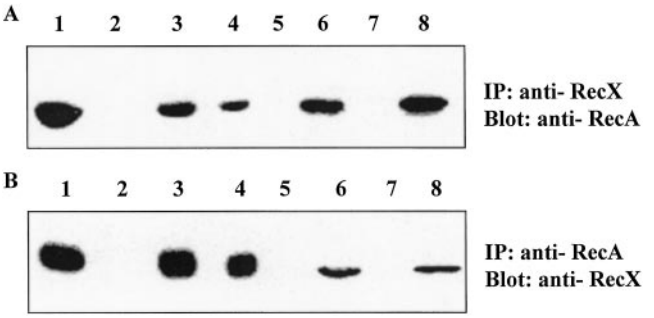

Fig. 2. RecX is associated with RecA *in vivo*. (*A*) RecA was immunoprecipitated (IP) with anti-RecX antibodies and immunoblotted (Blot) with anti-RecA antibodies. Lane 1, MtRecA. Immunoprecipitates from cell lysates of *M. tuberculosis* H37 Rv (lane 2) or *M. smegmatis* mc^{2}155 (lane 5) with preimmune serum. The remaining lanes represent immunoprecipitates from cell lysates of indicated strains with anti-RecX antibodies. Lane 3, *E. coli* bearing *M. tuberculosis recX;* lane 4, *M. tuberculosis* H37 Rv; lane 6, *M. smegmatis* mc^{2}155; lane 7, *M. smegmatis* mc^{2}155 Δ*recA*; lane 8, DNase I-treated cell lysate from *M. smegmatis* mc^{2}155. (*B*) RecX was immunoprecipitated with anti-RecA antibodies and then immunoblotted with anti-RecX antibodies. Lane 1, purified RecX. Immunoprecipitates from cell lysates of *M. tuberculosis* H37 Rv (lane 2) or *M. smegmatis* mc^{2}155 (lane 5) with preimmune serum. The remaining lanes contained immunoprecipitates of cell lysates of indicated strains using anti-RecA antibodies. Lane 3, *E. coli* bearing *M. tuberculosis recX*; lane 4, *M. tuberculosis* H37 Rv; lane 6, *M. smegmatis* mc^{2}155; lane 7, *M. smegmatis* mc^{2}155 Δ*recA*; lane 8, DNase I-treated cell lysate from *M. smegmatis* mc^{2}155.

siently generated during DNA replication under normal growth conditions (2, 19). The existence of a complex of RecA–RecX was ascertained by two parallel experiments. In one experiment, cell lysates were immunoprecipitated with anti-RecX antibodies and the immunoprecipitates were analyzed for the presence of RecA by immunoblotting with anti-RecA antibodies. In the complementary approach, immunoprecipitation was performed with anti-RecA antibodies and then immunoblotted with anti-RecX antibodies. We found that RecX and RecA could be coimmunoprecipitated from cell lysates of *M. tuberculosis* with anti-RecA or anti-RecX antibodies (Fig. 2). Similarly, immunoprecipitation of cell lysates from *M. smegmatis* or *E. coli* expressing *M. tuberculosis* recX with anti-RecA or anti-RecX antibodies indicated that RecX was associated with RecA. More importantly, these experiments revealed that RecX was able to associate with endogenous *E. coli* RecA (Fig. 2, lane 3). The interaction was specific, because neither RecX nor RecA were recovered from cell lysates of *M. smegmatis* Δ*recA* or wild-type strains incubated with preimmune serum. Coimmunoprecipitation of RecA and RecX was not affected by DNase I, thus excluding the possibility of their association via a DNA bridge (Fig. 2, lane 8).

