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Bacillus subtilis RecU Holliday-junction resolvase modulates RecA activities

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ABSTRACT

The *Bacillus subtilis* RecU protein is able to catalyze *in vitro* DNA strand annealing and Holliday-junction resolution. The interaction between the RecA and RecU proteins, in the presence or absence of a single-stranded binding (SSB) protein, was studied. Substoichiometric amounts of RecU enhanced RecA loading onto single-stranded DNA (ssDNA) and stimulated RecA-catalyzed D-loop formation. However, RecU inhibited the RecA-mediated three-strand exchange reaction and ssDNA-dependent dATP or rATP hydrolysis. The addition of an SSB protein did not reverse the negative effect exerted by RecU on RecA function. Annealing of circular ssDNA and homologous linear 3′-tailed double-stranded DNA by RecU was not affected by the addition of RecA both in the presence and in the absence of SSB. We propose that RecU modulates RecA activities by promoting RecA-catalyzed strand invasion and inhibiting RecA-mediated branch migration, by preventing RecA filament disassembly, and suggest a potential mechanism for the control of resolvasome assembly.

INTRODUCTION

All cells need to ensure the stability of their genetic material for survival, since unrepaired DNA lesions can lead to double-strand breaks (DSBs). In *Escherichia coli*, the resulting DSBs would be funneled into the RecBCD pathway for repair via homologous recombination. The *E.coli* RecBCD ($RecBCD_{Eco}$) (functional homolog of *Bacillus subtilis* AddAB) enzyme processes the DNA ends and directs $RecA_{Eco}$ to the resulting 3′-single-stranded DNA (3′-ssDNA) ends, facilitating the formation of the $RecA_{Eco}$ nucleoprotein filaments (pre-synaptic complex) (1,2). The $RecFOR_{Eco}$ (functional homolog of *B.subtilis* RecFLOR) complex stimulates replacement of SSB_{Eco} by $RecA_{Eco}$ on ssDNA and limits $RecA_{Eco}$ filament extension on duplex DNA (3,4). The $RecA_{Eco}$ nucleoprotein complex (1 $RecA_{Eco}$ monomer/3 nt) invades a homologous double-stranded DNA (dsDNA) molecule to form a recombination intermediate (a D-loop) (1,5,6). The unidirectional movement of the strand transfer produces a four-strand exchange intermediate or Holliday junction (HJ) (1,7). HJ intermediates can be processed by several enzymes ($RecA_{Eco}$, or the branch migration helicases, $RuvAB_{Eco}$ or $RecG_{Eco}$) and can be cleaved by the $RuvC_{Eco}$ (functional analogue of *B.subtilis* RecU) resolvase. However, branch migration by $RecA_{Eco}$ protein is relatively slow, at the rate of 6 bp/s and its activity is reduced by heterologous sequences (8,9). Furthermore, previous studies showed that the HJ-binding protein, $RuvA_{Eco}$, inhibited $RecG_{Eco}$-catalyzed branch migration of HJs, even when $RecG_{Eco}$ was in considerable molar excess over $RuvA_{Eco}$ (10). Therefore, the candidate for catalyzing HJ branch migration *in vivo* would be the $RuvAB_{Eco}$ complex, which catalyzes branch migration of a single HJ at a rate of 43 bp/s (11) to 98 bp/s (12). In *B.subtilis* cells, there is an extra product in the same epistatic group (the RecD protein) whose activity is poorly understood (13). The $RuvC_{Eco}$ (RecU) resolvase, assembled with the $RuvAB_{Eco}$ ('RuvAB-RecD' in *B.subtilis*) complex, will scan for its preferred consensus cleavage sequence and selectively promote resolution by introducing symmetric nicks on their cognate sites [reviewed in (14)]; therefore, the replication fork will be re-established. Unless otherwise stated, the indicated genes and products are of *B.subtilis* origin.

In both bacteria and eukaryotes, RecA and Rad51 are the central recombination proteins and the importance of controlling their activities is well documented. In many bacteria, RecX and DinI proteins are competing modulators of RecA function (15). $RecX_{Eco}$, at stoichiometric amounts, inhibits rATPase and recombinase activities of $RecA_{Eco}$ (16–18). The $DinI_{Eco}$ protein, whose presence in Gram-positive bacteria

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is not obvious, at stoichiometric amounts stabilizes the $RecA_{Eco}$ nucleofilaments and destabilizes them when present at a 20- to 100-fold excess relative to $RecA_{Eco}$ (19,20). The $RecBCD_{Eco}$ (AddAB) and the $RecFOR_{Eco}$ (RecFLOR) complexes also modulate the RecA function (3,4,21), but the eukaryotic counterparts for these protein complexes remain unclear. Rad51 or RecA filamentation is strongly stimulated by a single-stranded binding protein (SSB or RPA), which melts out secondary structures in the ssDNA and allows the recombinase to assemble more efficiently on the ssDNA (22–24). In both bacteria and eukaryotes, some DNA helicases have been shown to be directly involved in the disruption of the RecA or Rad51 filaments, and in the disruption of RecA- and Rad51-promoted HJs (25–29).

In vertebrates, five Rad51 paralogs (Rad51B, Rad51C, Rad51D, Xrcc2 and Xrcc3) that share ~20% identity with Rad51 are found (30,31). Different complexes between the human Rad51 paralogs have been described as follows: the Rad51B–Rad51C–Rad51D–Xrcc2 (termed here BCDX2) complex, the Rad51B–Rad51C (termed BC) complex and the Rad51C–Xrcc3 (termed CX3) complex (32–35). The BC complex might be required for the assembly of the Rad51–ssDNA nucleoprotein filaments (33). Furthermore, two forms of Rad51C, alone and in the CX3 complex, were purified and they exhibited homologous pairing activities (34,35). All these studies suggested a pre-synaptic role for the Rad51 paralogs. However, recent data support the proposal that the Rad51 paralogs play a role at later stages of homologous recombination: Rad51B, which specifically binds to HJs, could potentially target the BCDX2 complex to DNA structures that are formed late during recombination (36,37). The DX2 subcomplex stimulates HJ disruption by the BLM helicase (38). Furthermore, Rad51C alone or in the CX3 complex is involved in HJ resolution or is a resolvase activator (39), and a role for XRCC3 in HJ resolution at human telomeres has been recently described (40). Many of the activities associated with both the BCDX2 and the CX3 complex are also exhibited by the RecU resolvase (13).

Expression of RecU in *E.coli ruvC* cells restores the resistance to UV light (G. Sharples and J. C. Alonso, unpublished data). Several activities have been described for the *B.subtilis* RecU protein *in vitro* (13): (i) RecU, which preferentially binds three- and four-strand junctions, binds ssDNA with ~3-fold higher affinity than dsDNA, and the binding is modulated by Mg^{2+} cations; (ii) in a nucleotide-independent and Mg^{2+}-dependent reaction, RecU promotes the accumulation of joint molecules (jm) between a circular ssDNA and a linear dsDNA with a 3′-ssDNA tail longer than ~100 nt (3′-tailed dsDNA) with only limited DNA strand exchange activity; (iii) RecU anneals linear ssDNA and a homologous supercoiled dsDNA substrate (D-loop formation) in the absence of a nucleotide cofactor, but in the presence of 1 mM Mg^{2+}; and (iv) RecU, at high Mg^{2+} concentrations (10 mM), cleaves synthetic four-strand intermediates. The mechanism by which RecU coordinates homologous pairing and HJ resolution is poorly understood (13). Recently, the structure of the RecU HJ resolvase was determined (41). The RecU fold reveals a striking similarity to a class of resolvase enzymes found in archaeal sources and members of the type II restriction endonuclease family to which they are related (41).

In this paper, we show that RecU, which is highly conserved among Gram-positive bacteria with low dG + dC content in their DNA, helps to load RecA onto ssDNA and stimulates RecA-promoted D-loop formation. However, RecU inhibits the RecA dATPase or rATPase activity and the completion of the three-strand exchange reaction, without apparent destabilization of the RecA filaments. Our results suggest that RecU might work at the pre-synaptic stage by helping RecA to promote strand invasion. At later stages, RecU might redirect branch migration toward the 'RuvAB–RecD' complex and inhibit RecA-mediated strand exchange by preventing RecA filament disassembly.

MATERIALS AND METHODS

Bacterial strain and plasmids

E.coli BL21(DE3)[pLysS] and XL1-Blue, and *B.subtilis* YB886 strains were used (13). Plasmids pCB568 (13), pCB596 (42), pBT61 (43) and pGEM3Zf(+) (Promega) have been described previously.

Protein purification

The SSB_{Eco} protein was purchased from Amersham Biosciences. *E.coli* BL21(DE3)[pLysS] cells bearing pCB568-borne *rec*U gene or pCB596-borne bacteriophage SPP1-encoded gene 36 (also termed *ssb* gene) were overexpressed and the proteins purified as described previously (13). The RecU protein, which was >99% pure based on staining after SDS–PAGE and Edman degradation, was free of $RecA_{Eco}$, $RecO_{Eco}$, $RecT_{Eco}$ proteins and nucleases. The SSB protein, which shares 54% identity to host-encoded SSB (44) and is >99% pure, was free of SSB_{Eco} protein and nucleases.

