Nucleic Acids Research, 2005, Vol. 33, No. 7 ***2343–2350***
doi:10.1093/nar/gki533

Bacillus subtilis RecN binds and protects 3′-single-stranded DNA extensions in the presence of ATP

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Received January 31, 2005; Revised March 23, 2005; Accepted April 6, 2005

ABSTRACT

Bacillus subtilis **RecN appears to be an early detector of breaks in double-stranded DNA. *In vivo*, RecN forms discrete nucleoid-associated structures and *in vitro* exhibits Mg^{2+}-dependent single-stranded (ss) DNA binding and ssDNA-dependent ATPase activities. In the presence of ATP or ADP, RecN assembles to form large networks with ssDNA molecules (designated complexes CII and CIII) that involve ATP binding and requires a 3′-OH at the end of ssDNA molecule. Addition of dATP–RecA complexes dissociates RecN from these networks, but this is not observed following addition of an ssDNA binding protein. Apparently, ATP modulates the RecN–ssDNA complex for binding to ssDNA extensions and, *in vivo*, RecN–ATP bound to 3′-ssDNA might sequester ssDNA ends within complexes that protect the ssDNA while the RecA accessory proteins recruit RecA. With the association of RecA to ssDNA, RecN would dissociate from the DNA end facilitating the subsequent steps in DNA repair.**

INTRODUCTION

Cells are constantly facing the challenge of repairing numerous alterations in their genetic material that occur under normal physiological conditions, or in cells that suffer the effect of genotoxic agents or irradiation. A range of mechanisms has apparently evolved to repair double-stranded (ds) DNA breaks (DSBs) [reviewed in (1–5)].

In eukaryotes and bacteria, the assembly of multiprotein complexes into discrete foci at sites of unrepaired DSBs was observed [reviewed in (6)]. These large protein assemblies could facilitate the aggregation of repair proteins at a lesion, and the localization of multiple lesions to one or few foci (6). In the yeast *Saccharomyces cerevisiae*, in response to DSBs, the Mre11, Rad50 and Xrs2 (Nbs1 is the functional homolog in human) complex (termed MRX/N) directly binds to DNA ends and appears to be the earliest sensor of a DSB (7–9). It was shown *in vitro* that the DNA ends were transiently stabilized and tethered together by the action of Rad50 (10). Upon DSB recognition by the MRN/X complex, and before the 5′ resection of the DNA ends and checkpoint activation, the choice between homologous recombination (HR) and non-homologous end joining takes place [reviewed in (6)].

Viability of both *Escherichia coli* and *Bacillus subtilis* after exposure to ionizing radiation or mitomycin C (MMC) is severely compromised in *recN* mutants (11–14). Exposure of exponentially growing *B.subtilis* cells to MMC treatment or X-ray radiation results in random DSBs, inhibition of cell division, decreased growth and RecN assembly at typically one visible focus (rarely, two or three foci form on the genome) (14). Similar results were observed upon induction of the HO endonuclease and generation of site-specific DSBs (P. L. Graumann, personal communication). RecN assembly occurs within 15–30 min of DSBs induction (14), and these foci are frequently found close to the cells 'replication factory' (15), although they are separated from DNA polymerase III (14). Genetic analyses linked to cytological studies have helped to define the sequence of events involved in DSB repair in *B.subtilis*, with RecN foci accumulating in cells defective in DNA end processing (e.g. an *addAB recJ* strain), RecA loading (mutations in *addAB* or *recO* cells) or processing of recombination intermediates [mutations in *recG* or *recU* cells) (14) and H. Sanchez, D. Kidane, P. L. Graumann and J. C. Alonso, unpublished data]. Although the RecN concentration *in vivo* increases several fold after SOS induction, RecN foci form even in the absence of RecA, consistent with a very early role in the organization of repair complexes at sites of DSBs (14).

B.subtilis RecN (64.4 kDa; 576 amino acids) and *E.coli* RecN ($RecN_{Eco}$) (63.6 kDa, 567 amino acids long) resemble eukaryotic SMC-like proteins. They have two coiled coil

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domains and a central hinge domain that separate the N-terminal (lobe I) domain from the C-terminal (lobe II) domain, which contain Walker A and Walker B motifs, respectively (16,17). To gain insights into the function of RecN, the protein was purified from the soluble fraction of a *B.subtilis* extract and biochemically characterized. In the presence of Mg^{2+}, RecN exhibits single-stranded (ss) DNA-dependent ATP hydrolysis and ssDNA binding activity; it binds ssDNA independently of nucleotide cofactors, but also binds specifically to 3′-ssDNA extensions in a nucleotide-dependent manner. The hydrolysis of ATP drives the formation of a large nucleoprotein complex. In the presence of ATP or dATP, large ATP–RecN–ssDNA networks assemble *in vitro* that contain more than one ssDNA molecule per RecN molecule. These are disassembled by the addition of dATP–RecA but not by the addition of an ssDNA binding protein (SSB). These results are consistent with RecN binding 3′-ssDNA extensions of DSBs and protecting these regions from nuclease degradation before RecA-dependent repair.

MATERIALS AND METHODS

Enzymes, reagents and DNA substrates

The RecN protein was purified as described previously (14). The SPP1-encoded SSB protein (also termed G*36*P), which shares 46% identity with *B.subtilis* SSB protein and *B.subtilis* RecA protein, was provided by B. Carrasco (Centro Nacional de Biotecnología, CSIC). The exonuclease VII (ExoVII) and *E.coli* SSB protein were purchased from USB. DTT, EDTA and MMC were purchased from Sigma. Gel filtration chromatography was performed as described previously (14).