RecX Interacts with MtRecA, as Well as MsRecA and EcRecA, *in Vitro*. To corroborate interaction between RecX and RecA *in vivo*, we performed affinity chromatography by using His-tagged RecX tethered to Ni^{2+}-agarose. RecA from the indicated source was chromatographed over the tethered RecX to abet their interaction. Analysis of bound proteins revealed that MtRecA was retained on the column and coeluted with RecX (Fig. 3*A*). MsRecA and EcRecA also bound to tethered RecX, and coeluted in a similar fashion (Fig. 3 *B* and *C*). As a control for the specificity of interaction, we tested the ability of MtSSB to bind MtRecX. However, MtSSB was recovered in the flow-through and column wash, indicating its inability to bind RecX (Fig. 3*D*). These results suggest specific interaction of RecX with MtRecA, as well as EcRecA and MsRecA, *in vivo* and *in vitro*. By SPR measurements, we determined the affinity of RecX for RecA

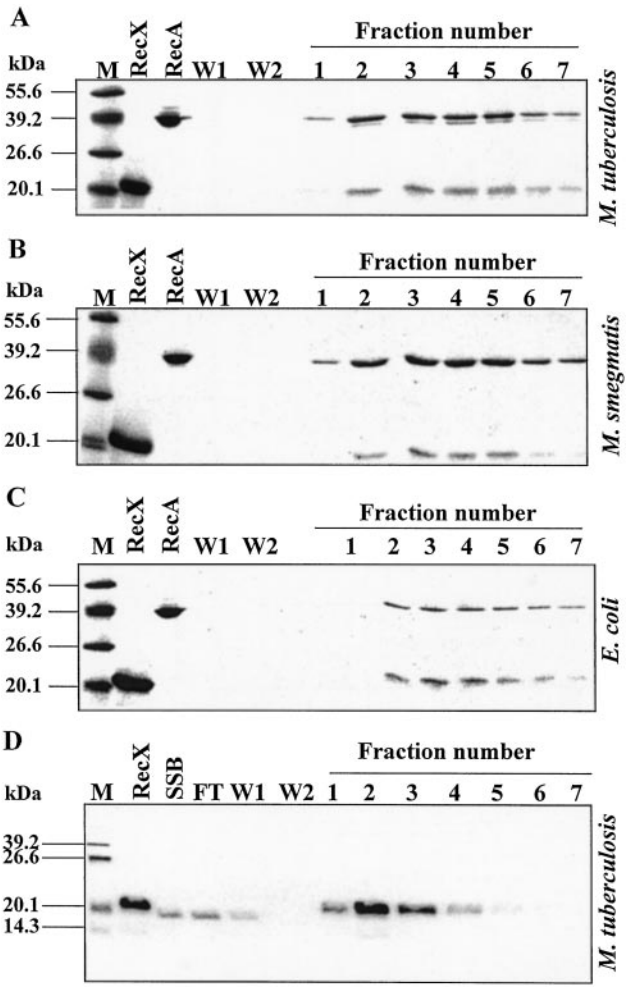

Fig. 3. Interaction of RecX with RecA or SSB. RecX-Ni^{2+} agarose was incubated with the indicated RecA or SSB, and analyzed as described in *Materials and Methods*. Lanes marked FT, W1, and W2 correspond to flow-through (FT) and wash (fractions 1 and 2), respectively, from the column before elution. Lane M contained molecular mass markers, whose position (kDa) is indicated on the left. Under these conditions, SSB or RecA failed to interact with resin in the absence of tethered RecX.

under equilibrium conditions. The values obtained for K_A were in the range of (1–3) $\times$ 10^5 M^{-1} (data not shown).

RecX Neither Bound DNA Nor Prevented the Binding of RecA or SSB to ssDNA. To gain insights into the mechanism of quelling of deleterious effects of *recA* overexpression by *recX*, we asked whether purified RecX could affect DNA-binding properties of RecA or SSB. Reactions were performed with stoichiometric amounts of MtRecA and ^{32}P-labeled 40-mer DNA in the absence or presence of varying amounts of RecX. In the absence of RecX, RecA or SSB formed protein–DNA complexes that displayed retarded mobility compared with free DNA. Analysis of the products of the competition assay revealed that RecX failed to inhibit binding of either RecA (Fig. 4*A*) or SSB (Fig. 4*B*) to ssDNA. Separately, we examined the ability of RecX to bind single- or linear double-stranded DNA, the substrates used in strand exchange reactions. Incubation of increasing concentrations of RecX with a fixed amount of DNA failed to alter their mobility, indicating lack of binding of RecX to both the DNA substrates under these conditions (Fig. 4*C*).