B.subtilis YB886 cells bearing pBT61-borne *rec*A gene (43) were grown to middle exponential phase, and the expression of RecA was induced by the addition of mitomycin C to a final concentration of 250 ng/ml for 2 h. Cells were harvested, resuspended in buffer A (50 mM Tris–HCl, pH 7.5, 1 mM DTT, 2 mM EDTA and 15% glycerol) containing 300 mM NaCl and disrupted by lysozyme addition (0.1 mg/ml) followed by sonication. The 38 kDa RecA protein was found in the soluble fraction. Polyethylenimine was added and precipitated RecA was recovered from the pellet with buffer A containing 150 mM ammonium sulfate. The supernatant was subjected to ammonium sulfate precipitation (60% saturation). The pellet was resuspended in buffer A and loaded onto a hydroxylapatite column (Bio-Rad). RecA was eluted with a 125–175 mM phosphate gradient and loaded onto a Blue-Sepharose (Bio-Rad) column, which was eluted by a step gradient from 50 to 200 mM NaCl. The RecA protein, which was ~99% pure as judged by staining after SDS–PAGE and Edman degradation, and free of nucleases, was stored at −20°C.

The molar extinction coefficients of RecA, SSB and RecU were calculated to be 15 200, 15 340 and 24 900 M^{-1} cm^{-1}, respectively, as described previously (45). These molar extinction coefficients were used to determine protein concentrations. RecA is expressed as mol of protein monomers, SSB and RecU as dimers, and SSB_{Eco} as tetramers.

DNA manipulations

Linear 3.2 kb KpnI-pGEM3Zf(+) dsDNA and circular 3.2 kb pGEM3Zf(+) ssDNA were used. Linear pGEM3Zf(+) dsDNA with 3′ ssDNA termini of ~140 nt (3′-tailed dsDNA) was prepared as described previously (42). The ~200 nt ssDNA was obtained after hybridizing to EcoRI-linearized M13mp18 dsDNA the 5′-TGCGCAACTGTTGGGAAGGGCG-3′ oligonucleotide (which contains the FspI site) and extension by PCR. The amplified product was gel purified as described previously (42).

The DNA concentrations are expressed as mol of nucleotides. For electrophoretic mobility-shift assay (EMSA), the linear 200 nt [γ-^{32}P]ssDNA (0.75 μM) was pre-incubated with RecA (400 or 200 nM) or with increasing concentrations of RecU (12–48 nM) for 10 min at 37°C in buffer B [50 mM Tris–HCl, pH 7.5, 1 mM DTT, 50 mM NaCl, 10 mM magnesium acetate ($MgAc_2$), 50 μg/ml BSA and 5% glycerol] containing 2 mM dATP, rATP or adenosine 5′-(β,γ-imido) triphosphate (AMP-PNP). Then, increasing concentrations of RecU (12–48 nM) or RecA (400 or 200 nM) were added and the samples were further incubated for 10 min at 37°C. As a control, the 200 nt ssDNA was incubated for 20 min at 37°C in buffer B with increasing concentrations of RecU (12–48 nM) or RecA (400 or 200 nM). The reaction was stopped and subjected to 0.8% agarose gel electrophoresis at 4°C. The gels were dried and analyzed by autoradiography.

For the DNA three-strand exchange reaction, circular pGEM3Zf(+) ssDNA (10 μM) was incubated at the indicated time and concentration of RecA, RecU or SSB in buffer B containing 2 mM dATP or rATP at 37°C. Then, the indicated concentration of RecU, SSB or RecA (second protein) and homologous KpnI-cleaved or 3′-tailed dsDNA (20 μM) were added and the reaction mixture was incubated for the indicated time. The samples were deproteinized as described previously (42,46) and fractionated through 0.8% agarose gel electrophoresis with ethidium bromide.

For D-loop formation, a homologous 200 nt [γ-^{32}P]ssDNA (0.12 μM) was pre-incubated with RecA (250 nM) or increasing concentrations of RecU (7.5–250 nM) for 10 min at 37°C in buffer B in the presence or absence of a nucleotide cofactor (2 mM dATP, rATP or AMP-PNP) to allow the formation of the protein–ssDNA complex. Homologous 3.2 kb supercoiled DNA (7.5 μM) was added and the samples incubated for further 10 min at 37°C. Then, increasing concentrations of RecU (7.5–250 nM) or a fixed amount of RecA (250 nM) were added, and after 15 min, the deproteinized products were analyzed by 0.8% agarose gel electrophoresis, with ethidium bromide. Gels were photographed, dried and exposed to autoradiography.

dATPase or rATPase activity measurement

Standard tests in buffer B (with a 100 000:1 ratio of labeled [α-^{32}P]dATP or [α-^{32}P]ATP) and 10 μM pGEM3Zf(+) ssDNA were assembled on ice. A constant amount of RecA or increasing concentrations of RecU were added and the samples incubated for 10 min at 37°C. Then, increasing concentrations of RecU or a constant amount of RecA were added, and the incubation was continued for an indicated time at 37°C in buffer B containing the indicated nucleotide cofactor. Aliquots were taken at several times. dATPase or rATPase activity was measured by thin layer chromatography using 200 mM potassium phosphate as eluent. The reaction products were quantified by PhosphorImager analysis (Molecular Dynamics).

Electron microscopy

Circular pGEM3Zf(+) ssDNA (10 or 5.6 μM) was pre-incubated with various amounts of RecA or RecU for 20 min at 37°C in buffer B containing 2 mM dATP. Then, RecU or RecA was added and incubated for 40 min at 37°C. DNA–protein complexes were visualized by negative staining with 1% uranyl acetate (47).

RESULTS

RecU stimulates RecA binding to ssDNA

To address the biological role of RecU in RecA-mediated recombination, we purified RecA protein from *B.subtilis* cells and analyzed the effect of RecU on RecA activities, i.e. ssDNA binding, nucleotide hydrolysis and strand exchange. Previously, it was shown that (i) RecU binding to ssDNA is modulated by Mg^{2+}, and at high Mg^{2+} (10 mM $MgAc_2$), the stoichiometry of binding corresponds to a site size of ~10 nt (13) and (ii) RecA assembly on ssDNA, with a site size of 1 RecA/3 nt, requires a nucleotide cofactor, but the 5′–3′ disassembly of the filaments requires rATP or dATP hydrolysis (48,49). To test the effect of RecU on RecA–ssDNA complex formation and *vice versa*, we used EMSA in the presence of 2 mM dATP.

A linear 200 nt [γ-^{32}P]ssDNA was pre-incubated with constant amounts of RecA (Figure 1, lanes 5–10) or various concentrations of RecU (Figure 1, lanes 13–18) for 10 min at 37°C in buffer B containing 2 mM dATP. Then, the second protein

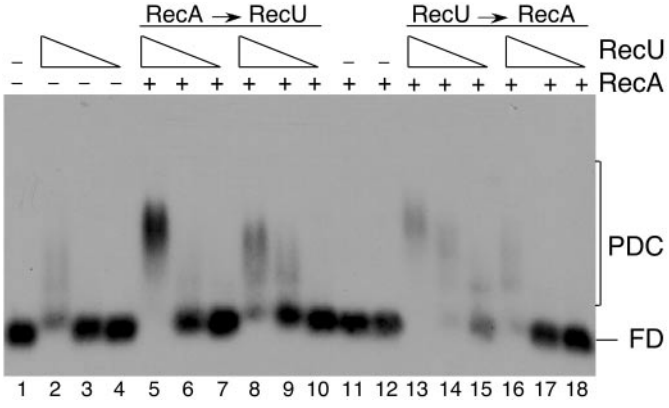

Figure 1. RecU stimulates binding of RecA to ssDNA. A linear 200 nt [γ-^{32}P]ssDNA (0.75 μM) was pre-incubated with RecA [400 nM (lanes 5–7) or 200 nM (lanes 8–10)] or with RecU [12–48 nM (lanes 15–13 and 18–16)] for 10 min at 37°C in buffer B containing 2 mM dATP. Then, RecU (12–48 nM, lanes 7–5 and 10–8)] or RecA [400 nM (lanes 13–15) or 200 nM (lanes 16–18)] were added and the reaction mixture was incubated for further 10 min at 37°C. As a control, the 200 nt ssDNA was incubated for 20 min at 37°C with increasing concentrations of RecU (12–48 nM, lanes 2–4) or RecA (400 nM in lane 11 and 200 nM in lane 12). Samples were analyzed by 0.8% agarose gel electrophoresis, and autoradiographs of the dried gels were subsequently taken. The order of protein addition is indicated. FD, free ssDNA; PDC, protein–DNA complexes.