[α-^{32}P]dATP, [α-^{32}P]ddATP and [γ-^{32}P]ATP were from Amersham Bioscience. All chemicals used were reagent grade, and nucleotides concentrations were measured spectrophotometrically using an extinction coefficient of 1.54×10^{-4} M^{-1} cm^{-1} at 260 nm. They were dissolved as concentrated stock solutions at pH 7.5. Linear oligonucleotide $ssDNA_{60}$ (5′-CTCCTATTATGCTCAACTTAAATGACCTACTCTAT-AAAGCTATAGTACTGCTATCTAATC-3′) and $dsDNA_{60}$, formed by annealing of $ssDNA_{60}$ with its complementary 60mer, were end-labelled at the 3′ end using [α-^{32}P]dATP or [α-^{32}P]ddATP and terminal transferase (Roche). They were 5′ end-labelled using [γ-^{32}P]ATP and polynucleotide kinase (New England Biolabs). Oligonucleotides $ssDNA_{15}$ and $ssDNA_{30}$ contained the first 15 and 30 nt of the $ssDNA_{60}$, respectively. DNA concentrations were determined using molar extinction coefficients of 8780 and 6500 M^{-1} cm^{-1} at 260 nm for ssDNA and dsDNA, respectively. Unless otherwise stated, DNA concentrations were given as moles of DNA molecules.

Glycerol gradient centrifugation

RecN (10 μg) was applied onto a 5 ml linear gradient of 15–30% glycerol that was prepared in buffer A (50 mM Tris–HCl, pH 7.5, 50 mM NaCl, 2 mM $MgCl_2$ and 1 mM DTT) performed as described previously (14). Thyroglobulin (THY): 669 kDa, aldolase (ALD): 158 kDa and BSA: 69 kDa, not shown in Figure 3A were used as protein standards. After a 17 h centrifugation at 200 000 *g* at 4°C, 200 μl fractions were collected and separated by 10% SDS–PAGE detected by Coomassie blue staining.

ATPase assay

The DNA-dependent RecN ATPase activity was assayed by thin-layer chromatography. Reaction mixtures were incubated in buffer A with increasing concentrations of the different effectors [$ssDNA_{60}$, viral M13mp18 (ssDNA M13) and replicative form M13mp18 (dsDNA M13)] as described previously (18,19).

Electrophoretic mobility shift assay (EMSA) of RecN–DNA complexes

Aliquots of $ssDNA_{60}$ or $dsDNA_{60}$ (1 nM) were incubated with indicated concentrations of RecN in buffer B (50 mM Tris–HCl, pH 7.5, 50 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT and 2% PEG-6000) with or without ADP, ATP, dATP, AMP-PNP or ATPγS, at concentrations ranging from 0.01 to 1 M, for 30 min at 37°C. The presence of 2% PEG-6000 could be omitted with a significant reduction on protein–ssDNA complex formation.

The reactions products separated in a 10% non-denaturing PAGE (ndPAGE) run in TBE buffer. The labelled complexes were visualized by autoradiography and quantified with an image analyzer (Molecular Imager FX-BioRad).

Complexes preformed by incubation of RecN with $ssDNA_{60}$, RecN with ADP and $ssDNA_{60}$, and RecN with ATP and $ssDNA_{60}$ were incubated with increasing amounts of ExoVII, SSB (0.1 μM) or RecA (0.2 μM) in buffer B for 10 min at 37°C, and the reactions products separated, visualized and quantified as described above.

RESULTS

ssDNA stimulates RecN ATPase activity

RecN protein from *B.subtilis* cells was purified as described previously (14). The preparations were ∼99% pure based on staining after SDS–PAGE, with ∼1% of the preparation being GroEL based on N-terminal sequencing. A similar result was observed with *B.subtilis* RecN protein purified after expression in *E.coli* (20).

Purified RecN was found to hydrolyze ATP to ADP and P_i in a ssDNA-dependent manner with ∼8-fold stimulation of ATPase activity observed in the presence of a 60 nt ssDNA ($ssDNA_{60}$) without any potential secondary structure formation. However, when the ssDNA has a self-annealing or partial annealing potential (e.g. a short ssDNA fragment or viral M13mp18 ssDNA), only an ∼3-fold stimulation was observed (Figure 1A). This is consistent with the observation that linear or supercoiled dsDNA did not stimulate the RecN ATPase activity (Figure 1A).

Based on gel filtration chromatography, the ssDNA-dependent ATPase activity was present in a ∼500 kDa RecN complex (data not shown). Traces amounts of 30–50 nt long fragments of ssDNA co-purified with the RecN protein (14,20), and the low level of ATPase activity observed in the absence of added ssDNA may reflect RecN ATPase stimulation by this contaminating ssDNA. GroEL does also have an ATPase activity, but this is reduced $\sim 10^4$-fold in the absence of K^+ ions (21). GroEL is therefore almost certainly not the

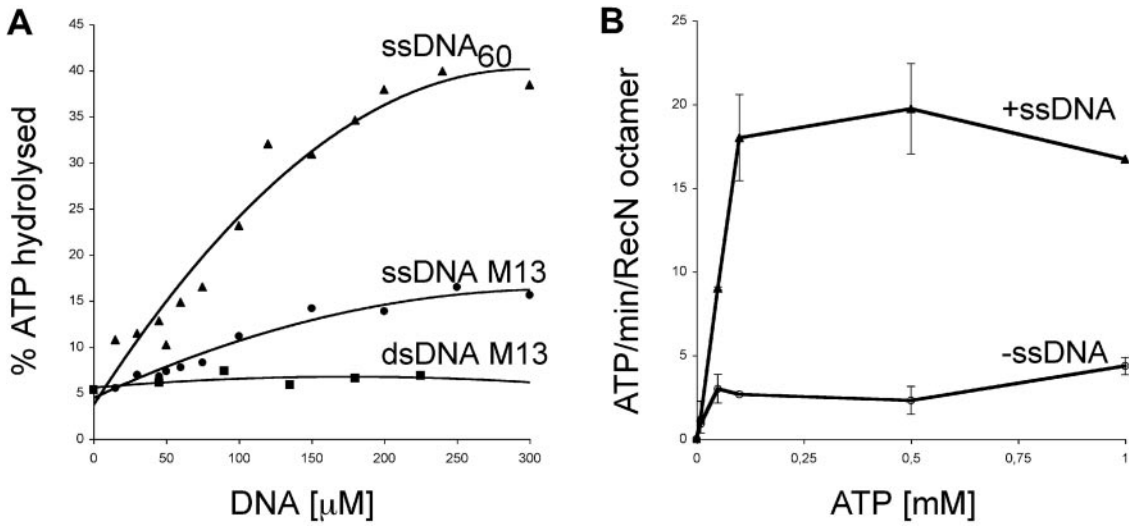

Figure 1. The ssDNA-dependent ATPase activity of RecN. (**A**) Stimulation of RecN ATPase activity by $ssDNA_{60}$ (filled triangles), M13 ssDNA (filled circles) and dsDNA (filled squares). (**B**) ATPase activity of RecN in the presence (filled triangles) and absence (empty circles) of $ssDNA_{60}$ at increasing ATP concentrations. The ATPase activity is expressed in terms of ATP molecules hydrolysed per min per RecN octamer.

source of the observed ssDNA-dependent ATPase activity. The basis for the association of GroEL with over-expressed RecN remains to be determined.

