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An SOS Inhibitor that Binds to Free RecA Protein: The PsiB Protein

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SUMMARY

The process of bacterial conjugation involves the transfer of a conjugative plasmid as a single strand. The potentially deleterious SOS response, which is normally triggered by the appearance of single-stranded DNA, is suppressed in the recipient cell by a conjugative plasmid system centered on the product of the *psiB* gene. The F plasmid PsiB protein inhibits all activities of the RecA protein, including DNA binding, DNA strand exchange, and LexA protein cleavage. The proteins known to negatively regulate recombinases, such as RecA or Rad51, generally work at the level of dismantling the nucleoprotein filament. However, PsiB binds to RecA protein that is free in solution. The RecA-PsiB complex impedes formation of RecA nucleoprotein filaments on DNA.

INTRODUCTION

Conjugation is a mechanism of horizontal gene transfer that allows asexually reproducing bacteria to diversify their genomes. Conjugative plasmids aid the spread of antibiotic resistance in bacterial strains (Aguero et al., 1984 and references therein), making the process of conjugation an important target for fighting disease (Lujan et al., 2007). In *Escherichia coli*, conjugation occurs between a donor cell harboring a conjugative plasmid and a recipient cell that lacks a similar plasmid (Frost et al., 1994). During conjugation, rolling circle replication ensues, and a single strand of the plasmid is spooled into the recipient (Wolkow et al., 1996 and references therein).

The single-stranded DNA (ssDNA) that enters the recipient cell can trigger the SOS response, involving induction of DNA repair genes that are under the control of the LexA transcription repressor protein (Roca and Cox, 1997; Walker et al., 2000). The LexA protein undergoes autocatalytic cleavage stimulated by RecA protein filaments bound to ssDNA and ATP (Little, 1991; Little et al., 1980; Roca and Cox, 1997; Slilaty and Little, 1987). During SOS, cell division halts (Walker et al., 2000), and the induction of the mutagenic DNA polymerase V is possible if the response is not abated (Sommer et al., 1993; Walker et al., 2000). Therefore, the induction of SOS during conjugation is potentially detrimental to the cell.

Some plasmids can suppress unwarranted SOS induction during conjugation. The Plasmid SOS Interference/Inhibition (psi) phenomenon was first observed when the conjugative plasmid R100.1 suppressed the temperature-sensitive constitutive SOS phenotype of *recA*441 (Bagdasarian et al., 1980). Further studies with the R6-5 plasmid showed that a region encoding the PsiB protein alone was sufficient to suppress the SOS response (Bailone et al., 1988).

PsiB expression varies as a function of the promoters present on a given conjugative plasmid and of the cellular circumstances. The native F plasmid *psiB* promoter appears to be activated only during conjugation and only in the recipient (Bagdasarian et al., 1992). Early transcription of PsiB is likely mediated by an element of secondary structure in the incoming single strand that mimics an RNA polymerase promoter recognized by the Frpo sigma factor (Masai and Arai, 1997).

The strength of PsiB inhibition of SOS depends on the RecA allele and on the concentration of PsiB in the host cell. Constitutive SOS at high temperatures is suppressed in *recA*441 (*tif-1*) cells harboring the R100.1 plasmid. However, the SOS response was still induced in response to DNA damage. The RecA441 mutant protein binds better than does wild-type RecA to ssDNA coated by single-stranded DNA binding protein (SSB) at high temperatures (Lavery and Kowalczykowski, 1990), and this characteristic may be significant in overcoming the inhibition by PsiB protein. In a separate experiment, plasmids derived from the R6-5 plasmid, containing a *psi* region, were able to suppress induction of wild-type *recA* gene after DNA damage (Bagdasarian et al., 1986). Thus, the PsiB protein is less effective in suppressing SOS induction by RecA441 than by wild-type RecA. The effect of PsiB is also dose dependent (Bailone et al., 1988).