[increasing amounts of RecU (Figure 1, lanes 5–10) or constant amounts of RecA (Figure 1, lanes 13–18)] was added, and the reaction mixture was incubated for 10 min at 37°C. As control, increasing concentrations of RecU (Figure 1, lanes 2–4) or two fixed amounts of RecA (Figure 1, lanes 11–12) were incubated with the labeled ssDNA and the products analyzed by agarose gel electrophoresis.

In the presence of suboptimal RecU concentrations (1 RecU/15 nt), a protein–DNA complex was observed (Figure 1, lane 2), but in the presence of RecA (1 RecA/4 nt or 2 nt) no stable protein–ssDNA complex was observed (Figure 1, lanes 11–12). When RecA was pre-incubated with ssDNA, and then incubated with RecU, a protein–ssDNA complex that migrated much slowly than the one formed by RecU alone was observed (see Figure 1, compare lanes 5 or 8 with lane 2). Such slow migrating complex cannot be due to a protein stabilization effect because all the reactions contain 50 μg/ml BSA (buffer B).

A maximal RecA binding to ssDNA occurred at RecU molar ratios of 0.12:1 (Figure 1, lane 5). However, when a suboptimal amount of RecU (1 RecU/30 nt) was pre-incubated with ssDNA and then RecA was added, a maximal RecA binding to ssDNA occurred at RecU molar ratios of 0.06:1, and a shift was observed even at RecU molar ratios of 0.03:1 (Figure 1, lanes 15–13). Since the mobility of the protein–ssDNA complexes observed in the presence of both RecA and RecU were different from the ones observed with RecU alone, and RecU binds with higher affinity than RecA to ssDNA, it is likely that RecU recruits and/or stabilizes the binding of RecA to ssDNA. Similar results were obtained when rATP was used, and when similar ssDNA:RecU:RecA ratios were used as the ones used in the strand exchange reactions (data not shown). It is likely that RecU stimulates the interaction of RecA with ssDNA, rather than inhibiting ssDNA binding. The 200 nt ssDNA segment used did not undergo spontaneous or protein catalyzed dimerization, because the mobility of the free ssDNA was the same when the sample was not treated or when an aliquot of it was heated at 95°C for 10 min and rapidly cooled on ice prior to electrophoresis. Therefore, the putative enhancement of binding of RecA to dsDNA and not to ssDNA by the presence of RecU was discarded. However, we failed to demonstrate a stable physical interaction between the RecU and RecA proteins in the absence of ssDNA (data not shown).

RecU inhibits the ssDNA-dependent RecA-catalyzed dATP or rATP hydrolysis

Previously, it was shown that *B.subtilis* RecA preferentially catalyzes ssDNA-dependent dATP hydrolysis and promotes dATP-dependent homologous paring between a circular ssDNA and a linear dsDNA, with the accumulation of recombination intermediates, [joint molecules (jm)], and fully strand exchanged products [nicked circular (nc) and linear ssDNA (lss) products] [(50), see below]. However, a later report showed that RecA catalyzes the hydrolysis of both dATP and rATP and can perform strand exchange using either dATP or ATP as a cofactor (51). We performed ATPase and dATPase assays as well as three-strand exchange assays with RecA protein purified from *B.subtilis* cells and found that, in the absence of an SSB protein, it catalyzed nucleotide hydrolysis and the three-strand exchange reactions with

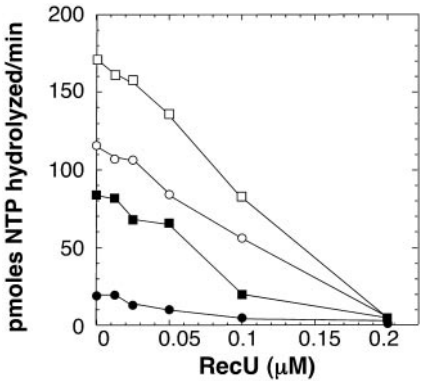

Figure 2. Effect of RecU on ssDNA-dependent RecA-catalyzed dATP and rATP hydrolysis. Circular ssDNA (10 μM) was pre-incubated with RecU (12–200 nM) in buffer B containing 0.15 or 0.6 mM dATP or rATP for 10 min at 37°C. Then, RecA (1.3 μM) was added and the reaction mixture incubated for 15 min at 37°C. Reactions were stopped by addition of EDTA, and the rate of dATP or rATP hydrolysis by RecA (denoted in pmoles of dATP or rATP hydrolyzed/min) was measured. Empty squares, RecA dATPase in the presence of 0.6 mM; filled squares, 0.15 mM dATP. Empty circles, RecA rATPase in the presence of 0.6 mM; filled circles, 0.15 mM rATP.

1.5- to 3-fold higher efficiency in the presence of dATP than with rATP (Figure 2, B. Carrasco, unpublished data).

The effect of RecU on the ssDNA-dependent RecA catalyzed rATP or dATP hydrolysis was assayed using two different rATP and dATP concentrations (0.15 or 0.6 mM, Figure 2). Circular ssDNA (10 μM) was pre-incubated with increasing concentrations of RecU (12–200 nM) for 10 min at 37°C in buffer B containing 0.15 or 0.6 mM rATP or dATP. Then, RecA (1.3 μM, 1 RecA monomer/7 nt) was added and the reaction incubated for 15 min at 37°C. RecA was more active in the presence of 0.6 mM than 0.15 mM dATP or rATP. At 0.6 mM, the amount of rATP hydrolyzed was only ∼1.5-fold lower when compared with dATP, which is consistent with the previous observation that RecA catalyzes dATP and rATP hydrolysis with similar efficiency (51). However, at low concentrations (0.15 mM), the RecA rATPase is ∼4-fold lower when compared with the presence of 0.15 mM dATP (Figure 2). Unexpectedly, an increase in RecA binding to ssDNA by the presence of RecU (Figure 1) did not correlate with an increase in the observed rate of ssDNA-dependent dATP or rATP hydrolysis. RecU at a molar ratio of 1:13 (RecU:RecA) and 1:16 decreased the dATPase of RecA to 50% in the presence of 0.6 and 0.15 mM dATP, respectively (Figure 2). The dATPase activity of RecA was completely abolished at molar ratios of 1:6 in the presence of both 0.6 and 0.15 mM dATP. A similar ratio of RecU:RecA was required to reduce the rATPase of RecA to 50% (Figure 2).

We examined the effect of RecU on the ssDNA-dependent dATPase activity of RecA as a function of the time and in the protein:ssDNA:dATP ratios used in a three-strand exchange reaction (see below). Circular ssDNA was pre-incubated with a constant amount of sub-saturating concentrations of RecA (1 RecA/7 nt, Figure 3A) or increasing concentrations of RecU (Figure 3B) in buffer B containing 2 mM dATP, and subsequently, increasing concentrations of RecU (Figure 3A) or a constant amount of RecA (Figure 3B) were added. Addition of RecU resulted in an inhibition of the RecA catalyzed