To evaluate kinetics parameters, increasing concentrations of ATP were incubated with RecN and ssDNA (Figure 1B). The RecN ATPase activity, in the presence of ssDNA, reached a plateau ($K_m = 0.05$ mM and $k_{cat} = 18$ min^{-1}) by 0.1 mM ATP (Figure 1B). In the absence of ssDNA, $k_{cat} = 3$ min^{-1}. Similar results were obtained when the reaction was performed in the presence of an ATP regeneration system (data not shown).

RecN forms multiple complexes with ssDNA

RecN binding to linear ssDNA and dsDNA was investigated by EMSAs using ndPAGE. In the absence of a nucleotide cofactor, RecN bound ssDNA to form a complex designated CI, with the amount of ssDNA bound into CI increasing from 25 to 40% as the RecN–DNA molar ratios increased from 7.5:1 to 60:1 (Figure 2A). CI formation was inhibited by the presence of 5 mM EDTA (data not shown). When ATP or ADP was present, two additional RecN–ssDNA complexes were formed, designated CII and CIII (Figure 2A, see below). Similar results were obtained when dATP was used (see below). However, when circular M13 ssDNA was used, only CI type complexes were observed (data not shown).

Addition of EDTA to the reaction mixture or removal of Mg^{2+} blocked the RecN binding to ssDNA. Incubation of the ssDNA with ATP or ADP, in the absence of RecN, had no effect on the electrophoretic mobility of the ssDNA (Figure 2A). Using identical reaction and EMSA conditions, RecN binding to dsDNA was not detected (data not shown).

The ATP and ADP concentrations needed to assemble CII and CIII were established in reaction mixtures that contained RecN and ssDNA at a 10:1 molar ratio. At 0.01 mM ATP, only CI was formed whereas both CI and CII were formed in the presence of 0.01 mM ADP, and 80% of ssDNA was assembled into CIII when the ADP was increased to 0.05 mM (Figure 2B). In the presence of the non-hydrolysable nucleotide analogue AMP-PNP, only CI was formed (Figure 2B), although with the weakly hydrolysable analogue (ATPγS) present, CII and CIII formation was observed (see below).

A RecN oligomer binds to $ssDNA_{60}$

Analytical ultracentrifugation and gel filtration chromatography indicate that RecN is an octamer, and glycerol gradient centrifugation shows that RecN is assembled into octamers, tetramers and dimers, both in the presence and absence of ATP (14). It is likely that the mild denaturing activity of glycerol might account for the reported differences and RecN monomers are not detected (see below).

To learn whether the octameric or dimeric form was able to bind ssDNA, RecN molecules that co-sedimented through glycerol gradients with THY (669 kDa, fraction 1), or with ALD (158 kDa, fraction 12) (Figure 3A) were incubated with ssDNA in the presence of 1 mM ADP or ATP. The complexes formed were separated by 10% ndPAGE revealing that the RecN species with a high sedimentation coefficient (presumably an octamer) formed complexes with ssDNA indistinguishable from CIII (Figure 3B), although the presence of ~15% glycerol reduced the ssDNA binding efficiency ~3-fold. In contrast, the RecN species with a low sedimentation coefficient (presumably a dimer) did not form a complex with $ssDNA_{60}$ detectable by EMSA (Figure 3B, lanes 4–6).

ssDNA parameters required for RecN binding

RecN, at a fixed ratio of 10:1, did not form a complex detectable by EMSA with a 15 nt ssDNA ($ssDNA_{15}$); formed a detectable but unstable complex with $ssDNA_{30}$ segment but bound to form a CI complex with all $ssDNA_{60}$ (Figure 4) or longer (data not shown). RecN bound with similar affinity to $ssDNA_{60}$ labelled with [^{32}P] at either the 3′- or 5′-terminus and without a 5′-terminal phosphate (Figure 4A and C). Similar results were observed in the absence (Figure 4A) or in the presence of a phosphorylated 5′-terminus (Figure 2). However, when a 2′,3′-dideoxyadenosine 5′ monophosphate is present at