Biochemical characterization of the *psiB* gene product has been very limited. PsiB does not inhibit SOS in LexA-deficient cells, ruling out the possibility that it is a transcription repressor (Bagdasarian et al., 1986). On the basis of lack of sequence homology, it has been argued that PsiB is unlikely to act as a LexA mimic, competing directly for the RecA nucleoprotein filament necessary for LexA cleavage (Bailone et al., 1988). Other proposals include a direct PsiB inhibition of RecA function by competing for DNA-binding sites, stabilizing a protein that binds to DNA and competes with RecA (e.g., SSB), or directly binding to RecA and preventing it from binding to DNA (Bagdasarian et al., 1986; Bailone et al., 1988). On the basis of all results, particularly on the unpublished effects of certain RecA mutant proteins, Devoret and colleagues favored an inhibition mediated

by a direct interaction between PsiB and RecA in solution (Bailone et al., 1988). However, biochemical data relevant to the PsiB mechanism have not appeared, and a direct interaction between PsiB and RecA has never been demonstrated.

The direct interaction of RecA and PsiB would represent a previously undescribed mechanism for the regulation of RecA and related recombinases. The strict requirements for targeted recombination in eukaryotic cells have led to a search for eukaryotic recombinase modulators, with a focus on modulators of yeast and human RecA homologs. These efforts have revealed a network of regulators, including BRCA2, p53, Rad52, Rad54, Rad55/57, Rad54b, TGFβ1, the Srs2 helicase, and c-Abl (Busygina et al., 2008; Kanamoto et al., 2002; Krejci et al., 2001; Marmorstein et al., 1998; Tanaka et al., 2000; Yuan et al., 1998). None of these modulators is known to exert its primary effect on Rad51 protein by forming a complex with it in the absence of DNA. Modulators of the bacterial RecA protein have also drawn more attention recently. Proteins that aid, direct, and stabilize RecA binding include RecBCD, RecFOR, and DinI (Anderson and Kowalczykowski, 1997; Lusetti et al., 2004b; Morimatsu and Kowalczykowski, 2003; Sakai and Cox, 2009). Negative regulation of RecA activity, mediated by the UvrD and RecX proteins, is also important (Drees et al., 2006; Veaute et al., 2005). As in the case of the eukaryotic recombinase modulators, none of these regulators exerts its primary effects by forming a complex with RecA in the absence of DNA. Phosphorylation prevents the human Rad51 protein from catalyzing DNA strand exchange (Yuan et al., 1998), but RecA is not a kinase substrate (Marcandier et al., 1994).

Mechanisms of RecA inhibition can guide the search for undiscovered mechanisms of recombinase modulation in eukaryotes. In this article, we demonstrate that the PsiB protein from the F conjugative plasmid binds directly to RecA protein that is free in solution, forming a complex that inhibits RecA filament formation on SSB-coated ssDNA.

RESULTS

We used several methods to characterize PsiB activity. Throughout, PsiB concentrations are reported both in terms of monomers and dimers. Analytical ultracentrifugation showed a homogeneous population of dimers (V.P., unpublished data). It is possible that each subunit has an independent interaction site, which makes the concentration of PsiB monomers potentially relevant. In many experiments, SSB was bound to the ssDNA prior to RecA protein addition. As we show below, PsiB appears to inhibit RecA filament formation on SSB-coated ssDNA.

PsiB Inhibits RecA Filament Formation on Circular ssDNA

ATP hydrolysis by the RecA protein is highly DNA dependent (Cox, 2003). This property of the RecA protein allows an indirect measurement of RecA binding to DNA by following the rate of ATP hydrolysis in a coupled spectrophotometric assay (Drees et al., 2004; Lindsley and Cox, 1990). To assess how PsiB affects RecA-mediated ATP hydrolysis in the presence of M13mp18 circular ssDNA (cssDNA) bound by SSB protein, PsiB was titrated into a reaction containing 2 μM RecA and 3 μM ssDNA (1 μM available DNA-binding sites at 3 nucleotides/site). The rate of ATP hydrolysis of RecA was inhibited proportionally to the concentration of PsiB (Figure 1A) and was almost completely abolished in the presence of 18 μM PsiB monomers or 9 μM PsiB dimers.