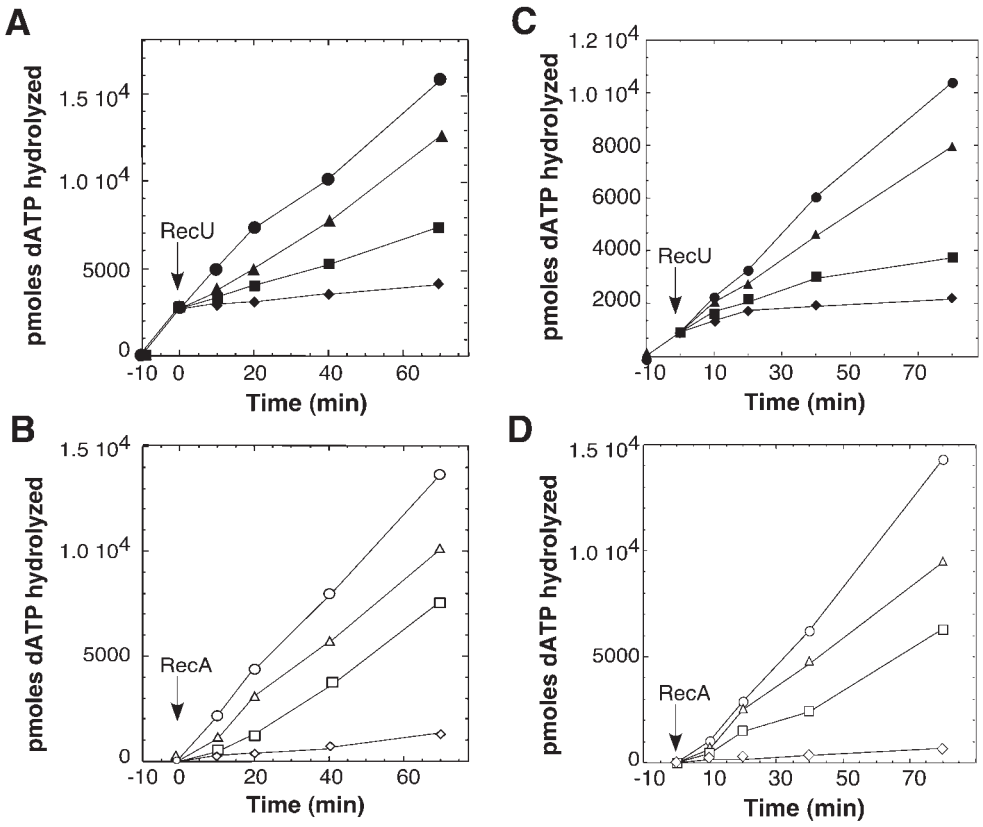

Figure 3. Effect of RecU on ssDNA-dependent RecA catalyzed dATP hydrolysis. Circular ssDNA (10 μM) was pre-incubated with RecA (1.3 μM) [time −10 in (**A**)] or increasing concentrations of RecU [50, 100 and 200 nM, in (**B**)] in buffer B containing 2 mM dATP for 10 min at 37°C. Then, the second protein {RecU [50, 100 or 200 nM, in (A)] or RecA [1.3 μM, in (B)], denoted by arrowheads} was added and the reaction mixture incubated for further 60 min at 37°C. In (**C** and **D**), the order of protein addition and concentrations are the same as in (A and B), respectively, but circular ssDNA (10 μM) and KpnI-linearized dsDNA (20 μM) were present. Aliquots were taken and the rate of dATP hydrolysis by RecA (denoted in pmoles of dATP hydrolyzed) was measured. RecA alone, circles; RecA and 50 nM RecU, triangles; RecA and 100 nM RecU, squares; RecA and 200 nM RecU, diamonds.

dATP hydrolysis (Figure 3A and B). A slightly more drastic effect was observed when the ssDNA was pre-incubated with high concentrations of RecU (200 nM) and then RecA was added (Figure 3B). The inhibition of RecA-catalyzed dATP hydrolysis by RecU in the presence of saturating amounts of ssDNA and independent of the order of addition of RecU suggests that the mechanism of inhibition is not merely the competition for ssDNA, what is consistent with the results obtained in Figure 1, where a recruitment or stabilization was observed. To see whether the addition of homologous duplex DNA, to which RecU could also bind (13), could rescue the dATPase activity of RecA, circular ssDNA (10 μM) and linear KpnI-linearized dsDNA (20 μM) were pre-incubated with RecA (1.3 μM, Figure 3C) or with increasing concentrations of RecU (50–200 nM, Figure 3D) for 10 min at 37°C in buffer B containing 2 mM dATP. Then, increasing concentrations of RecU (50–400 nM, Figure 3C) or constant amounts of RecA (1.3 μM, 1 RecA/7 nt, Figure 3D) were added, and the reaction was incubated for a variable time. The inhibition was similar to the one observed in the presence of ssDNA alone, suggesting that binding of RecU to dsDNA does not reverse the exerted inhibition in the RecA dATPase activity.

The ssDNA-dependent rATP hydrolysis catalyzed by RecA was similarly inhibited by the presence of RecU (data not shown).

RecU does not promote RecA filament disassembly

Since RecU decreased RecA-catalyzed dATP or rATP hydrolysis even in the presence of an excess of free ssDNA, two possible regulatory modes for RecU are suggested: (i) RecU interaction with RecA alters the binding of the latter to ssDNA, and indirectly inhibits dATP or rATP hydrolysis, as it has been demonstrated for RecX (17,18) or DinI (20) and (ii) RecU does not affect the nucleation step of RecA filament assembly, but inhibits dATP hydrolysis and the 5′–3′ disassembly. This last hypothesis is consistent with the results obtained in Figure 1. To assess whether the RecU protein affects the disassembly of RecA filaments on ssDNA in the presence of dATP, electron microscopy studies were performed.

In the presence of ssDNA, RecA–dATP, at saturating or sub-saturating concentrations (1 RecA/2 nt or 1 RecA/7 nt, respectively), forms regular right-handed filaments of close to six monomers per turn (see Supplementary Figure S1, parts A and D). The RecU protein forms blobs in bush-like ssDNA, but protein filaments or ring-shaped structures on DNA were not observed (13,52).

When the ssDNA was pre-incubated with saturating amounts of RecA (1 RecA/2 nt) and then an excess of RecU (1 RecU/7 nt) was added, no disassembly of the filaments by the presence of the excess RecU could be observed

(Supplementary Figure S1, parts B and C), which argues against a block of the dATPase activity by filament disassembly, as it has been demonstrated for RecX (17,18) or DinI (20).

RecU (200 nM, 1 RecU/50 nt) was pre-incubated with circular ssDNA (10 μM) and then sub-saturating amounts of RecA (1 RecA/7 nt) were added. Discrete RecU blobs embedded in the RecA filaments were observed (Supplementary Figure S1, parts E and F). When RecU concentrations >200 nM were pre-incubated with the ssDNA and then RecA was added, RecA filament assembly was observed, but via RecU–RecU interactions, thread-like structures, as those envisaged at the central region of the circular molecule in Figure S1, part E, were observed (data not shown).

RecU inhibits the RecA-promoted three-strand exchange reaction

To address the biological role of RecU in RecA-promoted strand annealing, and its possible effect on RecA-promoted strand exchange, we focused on conditions that work for RecA-mediated three-strand exchange (e.g. 2 mM dATP and 10 mM $MgAc_2$) with two types of DNA substrates (e.g. KpnI-linearized dsDNA or linear dsDNA with a 3′-ssDNA tail) in the presence of both proteins (Figure 4), but the same type of results were obtained when rATP was used instead of dATP, or when an rATP regeneration system was used (data not shown).

Circular ssDNA and homologous KpnI-linearized dsDNA (Figure 4B) or linear duplex DNA with a 3′-ssDNA tail of ~140 nt (3′-tailed dsDNA) (Figure 4C) were pre-incubated with RecA (1 RecA/7 nt) or with increasing concentrations of RecU for 30 min at 37°C. When indicated, the second protein was added and the reaction incubated for 30 min at 37°C. The products of the reaction were deproteinized and analyzed by agarose gel electrophoresis.

The RecA protein assembled on ssDNA was capable of pairing with homologous linear dsDNA within a 30 min reaction (Figure 4B, lane 2), but accumulation of final products, nc, were detected with longer incubation periods (Figure 4A and B, lanes 3 and 8).

Under optimal RecA conditions, e.g. at high Mg^{2+} concentrations, RecU failed to promote three-strand exchange between a circular ssDNA strand and a homologous blunt ended or nearly blunt ended linear dsDNA, but promoted the co-aggregation of circular ssDNA and the homologous 3′-tailed dsDNA (jm formation) (Figure 4C, lanes 2–5, see below). Previously, it was shown, using electron microscopy, that the low mobility complexes formed by RecU between circular ssDNA and the homologous 3′-tailed dsDNA are bona fide jms (13).

The RecA-mediated strand exchange between a circular ssDNA molecule and a nearly blunt ended linear dsDNA molecule was not affected by the presence of 1 RecU dimer/200 nt [molar ratio of 0.03:1 (RecU:RecA)] (Figure 4B, lane 4). However, in the presence of 1 RecU/50 nt or 1 RecU/25 nt, a drastic decrease of RecA-mediated accumulation of nc products was observed (Figure 4B, lanes 6–7). Under this experimental condition, the presence of diffuse DNA bands at the jm position was observed, probably reflecting a different degree of transferred DNA. As revealed in Figure 4B, lanes 9–12, pre-incubation of ssDNA or both