RecX Inhibits ATP Hydrolysis Promoted by RecA. To test the functional significance of RecA–RecX interaction, we examined the

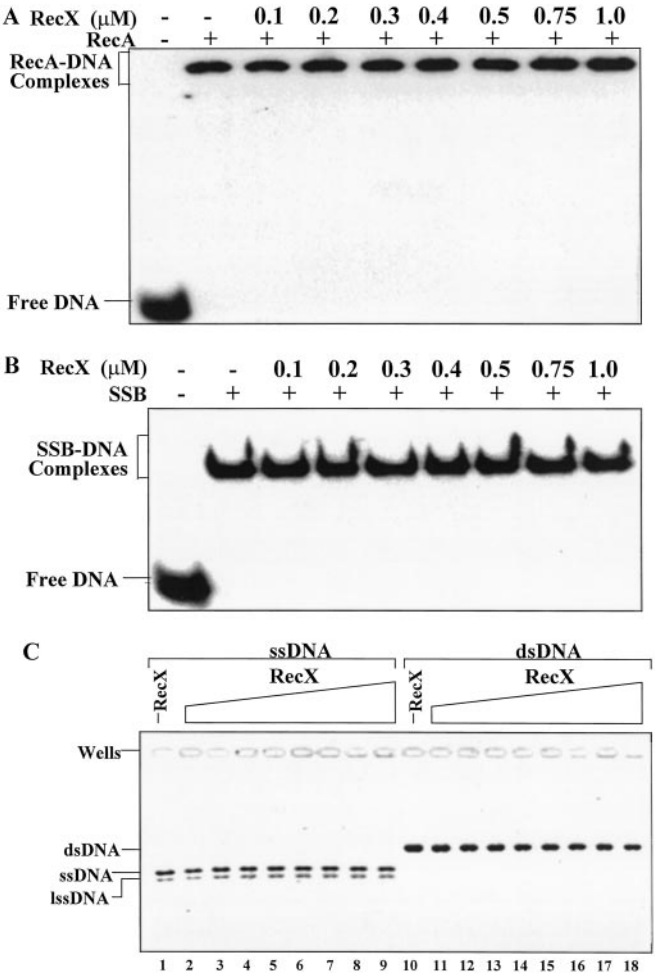

Fig. 4. Effect of RecX on binding of RecA or SSB to DNA. (*A*) RecX failed to inhibit binding of MtRecA to ssDNA. Reactions were performed in the absence or presence of indicated amounts of RecX as described in *Materials and Methods*. (*B*) RecX failed to inhibit binding of MtSSB to ssDNA. Reaction was performed with 0.2 μM MtSSB and increasing concentrations of RecX, as described above. (*C*) RecX failed to bind single- or double-stranded DNA. Reaction mixtures (20 μl) containing10 μM circular single- or linear double-stranded DNA were incubated for 1 h at 37°C. Samples were analyzed by gel mobility shift assays, and visualized by staining with ethidium bromide. Lanes 1 and 10, DNA alone; lanes 2–9 and 11–18, DNA plus RecX at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, and 4 μM, respectively. dsDNA, linear double-stranded DNA; lssDNA, linear single-stranded DNA