Fewer RecA Filaments Form on SSB-Coated DNA in the Presence of PsiB

The inhibition of ATP hydrolysis documented in Figure 1A could reflect either an inhibition of RecA filament formation or a reduction in the function of formed filaments. We visualized the RecA filaments formed in the presence and absence of PsiB to determine whether PsiB affected the amounts of RecA protein bound to DNA. The results are shown in Figures 1B and 1C. Under the conditions used, the rate of ATP hydrolysis is reduced by about 90% in the presence of the PsiB. By electron microscopy, the number of countable molecules in the presence of PsiB declined to approximately 70% of the number of molecules in the control lacking PsiB. Numbers of shorter/gapped filaments increased. Numbers of DNA molecules coated only with SSB more than doubled. However, a significant portion (11%–27%) of the molecules was twisted in a manner that precluded precise length measurements (Figure 2B). Although we cannot relate the observed amount of bound RecA to the ATPase results quantitatively, the EM results qualitatively support a mechanism in which PsiB prevents RecA from binding to DNA. A catalog of molecules observed in the electron microscopy is provided in Figure 1C.

PsiB Inhibits RecA-Catalyzed LexA Autocleavage

Figure 1A demonstrates an almost complete inhibition of RecA filament formation on SSB-bound cssDNA in the presence of a four-fold excess of PsiB dimers relative to RecA monomers. With 15,000 monomers per cell under normal growth conditions (Stohl et al., 2003), it is likely that RecA will be present in excess of PsiB dimers. In *E. coli* strains harboring the R6-5 plasmid, 6000 monomers of PsiB per cell have been reported (Bagdasarian et al., 1992). We examined the effects of PsiB on LexA cleavage in vitro, with about two PsiB dimers per seven RecA monomers. Under the conditions tested (including 7 μM RecA protein and 9 μM ssDNA), the LexA autocleavage was complete approximately 60 min after the start of the reaction in the absence of PsiB. In contrast, when 1.6 μM PsiB dimers were present, little LexA cleavage was evident after 90 min (Figure 2). Note that previous studies have documented a more robust RecA-mediated cleavage of LexA than is observed in the control experiment here. The reduced rate is due to the order of addition of reaction components in this experiment, with SSB preceding RecA and impeding RecA filament formation relative to the optimal conditions (RecA preceding SSB) used in earlier studies (e.g., Gruenig et al., 2008).

PsiB Moderately Inhibits RecA-Catalyzed Three-Strand Exchange

PsiB delayed nicked circular product (ncproduct) formation in a reaction in which RecA catalyzed strand exchange between cssDNA initially bound by SSB and a homologous ldsDNA molecule (Figure 3). The rate and extent of ncproduct formation