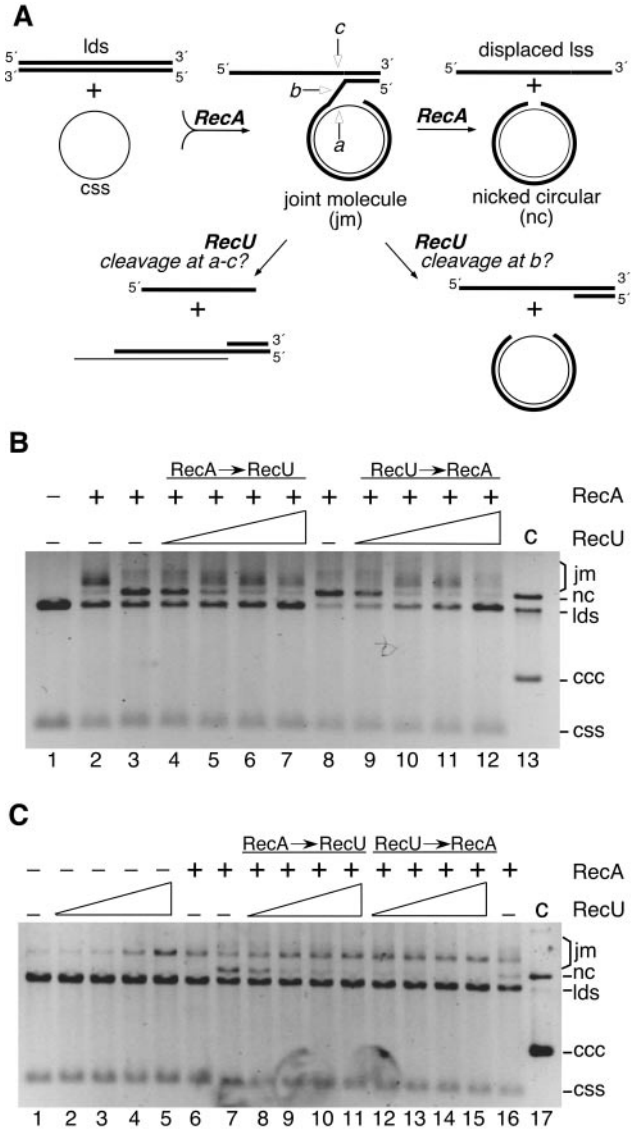

Figure 4. RecU inhibits three-strand exchange catalyzed by RecA. (**A**) Scheme of the three-strand exchange reaction, with the predicted products of RecU cleavage of the three-strand recombination intermediate, and the expected products after RecA catalyzed partial (jm) or full (nc and displaced lss) strand exchange. (**B** and **C**) Circular ssDNA (10 μM) was pre-incubated with a constant amount of RecA (1.3 μM) or increasing concentrations of RecU (50–400 nM) in the presence of homologous KpnI-linearized dsDNA (20 μM) (in B) or 3′-tailed dsDNA (20 μM) (in C) for 30 min at 37°C in buffer B containing 2 mM dATP. When indicated, increasing concentrations of RecU (50–400 nM) or RecA (1.3 μM) were added and the samples incubated for further 30 min at 37°C. (B) In lanes 2–7, RecA was pre-incubated with DNA, and in lanes 9–12 RecA was added after RecU. In lane 2, the reaction was stopped at 30 min and in lanes 3 and 8 at 60 min. (C) In lanes 8–11, RecA was pre-incubated with DNA, and in lanes 12–15 RecU was pre-incubated. In lane 6, the reaction was stopped at 30 min and in lanes 7 and 16 at 60 min. In lanes 2–5, the formation by RecU of jm with 3′-tailed dsDNA is shown. Positions of bands corresponding to circular ssDNA (css), linear, nicked and covalently closed dsDNA (lds, nc and ccc, respectively) and joint molecules (jm) are indicated. The order of protein addition is indicated. + and – denote the presence and absence of the indicated protein. C indicates the partially nicked dsDNA control.

DNA substrates with RecU leads to a higher inhibition of the RecA-promoted three-strand exchange reaction *in vitro*. At higher RecU:RecA molar ratio (0.3:1), RecA-mediated recombination was inhibited even after the formation of the

RecA nucleoprotein filament and the initial steps of jm formation (Figure 4B, lanes 7 and 12).

With the 3′-tailed substrate, RecA mediated the formation of jm and nc products with a similar kinetics as with KpnI-linearized dsDNA (compare Figure 4B, lanes 2–3 and Figure 4C, lanes 6–7). As revealed in Figure 4C (lanes 8–11), when RecU was added after RecA, accumulation of nc decreased ~3-fold at a molar ratio of 0.03:1, ~4-fold at a molar ratio of 0.07:1, and was completely inhibited at a molar ratio of 0.15:1 or 0.3:1. The pre-incubation of RecU with circular ssDNA and linear 3′-tailed dsDNA and subsequent addition of RecA inhibited RecA-mediated strand annealing or the accumulation of nc products even at a 0.03:1 ratio. However, the accumulation of jm was not affected (Figure 4C, lanes 12–15). The proteins that may be responsible for the formation of the jm observed at the highest RecU:RecA ratios used remains unknown, although it is tempting to speculate that these jms will be generated by RecU, since at this RecU:RecA ratios, with the KpnI substrate, the RecA catalyzed formation of jm was completely abolished (compare Figure 4B, lane 12 with Figure 4C, lane 15). The same results were obtained when a higher amount of RecA (3 μM, 1 RecA monomer/3 nt of ssDNA) was used (data not shown).

The accumulation of products of RecU catalyzed cleavage of the three-strand intermediate (either at *a* and *c* sites or at *b* site, see Figure 4A) was not detected. Furthermore, cleavage products were even not detected when ^{32}P-labeled substrates were used, but RecU efficiently cleaved RecA-generated four-stranded intermediates (data not shown).

SSB does not alleviate the RecU inhibition of RecA-mediated strand exchange

Bacterial RecA- and eukaryotic Rad51-mediated strand exchange are enhanced by adding, subsequent to the recombinase, SSB or RPA, respectively. SSB and RPA act pre-synaptically by removing secondary structures within the ssDNA, and post-synaptically by binding to the displaced lss product (23,24). In many cases, the SSB protein species can be substituted by another SSB protein, showing that the stimulatory role is not species specific (53). To address whether the inhibitory effect exerted by RecU on RecA-mediated strand exchange could be alleviated by the addition of an SSB protein, circular ssDNA and homologous KpnI-linearized dsDNA (Figure 5A) or 3′-tailed dsDNA (Figure 5B) were pre-incubated with RecA. The reaction mixture was then incubated with three different concentrations of RecU, and finally increasing concentrations of a SSB protein, at SSB:ssDNA ratios of 1:76, 1:38 and 1:19, were added. At low RecU (at ratios of 0.03:1), 2-fold inhibition in RecA-mediated strand exchange was observed (Figure 5A, compare lanes 3 and 5), but the addition of increasing amounts of SSB restored and even enhanced the yield of final recombination products (Figure 5A, lanes 6–7). It is likely that SSB (also termed SPP1-G36P), which shares 56% identity with *B.subtilis*-encoded SSB, can substitute for *B.subtilis* SSB. Similar results were obtained when SSB_{Eco} was used (data not shown).

As shown in Figure 5A, lanes 15–17, the absence of accumulation of RecA-mediated nc products, in the presence of RecU at ratios of 0.15:1, could not be alleviated by the addition

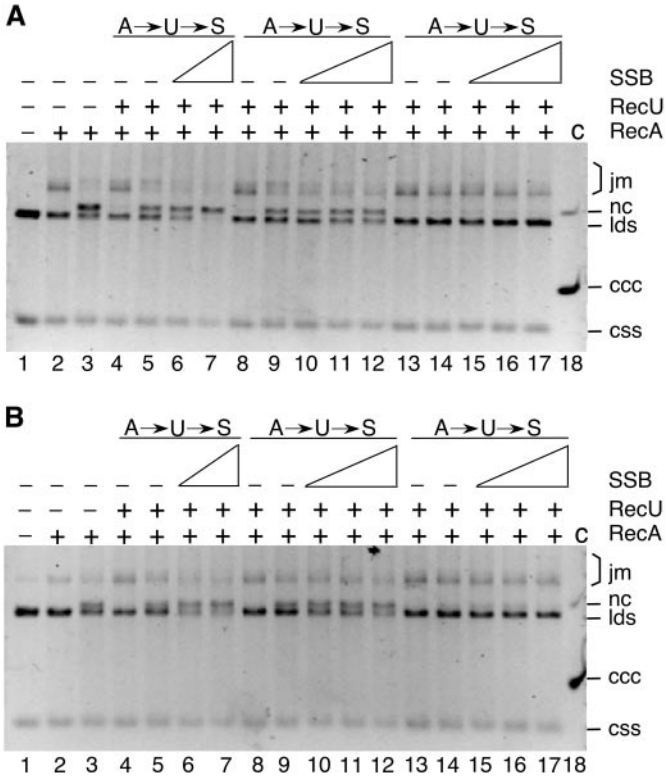

Figure 5. RecU inhibition of RecA-promoted DNA strand exchange is not reversed by SSB. Circular ssDNA (10 μM) was pre-incubated with a constant amount of RecA (1.3 μM, lanes 2–17) in the presence of homologous KpnI-linearized dsDNA (20 μM) (in **A**) or 3′-tailed dsDNA (20 μM) (in **B**) for 20 min at 37°C. RecU [50 (lanes 4–7), 100 (lanes 8–12) or 200 nM (lanes 13–17)] was added and the samples were incubated for 10 min at 37°C in buffer B containing 2 mM dATP. Then, increasing concentrations of SSB (270–550 nM, in lanes 6–7 or 130–550 nM, in lanes 10–12 and 15–17) were added and incubation was continued for further 30 min at 37°C. In lane 2, the reaction was stopped at 20 min, and in lanes 4, 8 and 13 after 30 min of incubation. Positions of the bands and the symbols are those of Figure 4.

of SSB. Furthermore, the negative effect of RecU on pre-formed RecA filaments was also observed with the dsDNA containing a 3′-ssDNA tail. Here, the addition of an excess of SSB (Figure 5B) or SSB_{Eco} protein did not reverse the negative effect of RecU in RecA-promoted recombination.