effect of RecX on the ATPase activity of EcRecA or MtRecA. To demonstrate this effect more clearly, we used 40-mer DNA because RecX failed to impede the binding of RecA or SSB to this substrate (Fig. 4), but served as an effective cofactor in ATPase assays (14); and M13 ssDNA. In controls, the rate of ATP hydrolysis obtained from triplicate experiments were similar to those reported previously (ref. 4; Table 1). Strikingly, addition of RecX resulted in robust inhibition of ATP hydrolysis promoted by EcRecA and MtRecA by 3- to 4-fold and 8- to 15-fold with 40-mer and ssDNA, respectively. Although the molecular basis underlying the differences between DNA lattices is obscure, it is possible that inhibition arises solely by interaction of RecX with RecA nucleoprotein filament or free RecA, rather than naked DNA. In parallel experiments, we ascertained the type of inhibition and K_i from double reciprocal plots (data not shown). The curves obtained correlated with uncompetitive type of inhibition and yielded a K_i of 52 nM.

To further test whether RecX influences binding of ATP to

Table 1. Effect of RecX on the rate of ssDNA-dependent ATP hydrolysis by MtRecA and EcRecA

	ATPase activity (μmol·min^{-1})			
	M13 ssDNA		40-mer DNA	
Proteins	−RecX	+RecX	−RecX	+RecX
No RecA	ND	ND	ND	ND
EcRecA	9.6	1.2	8.9	3.4
EcRecA + EcSSB	14.6	1.1	7.4	2.2
EcRecA + MtSSB	12.6	1.3	8.6	2.4
MtRecA	7.2	2.6	6.7	3.2
MtRecA + MtSSB	8.4	1.2	5.6	1.3
MtRecA + EcSSB	6.6	0.8	4.0	1.3

+ and − correspond to the presence or absence of RecX, respectively. ND, no detectable ATPase activity in the presence (+) or absence (−) of RecX.

RecA, ^{32}P-labeled ATP was incubated with MtRecA in the presence or absence of RecX, and bound ATP was subsequently crosslinked to RecA (4). The extent of crosslinking of ATP to MtRecA was not altered by incubation with increasing concentrations of RecX (Fig. 5). Under these conditions, RecX or MtSSB failed to bind detectable amounts of ^{32}P-labeled ATP.

RecX Prevents Strand Exchange Promoted by RecA. We next examined the effect of RecX on recombination promoted by homologous, as well as heterologous, RecA and SSB. In control reactions, we observed that EcRecA displayed maximal strand exchange in the presence of EcSSB (Fig. 6*A*, lane 3). Addition of increasing amounts of RecX led to marked inhibition of heteroduplex DNA synthesis (hDNA) by EcRecA with intermediates stalled at different stages of strand exchange (Fig. 6*A*, lanes 4–8). Similarly, MtRecA in the presence of EcSSB or MtSSB generated nearly equivalent amounts of hDNA and DNA networks that stayed in the wells (Fig. 6 *A*, lane 9, and *B*, lane 3). Addition of varying amounts of RecX resulted in robust inhibition of hDNA synthesis, although trace amounts of intermediates persisted at lower concentrations of RecX (Fig. 6 *A*, lanes 10–14, and *B*, lanes 4–9). Interestingly, addition of RecX to the reaction promoted by MsRecA led to complete inhibition of hDNA synthesis and intermediates (Fig. 6*B*, lanes 10–14). The amount of RecX required to suppress strand exchange promoted by EcRecA was 100-fold higher relative to MtRecA or MsRecA, presumably because of its heterologous source. These results also emphasize that RecX exerted its anti-recombinase effects through RecA, and not via DNA, because the amount of DNA in each of these reactions remained the same. Using SPR, we