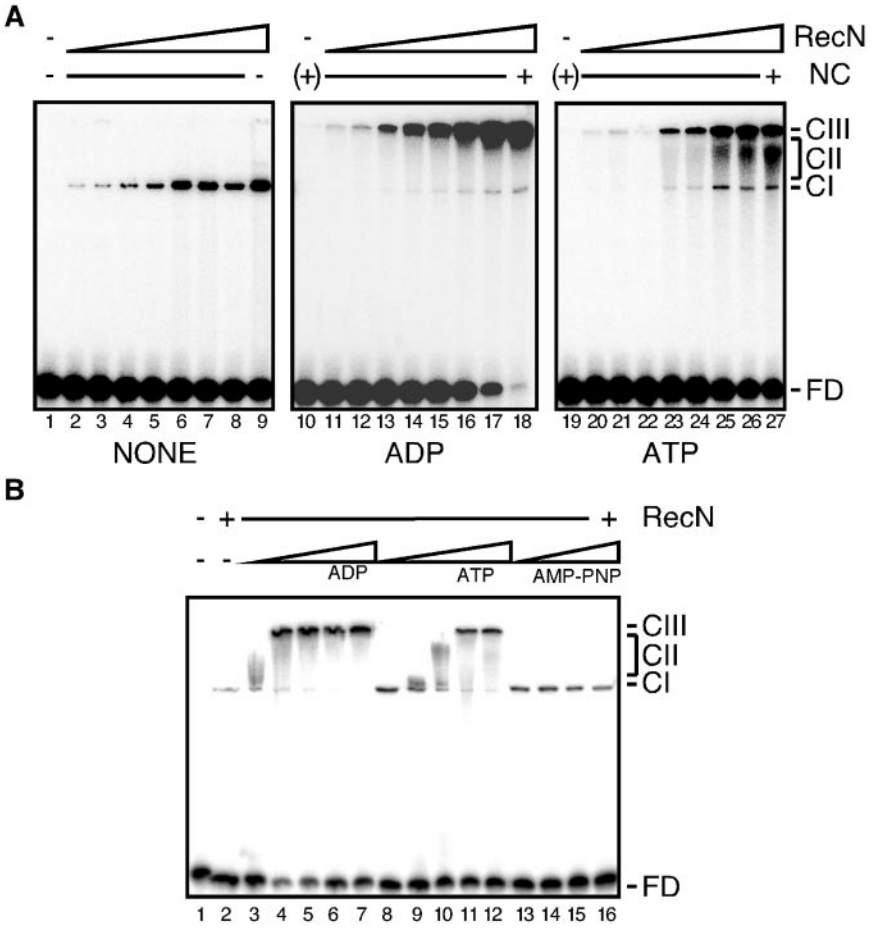

Figure 2. DNA binding by RecN. (**A**) [^{32}P]Labelled ssDNA$_{60}$ (1 nM), at the 5′-terminus, was incubated with RecN (0.4, 0.8, 1.7, 3.7, 15, 30 and 60 nM) for 30 min at 37°C in buffer B in the absence (lanes 1–9) or presence of 1 mM ADP (lanes 10–18) or 1 mM ATP (lanes 19–27). RecN was not added to the controls in lane 1, 10 and 19. In lanes 10 and 19, (+) denotes that identical results were observed in the presence or absence of a nucleotide cofactor. (**B**) [γ-^{32}P]Labelled ssDNA$_{60}$ (1 nM) and RecN (10 nM) were incubated and then ADP (0.01, 0.05, 0.1, 0.5 and 1 mM, lanes 3–7), ATP (0.01–1 mM, lanes 8–12) or AMP-PNP (0.01–1 mM, lanes 13–16) was added; incubation continued for 30 min at 37°C in buffer B. RecN was not added in lane 1. Complexes formed were separated by 10% ndPAGE and visualized by autoradiography. FD, free DNA; CI, complex I; CII, complex II; CIII, complex III; + and −, the presence or absence of the indicated factor.

the 3′-terminus, only CI was formed indicating that a hydroxyl group at the 3′-terminus was essential for CII and CIII formation (Figure 4B). These results are consistent with the observation that RecN bound to circular M13 ssDNA yields only CI formation.

Both the 3′-termini (labelled at 3′ end) and the 5′-termini (labelled at 5′ end) of ssDNA bound into CI were sensitive to ExoVII degradation. The 3′-termini of ssDNA molecules, however, bound into CII and CIII were resistant to degradation by ExoVII (data not shown). Apparently, therefore, RecN interacts with the backbone of the ssDNA to form CI; whereas, in the presence of ADP or ATP, RecN also interacts with the 3′-OH terminus, forming CII and CIII, and so protects the 3′-terminus from exonucleolytic attack.

Order of addition and complex disaggregation experiments

CII and CIII formation, after CI formation, was assayed by subsequent addition of ATP or ADP. Approximately 2 and 40% of the ssDNA was assembled into CIII, when ATP and ADP were added after CI formation, respectively (Figure 5A). The coincident addition of ATP or ADP plus a 50- to 500-fold excess of ssDNA$_{60}$ had no effect on this result. Apparently, therefore, RecN bound to the ssDNA in CI complexes remained bound to ssDNA during CIII assembly. However, when ATP or ADP was pre-incubated with RecN, before addition to the ssDNA, the amount of CIII formed was reduced ∼7-fold (Figure 5A).

RecN–ssDNA complexes formed in the presence of ATP (or dATP) were disrupted by incubation with 1 M urea (data not shown) or 1% SDS (Figure 5B, lanes 14–16), whereas the RecN–ssDNA complexes formed in the presence of ADP were resistant to incubation with urea or guanidinium hydrochloride up to 7 M (data not shown) or with 1% SDS (Figure 5B, lanes 11–13). The ADP-dependent complexes were also readily precipitated by low speed centrifugation and although resistant to proteinase K, they were disassembled by the combined action of proteinase K, SDS and phenol treatment (data not shown). The ssDNA$_{60}$ phenol extracted from these complexes was sensitive to ExoVII. Based on these results, it is apparent that CII and CIII formed in the presence of ATP do not contain

