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 Mg2+-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 (RecNEco) (63.6 kDa, 567 amino acids long) resemble eukaryotic SMC-like proteins. They have two coiled coil
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\textsuperscript{2+}, RecN exhibits single-stranded (ss) DNA-dependent ATP hydrolysis and ssDNA binding activity; it binds ssDNA independently of nucleotide co-factors, 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 G36P), 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 RecA protein, was provided by B. Carrasco (Centro Nacional de Biotecnología, CSIC). The SSB protein and RecN protein (SSB) were purchased from USB. DTT, EDTA and PEG-6000 were from nuclease degradation before RecA-dependent repair.

RESULTS

ssDNA stimulates RecN ATPase activity

The RecN protein was purified 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\textsubscript{i} in a ssDNA-dependent manner with ~8-fold stimulation of ATPase activity observed in the presence of a 60 nt ssDNA (ssDNA\textsubscript{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 ~10\textsuperscript{4}-fold in the absence of K\textsuperscript{+} ions (21). GroEL is therefore almost certainly not the...
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 ATP 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).

AMP-PNP, only CI was formed (Figure 2B), although with the weakly hydrolysable analogue (ATP$\gamma$S) 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 $\sim$15% glycerol reduced the ssDNA binding efficiency $\sim$3-fold. In contrast, the RecN species with a low sedimentation coefficient (presumably a dimer) did not form a complex with ssDNA$_{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$_{90}$ 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 1. The ssDNA-dependent ATPase activity of RecN.](https://academic.oup.com/nar/article-abstract/33/7/2343/2401391)

**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.
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 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 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 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

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Figure 2. DNA binding by RecN. (A) 

| Lane | RecN (nM) | ADP (mM) | ATP (mM) | CI | CII | CIII |
|------|-----------|-----------|-----------|----|-----|------|
| 1    | 0         | 0         | 0         | 0  | 0   | 0    |
| 2    | 0.4       | 0.4       | 0.4       | 0  | 0   | 0    |
| 3    | 0.8       | 0.8       | 0.8       | 0  | 0   | 0    |
| 4    | 1.7       | 1.7       | 1.7       | 0  | 0   | 0    |
| 5    | 3.7       | 3.7       | 3.7       | 0  | 0   | 0    |
| 6    | 15        | 15        | 15        | 0  | 0   | 0    |
| 7    | 30        | 30        | 30        | 0  | 0   | 0    |
| 8    | 60        | 60        | 60        | 0  | 0   | 0    |

No RecN was added in lane 1. Complexes formed were separated by 10% PAGE and visualized by autoradiography. FD, free DNA; CI, complex I; CII, complex II; CIII, complex III.

(B) 

| Lane | RecN (nM) | ADP (mM) | ATP (mM) | AMP-PNP (mM) | CI | CII | CIII |
|------|-----------|-----------|-----------|--------------|----|-----|------|
| 1    | 10        | 0         | 0         | 0            | 0  | 0   | 0    |
| 2    | 10        | 0.01      | 0         | 0            | 0  | 0   | 0    |
| 3    | 10        | 0.05      | 0         | 0            | 0  | 0   | 0    |
| 4    | 10        | 0.1       | 0         | 0            | 0  | 0   | 0    |
| 5    | 10        | 0.5       | 0         | 0            | 0  | 0   | 0    |
| 6    | 10        | 1         | 0         | 0            | 0  | 0   | 0    |

No RecN was added in lane 1. Complexes formed were separated by 10% PAGE and visualized by autoradiography. FD, free DNA; CI, complex I; CII, complex II; CIII, complex III.

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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.

**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,
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 5. ATP or ADP modifies the interaction of RecN with ssDNA. (A) RecN (10 nM) was pre-incubated with linear [γ-32P]labelled ssDNA 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-32P]labelled ssDNA (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-32P]labelled ssDNA 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% nPAGE (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.
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 (RecFLORE) (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 RecO 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.
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