SSB added prior to RecU does not reverse the inhibition of RecA-mediated strand exchange

The inhibition of RecA-mediated strand annealing by RecU might result from binding of RecU to ssDNA and sequestration of the substrate, or annealing in the opposite polarity. If this is the case, the addition of a protein with high affinity for ssDNA, as SSB, prior to RecU addition should displace RecU from the ssDNA and indirectly alleviate the inhibitory effect exerted by RecU on the RecA activity. To test this, circular ssDNA and homologous KpnI-linearized dsDNA (Figure 6A) or 3′-tailed dsDNA (Figure 6B) were pre-incubated with RecA, followed by SSB (at a ratio of 1 SSB/38 nt). Then, the samples were incubated with increasing concentrations of RecU.

The SSB protein by itself failed to promote jm formation between ssDNA and a 4 bp 3′-overhanged or a 3′-tailed

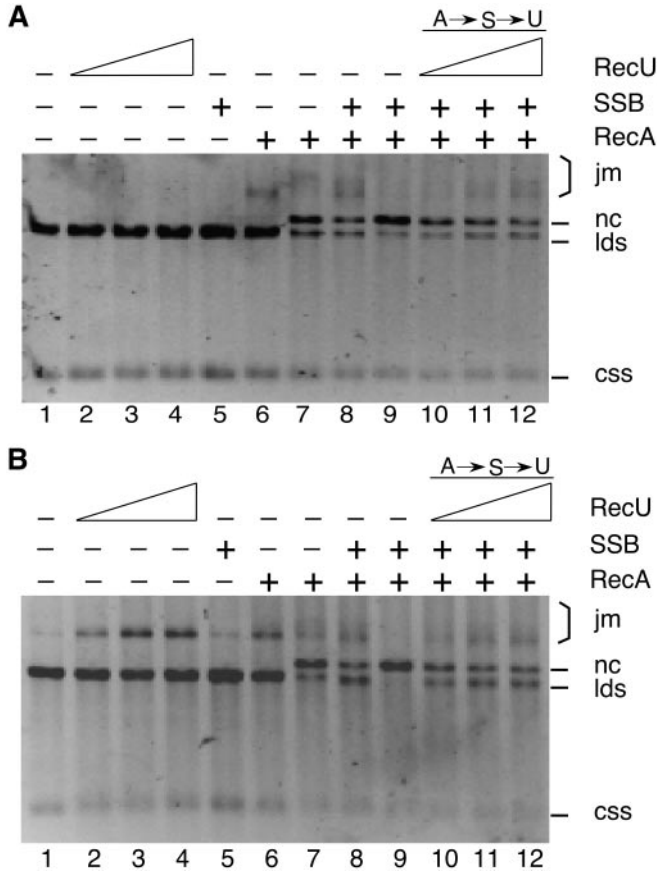

Figure 6. RecU inhibits DNA strand exchange catalyzed by RecA in the presence of SSB. Circular ssDNA (10 μM) was pre-incubated in buffer B containing 2 mM dATP with a constant amount of RecA (1.3 μM, lanes 6–12) in the presence of homologous KpnI-linearized dsDNA (20 μM) (in **A**) or 3′-tailed dsDNA (20 μM) (in **B**) for 20 min at 37°C. SSB (270 nM) was added and after incubation for 10 min at 37°C, increasing concentrations of RecU (50–200 nM) were added and the reaction incubated for further 30 min at 37°C. In lanes 6 and 8, the reaction was stopped at 30 min. In lanes 2–4, the DNA substrates were incubated with RecU alone. Position of the bands and the symbols are those of Figure 4.

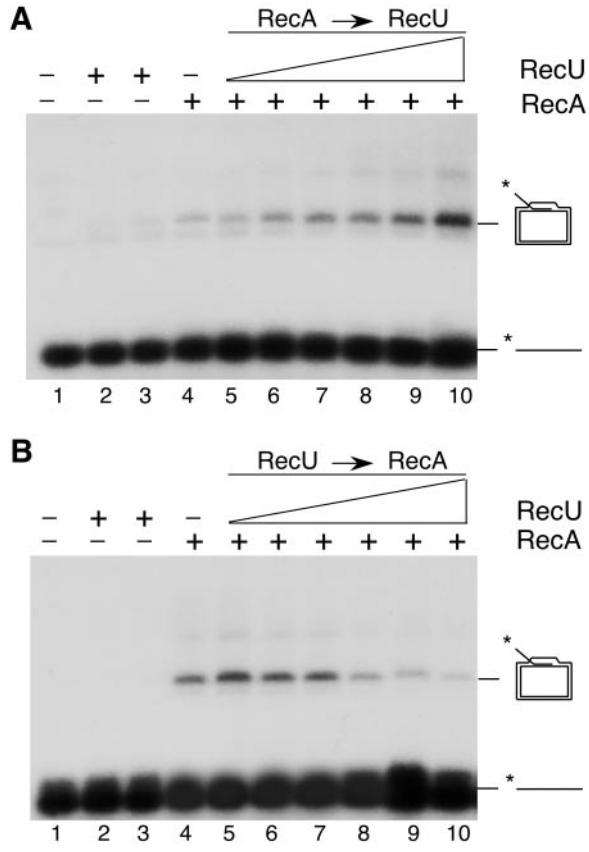

Figure 7. Effect of RecU on RecA-promoted strand invasion. A homologous 200 nt [γ^{32}P]ssDNA (0.12 μM) was pre-incubated with RecA [250 nM, in (**A**)] or with increasing concentrations of RecU [7.5–250 nM, in (**B**)] for 10 min at 37°C in buffer B containing 2 mM dATP. Supercoiled homologous DNA (7.5 μM) was added and the reaction incubated for 10 min at 37°C. Then, increasing concentrations of RecU [7.5–250 nM, in (A)] or a fixed amount of RecA [250 nM, in (B)] were added and after 15 min the deproteinized products were analyzed with 0.8% agarose gel electrophoresis; subsequently, autoradiographs of the dried gels were taken. As a control, the 200 nt ssDNA was incubated with supercoiled homologous DNA and 125 (lane 2) or 250 nM (lane 3) RecU or RecA (250 nM, lane 4) alone. The order of protein addition is indicated.

homologous dsDNA (Figure 6, lane 5), but stimulated the RecA-mediated reaction. In the absence of SSB, after 30 min <10% of the circular ssDNA and linear dsDNA was converted onto nc products by RecA, but 65% of nc products were observed in a 60 min reaction (Figure 6, lanes 6 and 7). Upon SSB addition, ∼50% of the linear DNA was converted to nc products within 30 min, and >85% in a 60 min reaction (Figure 6, lanes 8 and 9). The RecU protein inhibited the completion of the strand exchange reaction, although it was added after the SSB protein (Figure 6, lanes 10–12, compare them with the products in the absence of RecU, lane 9). Similar results were obtained with a higher excess of SSB or SSB_{Eco} (540 nM, data not shown).

RecU stimulates RecA-catalyzed D-loop formation

RecU modulates RecA-mediated strand exchange by enhancing RecA–ssDNA complex formation (Figure 1) and exerting a negative effect on dATP or rATP hydrolysis (Figures 2 and 3), with the subsequent inhibition of extensive strand exchange (Figures 4 to 6). It is likely that RecU stimulates RecA filament assembly, but exerts a negative effect in RecA filament disassembly (Supplementary Figure S1). To test this hypothesis, the effect of RecU on RecA-mediated D-loop formation, a process that requires dATP or rATP binding but not hydrolysis (48,49), was assayed. RecA-promoted D-loop formation in the presence of RecU was compared with similar reactions promoted by RecA or RecU alone. In the reaction, 200 nt [γ-^{32}P]ssDNA was pre-incubated with RecA (Figure 7A) or with increasing concentrations of RecU (Figure 7B) in the presence of 2 mM dATP followed by the addition of supercoiled homologous DNA. Then, increasing concentrations of RecU (Figure 7A) or a constant amount of RecA (Figure 7B) were added and the reaction mixtures were incubated for 15 min. All D-loops formed by these proteins migrated in the same position as supercoiled DNA (13).

Previously, it was shown that in 1 mM Mg^{2+} RecU anneals linear ssDNA and a homologous supercoiled dsDNA substrate in the absence of a nucleotide cofactor (13). At a concentration of 10 mM $MgAc_2$, the RecU-catalyzed strand invasion was low (Figure 7, lanes 2–4). The yield of D-loops catalyzed by

stoichiometric amounts of RecA was not affected at low RecU concentrations (Figure 7A, lane 5) and increased markedly in the presence of higher RecU concentrations (Figure 7A, lanes 6–10). Stoichiometric amounts of RecU stimulated RecA-mediated D-loop formation by >3-fold (Figure 7A). However, when RecU was pre-incubated with ssDNA a clear stimulation was observed at RecU molar ratios of 0.015:1, but at RecU molar ratios of 0.12:1 or higher, an inhibition of RecA-mediated D-loop formation was observed (Figure 7B, lanes 8–10). Similar results were observed when rATP or AMP-PNP was used; however, no reaction was observed when non-homologous superhelical DNA or homologous relaxed DNA was present in the reaction mixture (13).