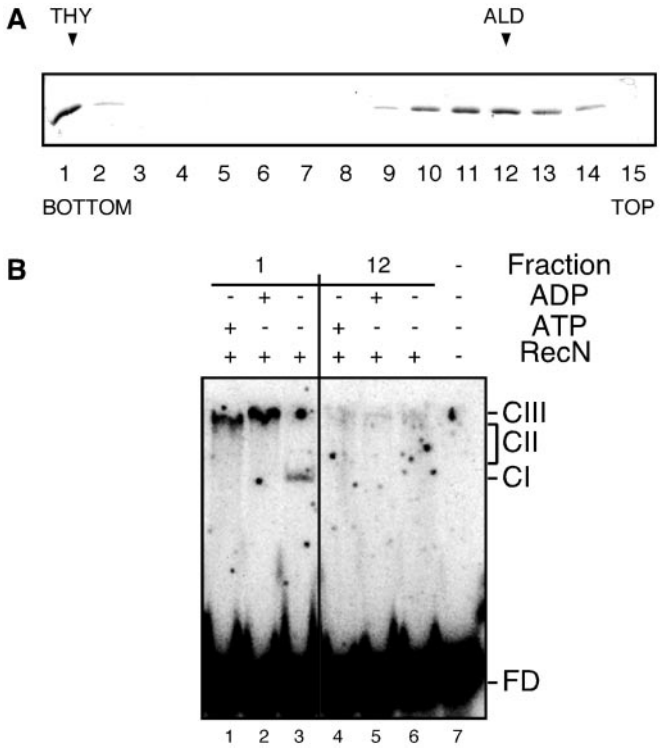

Figure 3. RecN octamers bind ssDNA$_{60}$. (**A**) RecN protein was subjected to glycerol gradient centrifugation and, after gradient fractionation, aliquots of protein in each fraction were subjected to 10% SDS–PAGE and detected by staining with Coomassie blue. The sedimentation locations of THY (669 kDa) and ALD (158 kDa), and the top and bottom of the gradient are indicated. (**B**) [^{32}P]Labelled ssDNA$_{60}$ (1 nM)), at the 5′-terminus, and RecN (10 nM) from fractions 1 or 12 (from A) were incubated, 1 mM ADP (lanes 1 and 4), ATP (lanes 2 and 5) or no nucleotide cofactor (lanes 3 and 6) then added and the mixture incubated in buffer B for 30 min at 37°C. The DNA–protein complexes formed were separated and visualized as described in Figure 2. FD, free DNA; CI, complex I; CII, complex II; CIII, complex III; + and −, the presence or absence of the indicated factor.

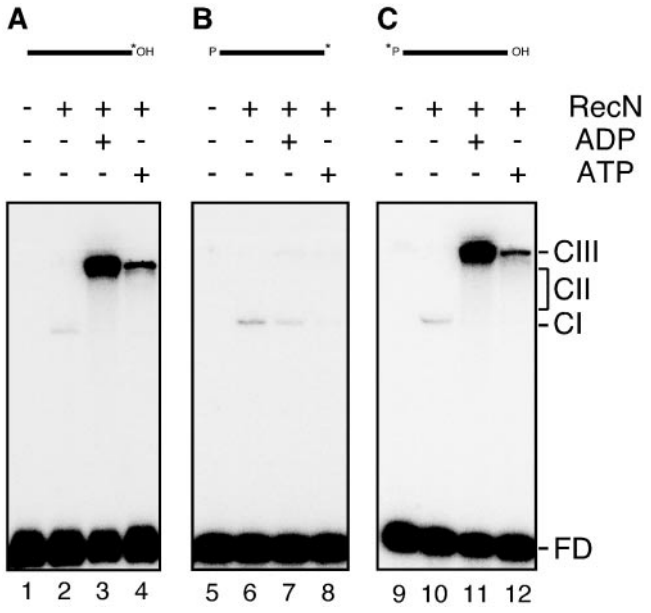

Figure 4. Specific binding of RecN to the 3′-OH terminus. Linear [^{32}P]-labelled ssDNA$_{60}$ (1 nM) with an un-phosphorylated 5′-terminus labelled at the 3′-terminus (**A**); phosphorylated 5′-terminus labelled at the 3′-terminus (**B**) or at the 5′-terminus, having a 3′-OH terminus (**C**) was incubated with RecN (10 nM). ADP, ATP (0.5 mM) or no nucleotide cofactor was added and incubated for 30 min at 37°C in buffer B. DNA–protein complexes formed were separated and visualized as described in Figure 2. FD, free DNA; CI, complex I; CII, complex II; CIII, complex III; + and −, the presence or absence of the indicated factor. The labelling is denoted by an asterisk.

covalent protein–DNA bonds. The biological significance of the RecN–ssDNA complexes in the presence of ADP remains to be unravelled.

To determine whether a *B.subtilis* SSB protein could displace RecN bound to ssDNA, preformed CI, CII and CIII were incubated with an excess of a SSB protein, and the reaction products were analyzed by 10% ndPAGE. The presence of 1 mM dATP (Figure 6) or ADP (data not shown) did not modify the migration of free DNA. In the absence of a nucleotide cofactor, RecN bound to ssDNA formed complex CI; also when ADP or dATP was present CIII and CII were formed (Figures 2A and 6, lanes 2–4). Addition of an SSB protein, at a concentration ∼10-fold higher than the apparent dissociation constant, K_{app}, for the ssDNA, displaced RecN from CI. Here, only SSB–ssDNA complexes were present (Figure 6A, lanes 5 and 6). In contrast, incubation with SSB, even at 10-fold higher than the K_{app} of SSB, did not disrupt CII or CIII assembled in the presence of ADP or dATP (Figure 6A, lanes 7 and 8). Similar results were obtained when *E.coli* SSB protein was used or ATP instead of dATP (data not shown).

Similar experiments were undertaken to determine whether RecA bound to ssDNA promoted the disassembly of RecN from CIII assembled in the presence of ATP, dATP or ADP. Previously, it has been shown that RecA shows some preferences for dATP over ATP (22); hence, dATP had been used in our assay. Under this experimental condition, the site size of RecA is 3 nt (22). Incubation with stoichiometric amounts of RecA (1 RecA/3 nt) resulted in the disassembly of CIII formed in the presence of dATP (Figure 6B, lane 8). Similar results were observed in the presence of ATP, but 2- to 3-fold higher RecA concentrations were required (data not shown). Incubation with RecA in the presence of ADP had little effect on the CIII formed (Figure 6B, lane 7).

DISCUSSION

The results presented in this paper provide insight into some biochemical characteristics of the RecN protein and into the early step of DSB repair. RecN forms homo-octamers that form stable complexes with ssDNA molecules >30 nt in length. This is consistent with the observation that RecN protein purified from the insoluble fraction of a heterologous *E.coli* expression system behaves as an octamer in analytical ultracentrifugation. Electron microscopy failed to detect species <150 kDa (e.g. RecN dimers), but revealed that RecN forms rod- and U-shaped, extended flexible rod-like molecules and oligomers interacting at both ends [(20), R. Lurz, personal communication).