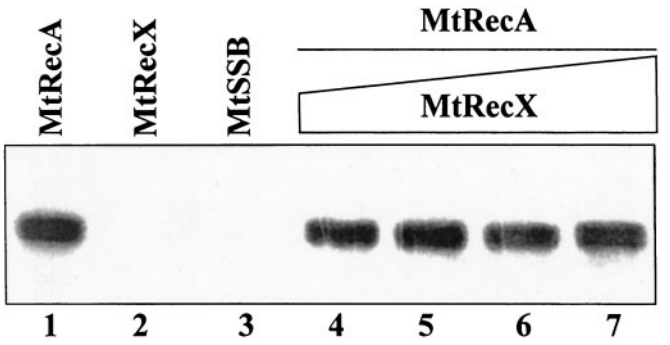

Fig. 5. RecX failed to inhibit binding of ATP by RecA. Reactions were performed and analyzed as described (4). Lane 1, RecA (0.75 μM); lane 2, MtRecX (1.5 μM); lane 3, MtSSB (1.5 μM). Lanes 4–7 contained 0.75 μM MtRecA and 0.75, 1, 1.2, and 1.75 μM RecX, respectively.

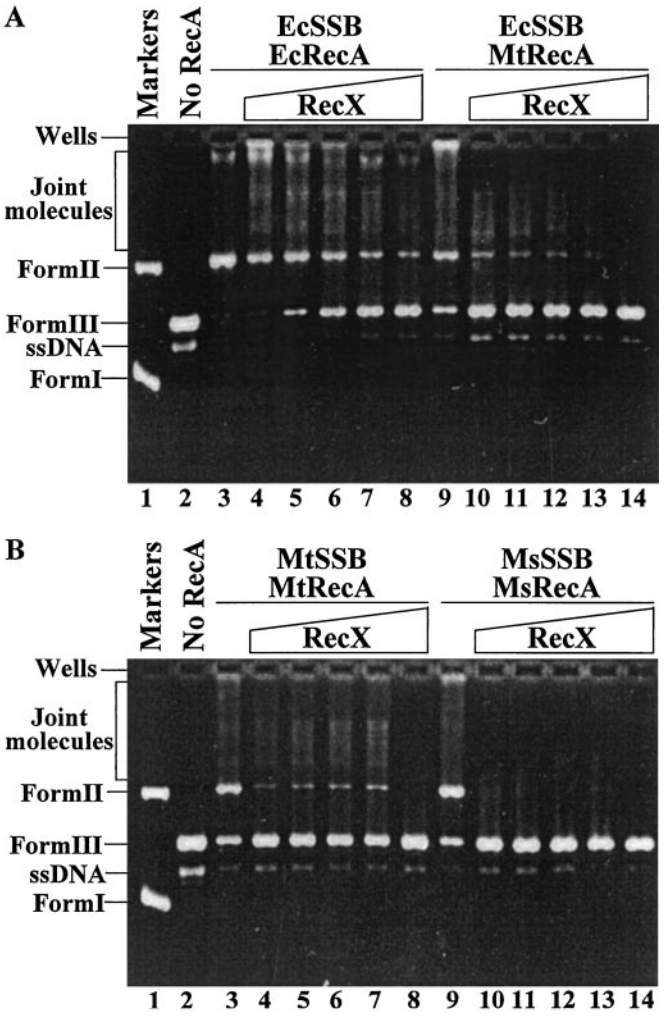

Fig. 6. RecX inhibits strand exchange promoted by RecA. (*A*) Effect of RecX on strand exchange promoted by EcRecA and EcSSB (lanes 3–8) or MtRecA and EcSSB (lanes 9–14). Lanes 3 and 9 represent controls in the absence of RecX. Lanes 4–8 represent reactions with EcRecA and EcSSB in the presence of 0.5, 1, 2.5, 5, or 7.5 μM RecX, and lanes 10–14 contained reactions with MtRecA and EcSSB in the presence of 0.025, 0.05, 0.075, 0.1, and 0.25 μM RecX, respectively. (*B*) Effect of RecX on strand exchange promoted by MtRecA and MtSSB (lanes 3–8) or MsRecA and MsSSB (lanes 9–14) in the absence (lanes 3 and 9) or presence (lanes 4–8 and 10–14) of 0.025, 0.05, 0.075, 0.1, and 0.25 μM RecX, respectively.