The three-strand intermediate (D-loop) generated by RecA is not a substrate for RecU-mediated cleavage, because the accumulation of a shorter ssDNA segment or a nicked plasmid, as shown in Figure 4A, was not observed.

DISCUSSION

The results presented in this study show that RecU modulates positively and negatively some of the RecA activities. Substoichiometric amounts of RecU enhance binding of RecA to ssDNA and stimulate RecA-catalyzed D-loop formation. The RecU enhancement of RecA-mediated D-loop formation takes place in the presence of dATP (Figure 7), rATP or the non-hydrolyzable analog AMP-PNP (data not shown). Substoichiometric amounts of RecU exert a negative effect on the completion of the RecA-mediated dATP-dependent three-strand exchange reaction. RecU inhibits RecA-mediated conversion of jm into nc products. The effects of RecU were amplified if it was added prior to RecA filament formation. The inhibition of the RecA-mediated formation of nc products by RecU took place even when SSB or SSB_{Eco} was added after RecA and before RecU. Substoichiometric amounts of RecU also exert a negative effect on the ssDNA-dependent RecA-catalyzed dATP or ATP hydrolysis, independently of the order of RecU addition, and at saturating ssDNA concentrations. RecA, in the presence of a nucleotide cofactor, however, did not affect RecU-promoted jm formation between circular ssDNA and linear 3′-tailed dsDNA. In the presence of dATP, $RecA_{Eco}$ can invade more secondary structure in native ssDNA and compete more efficiently with SSB_{Eco} for ssDNA than in the presence of rATP. In the absence of SSB_{Eco}, the rate of jm formation and product formation is also greater in the presence of dATP than rATP (48,49,54). We found a similar degree of inhibition/activation by RecU in the presence of both, dATP or rATP. Albeit, at least, in *E.coli* cells the rATP concentration is 10- to 20-fold higher than the dATP concentration (54).

Previously, it was shown that $RecA_{Eco}$, in the presence of a nucleotide cofactor, polymerizes on ssDNA by a slow initial nucleation step, followed by a rapid 5′–3′ extension in the absence of rATP or dATP hydrolysis, but the 5′–3′disassembly of the filaments requires hydrolysis of the nucleotide cofactor (48,49). The results presented in this report suggest that RecU favors RecA assembly on ssDNA, whereas the 5′–3′ disassembly of the RecA filament, promoted by the hydrolysis of dATP or rATP, is inhibited. This is consistent with electron microscopy experiments showing that RecA filamentation is not affected by the presence of RecU (Supplementary Figure S1). Therefore, the RecU protein will be a new kind of RecA modulator not described so far. We propose that in an initial step, RecU might help RecA to promote D-loop formation. RecU, which does not cleave three-stranded intermediates, will favor the production of a four-stranded intermediate or HJ. Then, three different events might take place: (i) by the inhibition of dATP (or rATP) hydrolysis, and subsequent inhibition of RecA redistribution, RecU halts extensive strand exchange catalyzed by RecA; (ii) RecU, specifically bound to the RecA-catalyzed HJ, cleaves it (data not shown) or (iii) RecU bound to HJ DNA enhances the assembly of the RuvAB–RecD complex. The outcome of all these putative events might be that RecU will be a barrier for the RecA-mediated branch migration reaction. The assembly of the RuvAB–RecD complex on the HJ favors the loading of a more effective branch migrating complex. RuvA alone, or as part of the 'RuvAB–RecD' complex, will bind to opposite faces of the four duplex DNA arms and unfold the junction into a square planar conformation that is energetically favorable to branch migration. The 'RuvAB–RecD' complex, in analogy with the *E.coli* system, should also enhance the RecU efficiency of HJ cleavage (55). Consequently, resolution in tracts of undamaged DNA, and therefore, reestablishment of a replication fork, may occur fast and without significant heteroduplex DNA formation.

Previous results and the ones presented here provide evidence that RecU and the human Rad51 paralogs might share many features in common: (i) both RecU and the BCDX2 complex bind specifically to HJs and help RecA and Rad51, respectively, to initiate DNA strand exchange [(13,35), this work], (ii) both RecU and Rad51C show synergy in their binding affinity with RecA and Rad51, respectively [(33,34), this work] and (iii) both RecU and the CX3 complex are involved in the processing and resolution of HJs (13,39). However, the suggested role of human Rad51 paralogs on the positive and negative modulation of the Rad51 function remains to be documented.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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Conflict of interest statement. None declared.

REFERENCES

1. Kowalczykowski,S.C. (2000) Initiation of genetic recombination and recombination-dependent replication. *Trends Biochem. Sci.*, **25**, 156–165.