RecN oligomers show an ssDNA-dependent ATPase in the presence of Mg^{2+}. It is likely that the two lobes that contain the Walker A and B motifs are placed in close proximity upon binding to ssDNA and ATP. In the absence of ATP or ADP,

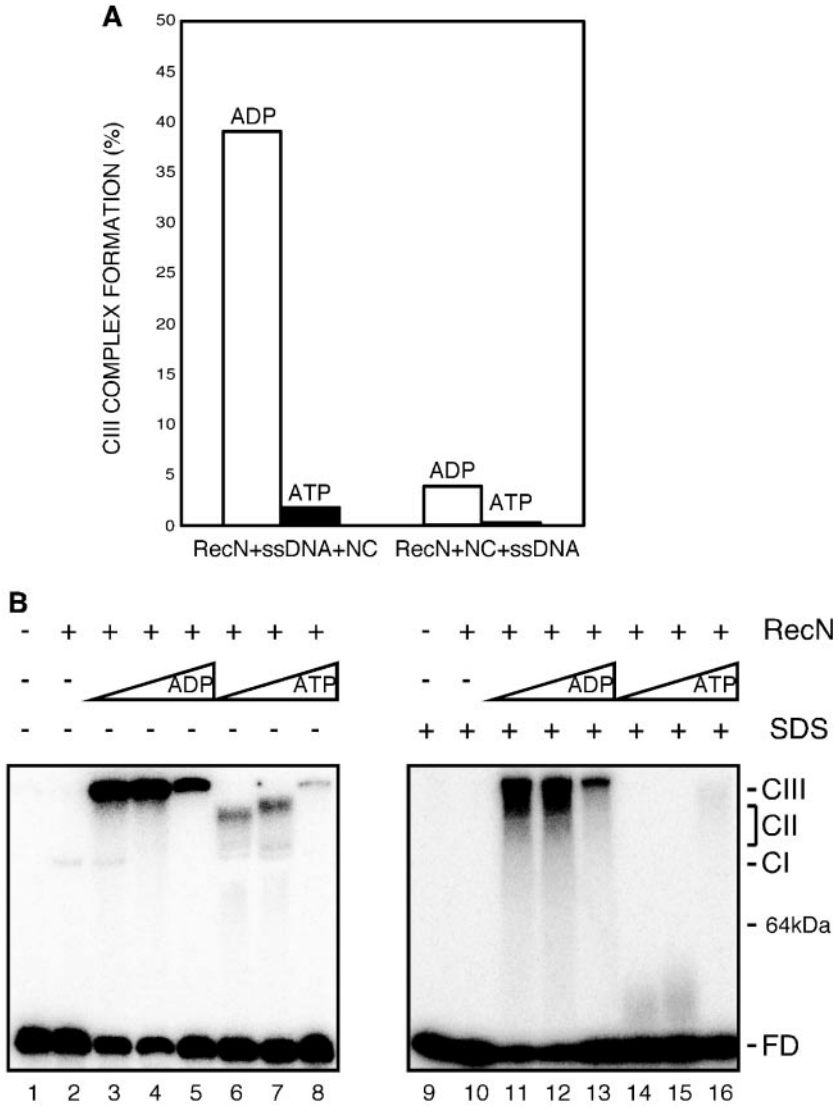

Figure 5. ATP or ADP modifies the interaction of RecN with $ssDNA_{60}$. (**A**) RecN (10 nM) was pre-incubated with linear [γ-^{32}P]labelled $ssDNA_{60}$, at the 5′-terminus (1 nM) or with 0.5 mM ADP or ATP in buffer B for 10 min at 37°C. A nucleotide cofactor (0.5 mM) or the [γ-^{32}P]labelled $ssDNA_{60}$ (1 nM) was then added, and incubation continued for 20 min at 37°C. The DNA–protein complexes formed were separated and visualized as described in Figure 2. (**B**) [γ-^{32}P]labelled $ssDNA_{60}$, at the 5′-terminus, was incubated with RecN (10 nM) in buffer B for 10 min at 37°C. Increasing concentrations of ADP (0.05, 0.5 and 5 mM, lanes 3–5, 11 and 12) or ATP (6–8 and 14–16) were added and incubated for 20 min at 37°C in buffer B. Half of each reaction mixture was then subjected to 10% ndPAGE (lanes 1–8), and the remaining half was incubated with 1% SDS and fractionated by 10% SDS–PAGE (lanes 9–16) (B). The gels were dried and visualized by autoradiography. The position of the RecN protein is denoted. FD, free DNA; CI, complex I; CII, complex II; CIII, complex III; + and −, the presence or absence of the indicated factor; NC, nucleotide cofactor.

a complex CI is formed that most likely contains one RecN octamer and one ssDNA molecule; whereas, larger complexes, CII and CIII, are formed when RecN and ssDNA are incubated together with ATP, ADP, dATP or ATPγS. These complexes apparently contain more than one RecN oligomer and more than one ssDNA molecule.

Pre-incubation of RecN with ATP reduces CII and CIII formation arguing for an ATP-dependent structural change in RecN, and when ATP is added to preformed CI it appears that bound RecN migrates to the 3′-OH of the DNA. Like observed with other ABC ATPases proteins, where ATP binding induces a protein–protein engagement (23), we proposed that the type CII complexes represent a repertoire of RecN–ssDNA intermediates and the stable CIII complex includes many RecN oligomers interacting with many ssDNA molecules. Apparently, RecN interacts with the backbone of the ssDNA to form CI; whereas, in the presence of ADP or ATP, RecN also interacts with the 3′-OH terminus, forming CII and CIII, and so protects the 3′-terminus from exonucleolytic attack.

RecN is not released from the DNA upon addition of ATP or ADP and an ∼500-fold excess of cold ssDNA, and the excess ssDNA has no effect on the accumulation of CII and CIII. A similar result, the apparent migration of protein bound to DNA, has been reported for Rad50–Mre11 (24).