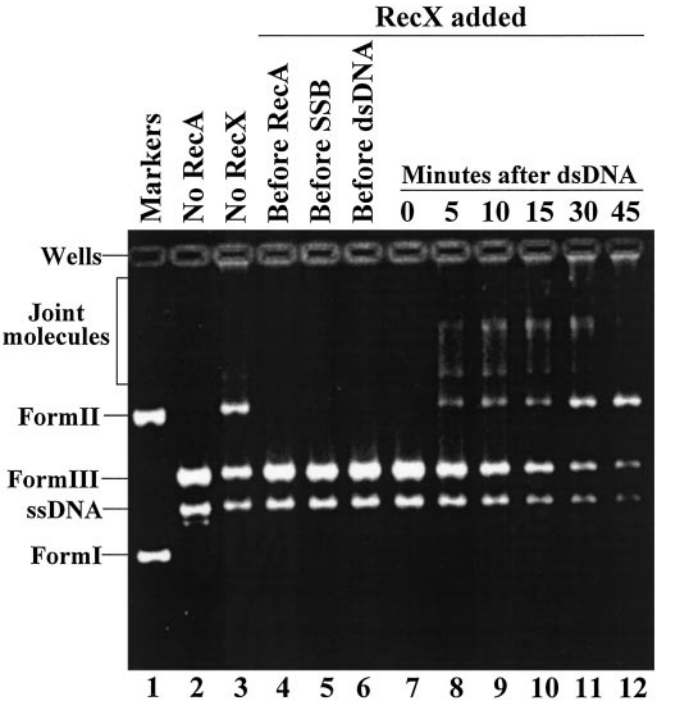

Fig. 7. RecX arrests strand exchange reaction in progress. Reactions were performed as described in the legend to Fig. 6, except that RecX (0.25 μM) was added to the reaction mixture at 0, 5, 10, 15, 30, and 45 min, respectively, after initiation of reaction by the addition of linear duplex DNA. All reactions were terminated after a 60-min incubation.

determined the affinity of EcRecA, MtRecA, or MsRecA for ssDNA under equilibrium conditions. We found that the K_A was in the range of 10^6 M^{-1} in the presence of ATP or dATP, indicating that the strength of binding of RecAs had no effect on the function of RecX (data not shown).

To test the effect of RecX on the progression of strand exchange, RecX was added to reaction promoted by MtRecA at the indicated time intervals. In the absence of RecX, MtRecA generated maximal amounts of hDNA (Fig. 7, lane 3). However, addition of RecX at various time intervals coincided with corresponding decline in the extent of formation of hDNA, as well as intermediates, depending on the time of its addition (Fig. 7, lane 3 vs. lanes 8–12). These results suggest that RecX can also impede the progression of strand exchange, leading to the accumulation of intermediates.

Discussion

In this study, we present evidence in favor of three conclusions. First, RecX forms a stable complex with RecA both *in vivo* and *in vitro*. Second, RecX was able to inhibit ATP hydrolysis promoted by RecA. Third, although RecX failed to impede binding of RecA or SSB to ssDNA, it abolished initiation and progression of strand exchange. Although these results provide evidence that direct interaction between RecA and RecX might be required for negative regulation of *recA* overexpression, it remains possible that RecX acts via DNA in some manner under stringent conditions. If the latter view is correct, then it suggests that RecX may quell the deleterious effects of *recA* overexpression by two independent mechanisms. We consider the implications of these findings in regard to the attenuation of deleterious effects induced by *recA* overexpression and homologous recombination.