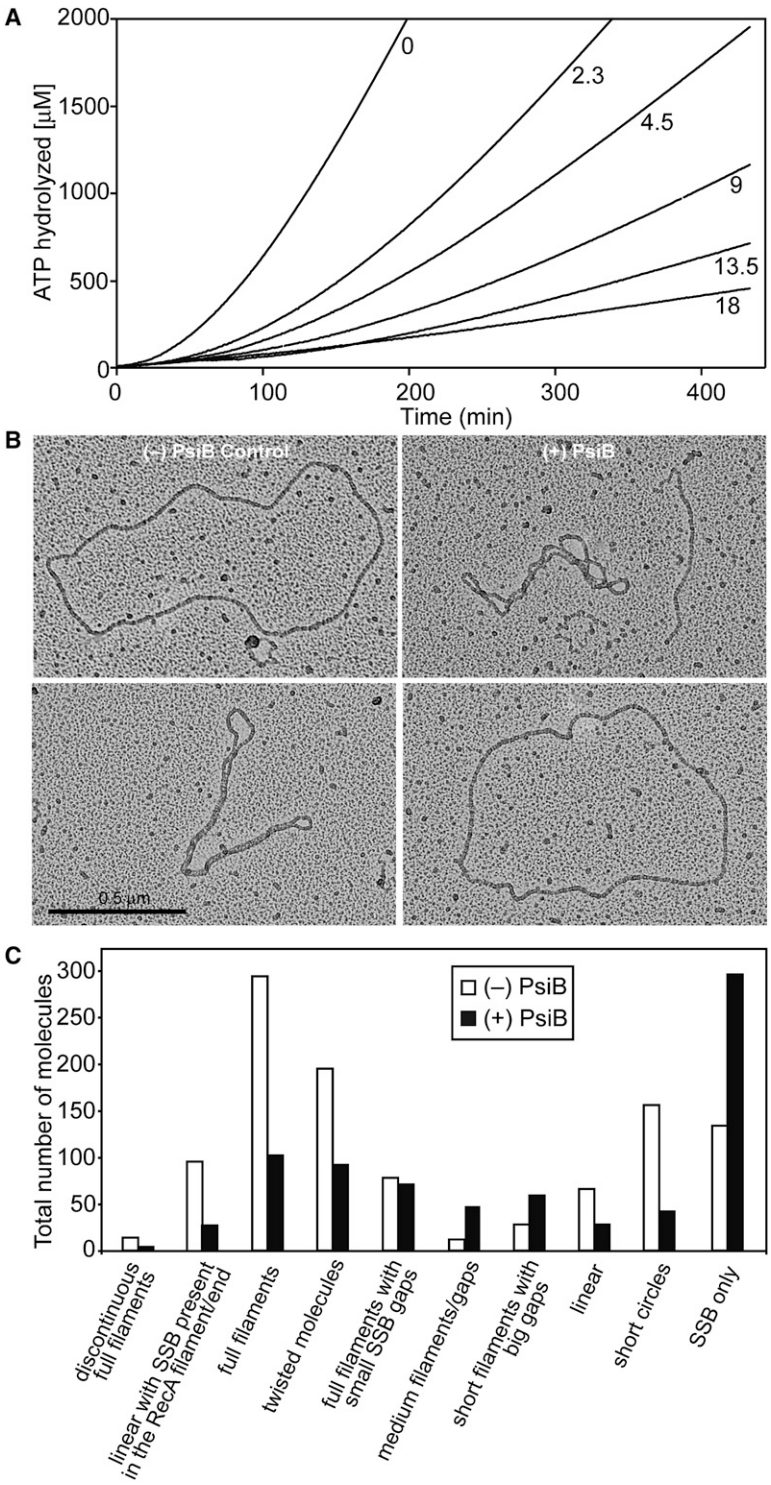

Figure 1. PsiB Inhibits RecA Filament Formation on SSB-Coated M13mp18 cssDNA

(A) RecA ATPase on SSB-coated DNA in the presence of PsiB. PsiB was incubated with M13mp18 ssDNA (3 μM nt) and SSB (0.5 μM) for 10 min at 37°C at concentrations of PsiB monomers shown by each curve. The reaction was initiated by addition of RecA (2 μM) at t = 0 min, and the rate of ATP hydrolysis was followed. Lower ATPase rate can be correlated with fewer RecA filaments on DNA.

(B) Electron microscopy images of RecA establishing filaments on SSB-coated DNA. The conditions were the same as in (A). The visualized samples were taken 100 min after the initiation of the reaction by addition of RecA and were adsorbed to a glow-discharge grid as described in Experimental Procedures. The (–) PsiB control and the (+) PsiB experimental panels each show a discontinuous full RecA filament, an SSB molecule, and a twisted molecule.