RecN binding requires a 3′-OH and protects the 3′-terminus of ssDNA molecules bound into CII and CIII—properties consistent with a role for RecN in binding and facilitating

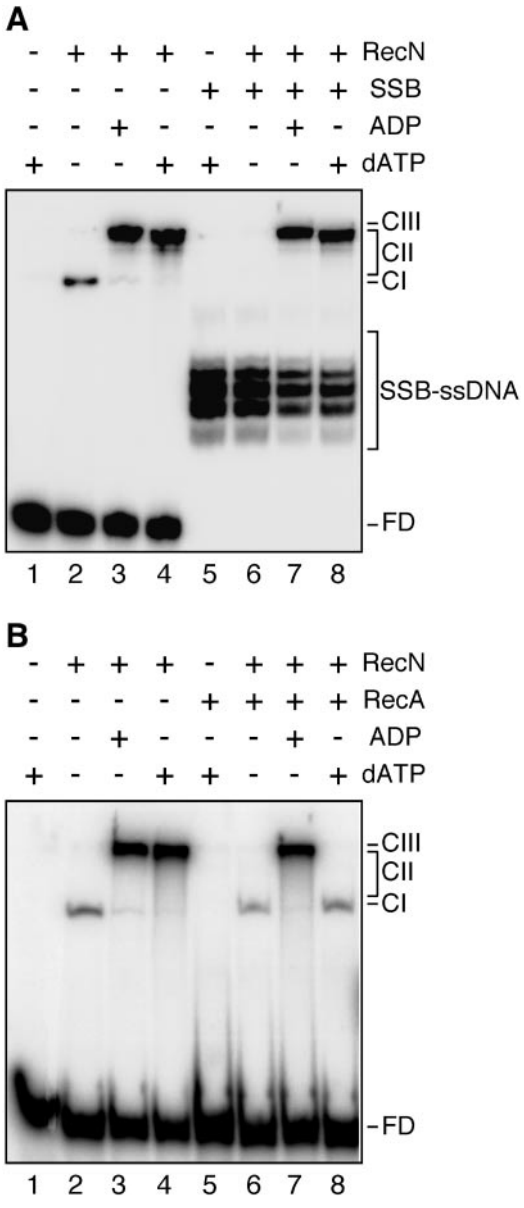

Figure 6. RecA promotes disassembly of the dATP–RecN–ssDNA$_{60}$ networks. [γ-^{32}P]Labelled ssDNA$_{60}$, at the 5′-terminus (1 nM) and RecN (10 nM), was incubated and then 1 mM ADP (lanes 3 and 7), dATP (1 mM, lanes 4 and 8) or no nucleotide cofactor (lanes 2 and 6) was added, and incubated for 10 min at 37°C in buffer B. In lane 1, labelled ssDNA$_{60}$ was incubated with 1 mM dATP and in lane 6 also with 1 mM dATP and SSB (in A) or RecA (in B). Similar results were observed in the presence of dATP or 1 mM ADP, thus only the former is shown. (**A**) SSB (0.1 μM) or (**B**) RecA protein (0.2 μM) was added to the RecN–ssDNA complexes and incubation continued for 20 min at 37°C. The DNA–protein complexes were fractionated and visualized as described above. FD, free DNA; CI, complex I; CII, complex II; CIII, complex III; + and −, the presence or absence of the indicated factor. The SSB–ssDNA complexes are indicated.

the close association of the termini of DNA molecules. RecN in CII and CIII is not displaced by SSB, but these complexes are dissociated by the assembly of a RecA filament on ssDNA. Under *in vivo* conditions, this assembly might be aided by the AddAB or indirectly by the RecA accessory proteins (RecFLOR) (see below). A similar architectural change has been proposed for human Rad50–Mre11 (10) and SMC–ScpA complex (25). RecN is thought, in both *B.subtilis* and *E.coli*, to play a role in DSB repair and genetic recombination (11–14,26–28) and specifically to participate in a step that generates or makes molecules with a 3′-ssDNA terminus accessible for DNA strand exchange (26). Based on previous observations (see Introduction) and the results presented here, the initial steps of HR appear to share many features common to all organisms, but a much simpler ensemble of enzymes is apparently required for DSB repair in bacteria than in eukaryotes (9). The results reported here suggest that RecN, like the eukaryotic MRN/X complex (9), could detect DSB and help facilitate the first step in HR by protecting 3′-ssDNA extensions. The AddAB, the RecJ and RecQ or the RecJ and RecS protein process the DNA ends (H. Sanchez, D. Kidane, P. L. Graumann and J. C. Alonso, unpublished data) and RecN–ATP binds and protects 3′-termini. RecN certainly promotes those activities *in vitro*. RecN binding might also promote assembly of RecO (14), and so indirectly also enhance RecA loading onto ssDNA generated at DSBs (29–31).

ACKNOWLEDGEMENTS

The authors are very grateful to B. Carrasco for providing SSB and RecA proteins, to R. Lurz for performing the RecN electron microscopy analysis, to G. Rivas for analytical ultracentrifugation of RecN, to P. L. Graumann for the communication of unpublished results on the localization of RecN foci upon HO-induced DSB, to P. Sung, T. Hirano, M. Cox and P. L. Graumann for helpful comments and suggestions on the manuscript and to J. Reeve and S. Kowalczykowski for critical reading of the manuscript. This work was supported by grant BMC2003-00150 from DGI-MEC and GR/SAL/0668/2004 from Comunidad de Madrid to J.C.A. Funding to pay the Open Access publication charges for this article was provided by GR/SAL/0668/2004.

Conflict of interest statement. None declared.

REFERENCES

1. Pâques,F. and Haber,J.E. (1999) Multiple pathways of recombination induced by double-strand breaks in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.*, **63**, 349–404.
2. Symington,L.S. (2002) Role of RAD52 epistasis group genes in homologous recombination and double-strand break repair. *Microbiol. Mol. Biol. Rev.*, **66**, 630–670.
3. 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.
4. Wilson,T.E., Topper,L.M. and Palmbos,P.L. (2003) Non-homologous end-joining: bacteria join the chromosome breakdance. *Trends Biochem. Sci.*, **28**, 62–66.
5. Michel,B., Grompone,G., Flores,M.J. and Bidnenko,V. (2004) Multiple pathways process stalled replication forks. *Proc. Natl Acad. Sci. USA*, **101**, 12783–12788.
6. Lisby,M. and Rothstein,R. (2004) DNA damage checkpoint and repair centers. *Curr. Opin. Cell Biol.*, **16**, 328–334.
7. Carson,C.T., Schwartz,R.A., Stracker,T.H., Lilley,C.E., Lee,D.V. and Weitzman,M.D. (2003) The Mre11 complex is required for ATM activation and the G_2/M checkpoint. *EMBO J.*, **22**, 6610–6620.
8. Uziel,T., Lerenthal,Y., Moyal,L., Andegeko,Y., Mittelman,L. and Shiloh,Y. (2003) Requirement of the MRN complex for ATM activation by DNA damage. *EMBO J.*, **22**, 5612–5621.