What could be the biochemical link between induction of deleterious effects and *recA* overexpression? The fact that the absence of *recX* leads to lethality, arising from *recA* overexpression, indicates that excessive amounts of RecA might cause defects in processes such as DNA repair, replication, or mutagenesis that are likely to have the greatest impact during normal DNA metabolism. The present studies suggest a model in which repression of ATP hydrolysis and strand exchange appears to be the potential mechanism of RecX function. Regardless of the mechanism, RecX was able to attenuate RecA at exceedingly lower concentrations. An alternative explanation would be that attenuation might be through binding of RecX to the *recA* promoter. A caveat of this interpretation is that *recA* expression in *recX* mutants of *P. aeruginosa* and *S. lividans* was similar to that of wild-type strains (8, 11). Similarly, the lack of binding of RecX to DNA suggests that *recA* promoter is unlikely to be the site of RecX action (Fig. 4).

How does RecX contribute to regulation of homologous recombination? The data presented in this study raise the possibility of negative regulation of recombination-like activities of RecA by RecX. Although the consequence of *recA* overex-

pression on *M. tuberculosis* Δ*recX* strain remains to be delineated, our *in vitro* results are consistent with genetic evidence linking attenuation of deleterious effects of *recA* overexpression to *recX* in many eubacteria including *M. smegmatis* (8, 10, 13). The *oraA* (*recX* homologue) of *E. coli* shares considerable sequence similarity with *P. aeruginosa recX*, but its function is unknown (24).

Stohl and Seifert (25) have reported an instance of positive genetic interaction between *recA* and *recX* in pilus variation in *Neisseria gonorrhoeae*. However, *recX* mutant displayed the same increases in phase variation under iron-starved conditions, raising the possibility that the positive effect of *recX* might be indirect. Consistent with this notion, efforts to identify association between gonococcal RecX with RecA have been unsuccessful (25).

A distinctive feature of RecX is its relatively high pI of 9.6. It therefore would seem that the positive charge of RecX would abet its interaction with negatively charged DNA. However, RecX failed to form protein–DNA complexes with ssDNA or linear double-stranded DNA at the concentrations used under RecA assay conditions. It should be noted that whereas the basic pI of RecX is expected to promote its interaction with DNA, there are a number of possible reasons why it does not appear to interact with DNA. One possibility is that the charge on its molecular surface could be different. The presence of helix–turn–helix motif is normally present in proteins that bind DNA; however, AsiA, the anti-Sigma factor that bears the helix-turn-helix motif does not interact with DNA (26). There are many examples of basic proteins that do not interact with nucleic acids.

Previous studies have shown that *E. coli* DinI modulates the biochemical activities of its cognate RecA protein. The genetic approach revealed that overexpression of DinI suppressed induction of SOS response and impaired cleavage of LexA and UmuD *in vivo* (27). Recent studies have shown that DinI physically interacts with RecA at the ssDNA-binding site, thereby precluding it from forming an active nucleoprotein filament (28, 29). DinI also binds to the RecA nucleoprotein filament, thus inhibiting RecA-mediated homologous pairing, coprotease, but not the ATPase activity (28, 29). The interplay between DinI-RecA differs from that of RecA–RecX interaction in several ways. The most notable difference is the relatively high concentration of DinI required for the inhibition of strand exchange activities of EcRecA. In contrast, RecX is required in exceedingly low levels to inhibit strand exchange activity of RecA. This level of sensitivity suggests a direct role for RecX in RecA-promoted homologous recombination. We also note that, unlike RecX, DinI inhibits only the initiation of strand exchange, but not strand transfer and its deletion has no phenotype under normal growth conditions (27, 29).

There is an interesting finding in this work that warrants further study. For inhibition of strand exchange, RecX was required at exceedingly lower concentrations, whereas higher amounts were required to achieve maximal inhibition of ATPase activity. It is known that all monomers in the RecA nucleoprotein filament hydrolyze ATP and contribute to net ATPase activity, whereas pairing and strand exchange involve localized regions that are covered by RecA (1–3). One intriguing possibility would be that binding of RecX to RecA at a localized region might be sufficient to cause inhibition, whereas higher concentrations are required to suppress ATPase activity. Finally, the negative regulation of ATPase and strand exchange promoted by RecA by RecX implies that these two proteins act as antagonistic partners in homologous recombination.

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