(C) Distribution of molecules observed in the reactions described in (B). In the presence of PsiB, there was a decrease in full-length RecA filaments and increase in DNA bound only with SSB.

catalyzed by RecA depended on the order of addition of SSB and RecA to DNA. When RecA was allowed to nucleate on cssDNA first and SSB was added second, there was robust joint molecule (jm) and ncproduct formation 15 min after the reaction was started with the addition of ldsDNA. However, when SSB was allowed to coat the cssDNA first, before RecA was added, the reaction was slowed. When PsiB was present in the reaction in which RecA had to nucleate on SSB-coated DNA, product formation was further delayed in both rate and extent. The ratios of DNA to RecA protein to SSB protein were similar to that in the ATPase experiments above. However, the total protein and DNA concentrations were greater in the strand exchange experiments.

The Effects of PsiB Are Diminished in the Absence of SSB

The inhibition of RecA filament formation on DNA by PsiB may be due to PsiB alone or PsiB association with an interaction partner. To begin to examine the potential interaction partners of PsiB that are necessary to achieve an inhibition of RecA binding to DNA, we omitted the SSB protein from the ATPase reactions. The SSB protein is necessary to melt secondary structure in cssDNA (Kowalczykowski and Krupp, 1987), which enables RecA to bind along the entire length of the DNA molecule. Therefore, we used linear poly(dT) DNA for this set of experiments. Again, PsiB inhibited the RecA ATPase activity, reflecting a reduction of RecA binding to DNA (Figure 4A). However, the effect saturated before complete inhibition occurred. The ATPase activity of RecA did not fall much below 50% of the rate observed in the control reaction without PsiB (Figure 4B) even in the presence of 33.7 μM PsiB dimers.

The effects of SSB on the scope of inhibition by PsiB (compare Figure 1A to Figure 4A) hint at further mechanistic complexity. To understand the role of SSB in the inhibition of RecA by PsiB, we decided to use the DinI protein as a tool. DinI stabilizes RecA nucleoprotein filaments (Lusetti et al., 2004b) and counteracts the effect of the RecX protein, another inhibitor of RecA (Lusetti

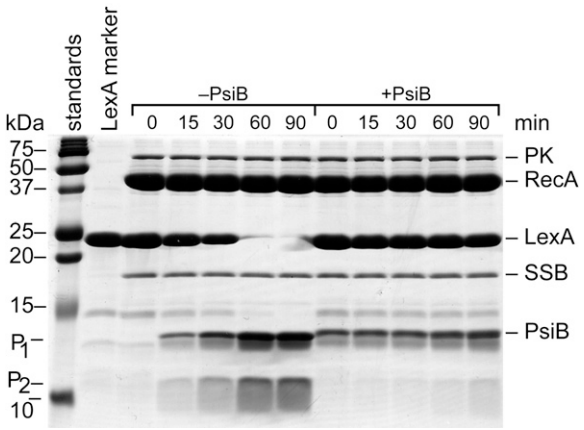

Figure 2. PsiB Inhibits RecA Coprotease Activity on LexA

The SSB protein (1.5 μM) was incubated for 10 min at 37°C with M13mp18 cssDNA (9 μM) and with PsiB protein (1.6 μM dimers or 3.2 μM monomers) or compensatory PsiB storage buffer. RecA (7 μM) was then added to the reaction and allowed to nucleate on the DNA for 10 min. The reaction was started with the addition of the LexA protein to 10 μM. Lane 1 shows a Bio-Rad Precision Plus protein ladder (250, 150, 100, 75, 50, 37, 25, 20, and 15 kDa.). Lane 2 contains pure LexA protein without any other proteins added. Lanes 3–7 contain aliquots of the control reaction withdrawn at the indicated times, whereas lanes 8–12 contain aliquots of the reaction containing the PsiB protein. The proteins present in the assay are labeled on the figure. PK is pyruvate kinase (a component of the ATP regeneration system), and P_1 and P_2 are the two products of LexA cleavage. Note that PsiB appears at the same position as P_1 on the gel.