9. Lisby,M., Barlow,J.H., Burgess,R.C. and Rothstein,R. (2004) Choreography of the DNA damage response: spatiotemporal relationships among checkpoint and repair proteins. *Cell*, **118**, 699–713.
10. de Jager,M., van Noort,J., van Gent,D.C., Dekker,C., Kanaar,R. and Wyman,C. (2001) Human Rad50/Mre11 is a flexible complex that can tether DNA ends. *Mol. Cell*, **8**, 1129–1135.
11. Sargentini,N.J. and Smith,K.C. (1983) Characterization of an *Escherichia coli* mutant (radB101) sensitive to gamma and UV radiation, and methyl methanesulfonate. *Radiat. Res.*, **93**, 461–478.
12. Picksley,S.M., Attfield,P.V. and Lloyd,R.G. (1984) Repair of DNA double-strand breaks in *Escherichia coli* K12 requires a functional *recN* product. *Mol. Gen. Genet.*, **195**, 267–274.
13. Sargentini,N.J. and Smith,K.C. (1986) Quantitation of the involvement of the *recA*, *recB*, *recC*, *recF*, *recJ*, *recN*, *lexA*, *radA*, *radB*, *uvrD*, and *umuC* genes in the repair of X-ray-induced DNA double-strand breaks in *Escherichia coli*. *Radiat. Res.*, **107**, 58–72.
14. Kidane,D., Sanchez,H., Alonso,J.C. and Graumann,P.L. (2004) Visualization of DNA double-strand break repair in live bacteria reveals dynamic recruitment of *Bacillus subtilis* RecF, RecO and RecN proteins to distinct sites on the nucleoids. *Mol. Microbiol.*, **52**, 1627–1639.
15. Lemon,K.P. and Grossman,A.D. (1998) Localization of bacterial DNA polymerase: evidence for a factory model of replication. *Science*, **282**, 1516–1519.
16. Walker,J.E., Saraste,M., Runswick,M.J. and Gay,N.J. (1982) Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.*, **1**, 945–951.
17. Hopfner,K.P., Karcher,A., Shin,D.S., Craig,L., Arthur,L.M., Carney,J.P. and Tainer,J.A. (2000) Structural biology of Rad50 ATPase: ATP-driven conformational control in DNA double-strand break repair and the ABC-ATPase superfamily. *Cell*, **101**, 789–800.
18. Ayora,S., Rojo,F., Ogasawara,N., Nakai,S. and Alonso,J.C. (1996) The Mfd protein of *Bacillus subtilis* 168 is involved in both transcription-coupled DNA repair and DNA recombination. *J. Mol. Biol.*, **256**, 301–318.
19. Ayora,S., Weise,F., Mesa,P., Stasiak,A. and Alonso,J.C. (2002) *Bacillus subtilis* bacteriophage SPP1 hexameric DNA helicase, G40P, interacts with forked DNA. *Nucleic Acids Res.*, **30**, 2280–2289.
20. Sabariegos-Jareño,M.R. (1998) Purificación y caracterización del mecanismo de acción de la proteína RecN de *Bacillus subtilis*. *PhD Thesis.,* Universidad Autónoma de Madrid, Madrid.
21. Viitanen,P.V., Lubben,T.H., Reed,J., Goloubinoff,P., O'Keefe,D.P. and Lorimer,G.H. (1990) Chaperonin-facilitated refolding of ribulosebisphosphate carboxylase and ATP hydrolysis by chaperonin 60 (groEL) are K^+ dependent. *Biochemistry*, **29**, 5665–5671.
22. 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.
23. Hopfner,K.P. and Tainer,J.A. (2003) Rad50/SMC proteins and ABC transporters: unifying concepts from high-resolution structures. *Curr. Opin. Struct. Biol.*, **13**, 249–255.
24. de Jager,M., Wyman,C., van Gent,D.C. and Kanaar,R. (2002) DNA end-binding specificity of human Rad50/Mre11 is influenced by ATP. *Nucleic Acids Res.*, **30**, 4425–4431.
25. Hirano,M. and Hirano,T. (2004) Positive and negative regulation of SMC–DNA interactions by ATP and accessory proteins. *EMBO J.*, **23**, 2664–2673.
26. Lloyd,R.G., Buckman,C. and Benson,F.E. (1987) Genetic analysis of conjugational recombination in *Escherichia coli* K12 strains deficient in RecBCD enzyme. *J. Gen. Microbiol.*, **133**, 2531–2538.
27. Lloyd,R.G. and Buckman,C. (1991) Overlapping functions of *recD*, *recJ* and *recN* provide evidence of three epistatic groups of genes in *Escherichia coli* recombination and DNA repair. *Biochimie*, **73**, 313–320.
28. Alonso,J.C., Stiege,A.C. and Luder,G. (1993) Genetic recombination in *Bacillus subtilis* 168: effect of *recN*, *recF*, *recH* and *addAB* mutations on DNA repair and recombination. *Mol. Gen. Genet.*, **239**, 129–136.
29. Umezu,K., Chi,N.W. and Kolodner,R.D. (1993) Biochemical interaction of the *Escherichia coli* RecF, RecO, and RecR proteins with RecA protein and single-stranded DNA binding protein. *Proc. Natl Acad. Sci. USA*, **90**, 3875–3879.
30. Shan,Q., Bork,J.M., Webb,B.L., Inman,R.B. and Cox,M.M. (1997) RecA protein filaments: end-dependent dissociation from ssDNA and stabilization by RecO and RecR proteins. *J. Mol. Biol.*, **265**, 519–540.
31. 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.