2. Chedin,F. and Kowalczykowski,S.C. (2002) A novel family of regulated helicases/nucleases from Gram-positive bacteria: insights into the initiation of DNA recombination. *Mol. Microbiol.*, **43**, 823–834.
3. Bork,J.M., Cox,M.M. and Inman,R.B. (2001) The RecOR proteins modulate RecA protein function at 5′ ends of single-stranded DNA. *EMBO J.*, **20**, 7313–7322.
4. Morimatsu,K. and Kowalczykowski,S.C. (2003) RecFOR proteins load RecA protein onto gapped DNA to accelerate DNA strand exchange: a universal step of recombinational repair. *Mol. Cell*, **11**, 1337–1347.
5. Cox,M.M., Goodman,M.F., Kreuzer,K.N., Sherratt,D.J., Sandler,S.J. and Marians,K.J. (2000) The importance of repairing stalled replication forks. *Nature*, **404**, 37–41.
6. Kuzminov,A. (1999) Recombinational repair of DNA damage in *Escherichia coli* and bacteriophage lambda. *Microbiol. Mol. Biol. Rev.*, **63**, 751–813.
7. Cromie,G.A. and Leach,D.R. (2000) Control of crossing over. *Mol. Cell*, **6**, 815–826.
8. Bedale,W.A. and Cox,M. (1996) Evidence for the coupling of ATP hydrolysis to the final (extension) phase of RecA protein-mediated DNA strand exchange. *J. Biol. Chem.*, **271**, 5725–5732.
9. Robu,M.E., Inman,R.B. and Cox,M.M. (2001) RecA protein promotes the regression of stalled replication forks *in vitro*. *Proc. Natl Acad. Sci. USA*, **98**, 8211–8218.
10. Mandal,T.N., Mahdi,A.A., Sharples,G.J. and Lloyd,R.G. (1993) Resolution of Holliday intermediates in recombination and DNA repair: indirect suppression of *ruvA*, *ruvB*, and *ruvC* mutations. *J. Bacteriol.*, **175**, 4325–4334.
11. Dawid,A., Croquette,V., Grigoriev,M. and Heslot,F. (2004) Single-molecule study of RuvAB-mediated Holliday-junction migration. *Proc. Natl Acad. Sci. USA*, **101**, 11611–11616.
12. Amit,R., Gileadi,O. and Stavans,J. (2004) Direct observation of RuvAB-catalyzed branch migration of single Holliday junctions. *Proc. Natl Acad. Sci. USA*, **101**, 11605–11610.
13. Ayora,S., Carrasco,B., Doncel,E., Lurz,R. and Alonso,J.C. (2004) *Bacillus subtilis* RecU protein cleaves Holliday junctions and anneals single-stranded DNA. *Proc. Natl Acad. Sci. USA*, **101**, 452–457.
14. West,S.C. (1997) Processing of recombination intermediates by the RuvABC proteins. *Annu. Rev. Genet.*, **31**, 213–244.
15. Lusetti,S.L., Drees,J.C., Stohl,E.A., Seifert,H.S. and Cox,M.M. (2004) The DinI and RecX proteins are competing modulators of RecA function. *J. Biol. Chem.*, **279**, 55073–55079.
16. Venkatesh,R., Ganesh,N., Guhan,N., Reddy,M.S., Chandrasekhar,T. and Muniyappa,K. (2002) RecX protein abrogates ATP hydrolysis and strand exchange promoted by RecA: insights into negative regulation of homologous recombination. *Proc. Natl Acad. Sci. USA*, **99**, 12091–12096.
17. Stohl,E.A., Brockman,J.P., Burkle,K.L., Morimatsu,K., Kowalczykowski,S.C. and Seifert,H.S. (2003) *Escherichia coli* RecX inhibits RecA recombinase and coprotease activities *in vitro* and *in vivo*. *J. Biol. Chem.*, **278**, 2278–2285.
18. Drees,J.C., Lusetti,S.L., Chitteni-Pattu,S., Inman,R.B. and Cox,M.M. (2004) A RecA filament capping mechanism for RecX protein. *Mol. Cell*, **15**, 789–798.
19. Lusetti,S.L., Voloshin,O.N., Inman,R.B., Camerini-Otero,R.D. and Cox,M.M. (2004) The DinI protein stabilizes RecA protein filaments. *J. Biol. Chem.*, **279**, 30037–30046.
20. Voloshin,O.N., Ramirez,B.E., Bax,A. and Camerini-Otero,R.D. (2001) A model for the abrogation of the SOS response by an SOS protein: a negatively charged helix in DinI mimics DNA in its interaction with RecA. *Genes Dev.*, **15**, 415–427.
21. Churchill,J.J., Anderson,D.G. and Kowalczykowski,S.C. (1999) The RecBC enzyme loads RecA protein onto ssDNA asymmetrically and independently of chi, resulting in constitutive recombination activation. *Genes Dev.*, **13**, 901–911.
22. Sung,P., Krejci,L., Van Komen,S. and Sehorn,M.G. (2003) Rad51 recombinase and recombination mediators. *J. Biol. Chem.*, **278**, 42729–42732.
23. Shibata,T., DasGupta,C., Cunningham,R.P. and Radding,C.M. (1980) Homologous pairing in genetic recombination: formation of D loops by combined action of RecA protein and a helix-destabilizing protein. *Proc. Natl Acad. Sci. USA*, **77**, 2606–2610.
24. Kowalczykowski,S.C. and Krupp,R.A. (1987) Effects of *Escherichia coli* SSB protein on the single-stranded DNA-dependent ATPase activity of *Escherichia coli* RecA protein. Evidence that SSB protein facilitates the binding of RecA protein to regions of secondary structure within single-stranded DNA. *J. Mol. Biol.*, **193**, 97–113.
25. Adams,D.E., Tsaneva,I.R. and West,S.C. (1994) Dissociation of RecA filaments from duplex DNA by the RuvA and RuvB DNA repair proteins. *Proc. Natl Acad. Sci. USA*, **91**, 9901–9905.
26. Iype,L.E., Inman,R.B. and Cox,M.M. (1995) Blocked RecA protein-mediated DNA strand exchange reactions are reversed by the RuvA and RuvB proteins. *J. Biol. Chem.*, **270**, 19473–19480.
27. Veaute,X., Jeusset,J., Soustelle,C., Kowalczykowski,S.C., Le Cam,E. and Fabre,F. (2003) The Srs2 helicase prevents recombination by disrupting Rad51 nucleoprotein filaments. *Nature*, **423**, 309–312.
28. Krejci,L., Van Komen,S., Li,Y., Villemain,J., Reddy,M.S., Klein,H., Ellenberger,T. and Sung,P. (2003) DNA helicase Srs2 disrupts the Rad51 presynaptic filament. *Nature*, **423**, 305–309.
29. Veaute,X., Delmas,S., Selva,M., Jeusset,J., Le Cam,E., Matic,I., Fabre,F. and Petit,M.A. (2005) UvrD helicase, unlike Rep helicase, dismantles RecA nucleoprotein filaments in *Escherichia coli*. *EMBO J.*, **24**, 180–189.
30. Bianco,P.R., Tracy,R.B. and Kowalczykowski,S.C. (1998) DNA strand exchange proteins: a biochemical and physical comparison. *Front. Biosci.*, **3**, D570–D603.
31. West,S.C. (2003) Molecular views of recombination proteins and their control. *Nature Rev. Mol. Cell Biol.*, **4**, 435–445.
32. Masson,J.Y., Tarsounas,M.C., Stasiak,A.Z., Stasiak,A., Shah,R., McIlwraith,M.J., Benson,F.E. and West,S.C. (2001) Identification and purification of two distinct complexes containing the five RAD51 paralogs. *Genes Dev.*, **15**, 3296–3307.
33. Sigurdsson,S., Van Komen,S., Bussen,W., Schild,D., Albala,J.S. and Sung,P. (2001) Mediator function of the human Rad51B–Rad51C complex in Rad51/RPA-catalyzed DNA strand exchange. *Genes Dev.*, **15**, 3308–3318.
34. Kurumizaka,H., Ikawa,S., Nakada,M., Eda,K., Kagawa,W., Takata,M., Takeda,S., Yokoyama,S. and Shibata,T. (2001) Homologous-pairing activity of the human DNA-repair proteins Xrcc3.Rad51C. *Proc. Natl Acad. Sci. USA*, **98**, 5538–5543.
35. Lio,Y.C., Mazin,A.V., Kowalczykowski,S.C. and Chen,D.J. (2003) Complex formation by the human Rad51B and Rad51C DNA repair proteins and their activities *in vitro*. *J. Biol. Chem.*, **278**, 2469–2478.
36. Yokoyama,H., Sarai,N., Kagawa,W., Enomoto,R., Shibata,T., Kurumizaka,H. and Yokoyama,S. (2004) Preferential binding to branched DNA strands and strand-annealing activity of the human Rad51B, Rad51C, Rad51D and Xrcc2 protein complex. *Nucleic Acids Res.*, **32**, 2556–2565.
37. Yokoyama,H., Kurumizaka,H., Ikawa,S., Yokoyama,S. and Shibata,T. (2003) Holliday junction binding activity of the human Rad51B protein. *J. Biol. Chem.*, **278**, 2767–2772.
38. Braybrooke,J.P., Li,J.L., Wu,L., Caple,F., Benson,F.E. and Hickson,I.D. (2003) Functional interaction between the Bloom's syndrome helicase and the RAD51 paralog, RAD51L3 (RAD51D). *J. Biol. Chem.*, **278**, 48357–48366.
39. Liu,Y., Masson,J.Y., Shah,R., O'Regan,P. and West,S.C. (2004) RAD51C is required for Holliday junction processing in mammalian cells. *Science*, **303**, 243–246.
40. Wang,R.C., Smogorzewska,A. and de Lange,T. (2004) Homologous recombination generates T-loop-sized deletions at human telomeres. *Cell*, **119**, 355–368.
41. McGregor,N., Ayora,S., Sedelnikova,S., Carrasco,B., Alonso,J.C., Thaw,P. and Rafferty,J.B. (2005) The structure of *Bacillus subtilis* RecU Holliday junction resolvase and its role in substrate selection and sequence specific cleavage. *Structure,* in press.
42. Ayora,S., Missich,R., Mesa,P., Lurz,R., Yang,S., Egelman,E.H. and Alonso,J.C. (2002) Homologous-pairing activity of the *Bacillus subtilis* bacteriophage SPP1 replication protein G35P. *J. Biol. Chem.*, **277**, 35969–35979.
43. Gassel,M. and Alonso,J.C. (1989) Expression of the *recE* gene during induction of the SOS response in *Bacillus subtilis* recombination-deficient strains. *Mol. Microbiol.*, **3**, 1269–1276.
44. Weise,F., Chai,S., Luder,G. and Alonso,J.C. (1994) Nucleotide sequence and complementation studies of the gene 35 region of the *Bacillus subtilis* bacteriophage SPP1. *Virology*, **202**, 1046–1049.
45. Gill,S.C. and von Hippel,P.H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.*, **182**, 319–326.

46. Ayora,S., Piruat,J.I., Luna,R., Reiss,B., Russo,V.E., Aguilera,A. and Alonso,J.C. (2002) Characterization of two highly similar Rad51 homologs of *Physcomitrella patens*. *J. Mol. Biol*., **316**, 35–49.
47. Steven,A.C., Trus,B.L., Maizel,J.V., Unser,M., Parry,D.A., Wall,J.S., Hainfeld,J.F. and Studier,F.W. (1988) Molecular substructure of a viral receptor-recognition protein. The gp17 tail-fiber of bacteriophage T7. *J. Mol. Biol*., **200**, 351–365.
48. Kowalczykowski,S.C. and Eggleston,A.K. (1994) Homologous pairing and DNA strand-exchange proteins. *Annu. Rev. Biochem*., **63**, 991–1043.
49. Lusetti,S.L. and Cox,M.M. (2002) The bacterial RecA protein and the recombinational DNA repair of stalled replication forks. *Annu. Rev. Biochem*., **71**, 71–100.
50. Lovett,C.M.,Jr and Roberts,J.W. (1985) Purification of a RecA protein analogue from *Bacillus subtilis*. *J. Biol. Chem*., **260**, 3305–3313.
51. Steffen,S.E. and Bryant,F.R. (1999) Reevaluation of the nucleotide cofactor specificity of the RecA protein from *Bacillus subtilis*. *J. Biol. Chem*., **274**, 25990–25994.
52. Doncel-Pérez,E. (2002) Caracterización de la proteína RecU de *Bacillus subtilis* 168. Estudio de su papel en la recombinación y segregación cromosómica, PhD, Thesis Universidad Autónoma de Madrid, Madrid, Spain.
53. Sugiyama,T., Zaitseva,E.M. and Kowalczykowski,S.C. (1997) A single-stranded DNA-binding protein is needed for efficient presynaptic complex formation by the *Saccharomyces cerevisiae* Rad51 protein. *J. Biol. Chem*., **272**, 7940–7945.
54. Menetski,J.P. and Kowalczykowski,S.C. (1989) Enhancement of *Escherichia coli* RecA protein enzymatic function by dATP. *Biochemistry*, **28**, 5871–5881.
55. Eggleston,A.K. and West,S.C. (2000) Cleavage of Holliday junctions by the *Escherichia coli* RuvABC complex. *J. Biol. Chem*., **275**, 26467–26476.