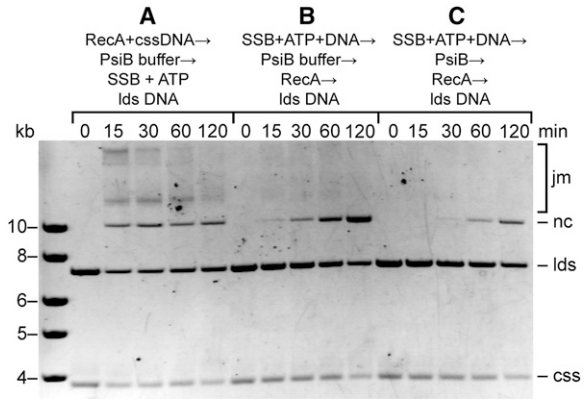

Figure 3. PsiB Inhibits RecA-Catalyzed Recombination of Homologous DNA In Vitro

The order of addition is shown above each time course. Each arrow indicated a10 min incubation at 37°C. The order of addition in reaction A (6.67 μM RecA was allowed to nucleate on 20 μM cssDNA before 3.3 μM SSB and 3 mM ATP were added to the reaction) was chosen to differentiate between joint molecule intermediates (jm) and nicked circular product (nc). Time course B shows the progress of nc formation when RecA had to nucleate on SSB-coated DNA. Reaction C follows the order of addition of the second, but includes PsiB at 6 μM monomers. Time point 0 is defined by the addition of the 20 μM homologous dsDNA.

et al., 2004a). We wanted to examine how the stabilization of the RecA filaments affects the ability of PsiB to inhibit RecA.

When DinI was added prior to RecA and at the same time as PsiB to ATPase reactions containing poly(dT) DNA and no SSB, DinI completely counteracted the inhibitory effect of PsiB (Figure 5A). If preventing RecA from binding to DNA had been the only way PsiB inhibited RecA, then the presence or absence of SSB should not be an issue. However, that was not the case. Figure 5B illustrates that, when SSB was present, RecA inhibition by PsiB on M13mp18 DNA was diminished in the presence of DinI but not completely abolished. This finding suggests that SSB has a significant role in the PsiB inhibition (see Discussion).

We investigated the dynamics of the PsiB-RecA interaction by testing whether DinI can disturb the inhibition of RecA by PsiB after the PsiB-RecA complexes are formed. RecA was incubated with PsiB and poly(dT) DNA, and after 15 min DinI was added, and the change in ATP hydrolysis was monitored. A restoration of the ATP hydrolysis was observed, indicating that the PsiB-RecA interaction was readily reversible (Figure 5C). In contrast, when a filament stabilized by DinI was challenged with PsiB, little effect was seen. This finding again indicates that PsiB has little effect on RecA protein once it is bound to ssDNA.

The PsiB Protein Does Not Interact with the LexA or SSB Proteins or with Linear ssDNA

FITC-labeled PsiB protein was titrated with LexA protein, and the potential interaction between the two proteins was examined using fluorescence anisotropy. No evidence for interaction was observed using LexA protein concentrations up to 5 μM (Figure 6A).

Because SSB affects how strongly PsiB inhibits RecA, PsiB and SSB may interact. We investigated the potential existence of direct interactions between the two proteins by fluorescence anisotropy. FITC-labeled PsiB protein was titrated with SSB protein. Figure 6B illustrates that PsiB did not interact with SSB in solution, because no significant change in PsiB anisotropy occurs even when SSB is present at a 100-fold molar excess. The absence of interaction between the two proteins obtained by fluorescence anisotropy was confirmed in an electrophoretic mobility shift assay (EMSA), monitoring SSB and PsiB in the presence DNA (Figure 6C). Therefore, PsiB did not interact with SSB either in solution or when SSB was bound to DNA under the conditions of these experiments.

Even though it has been reported that PsiB does not bind to ssDNA (Bailone et al., 1988), we decided to test whether an interaction between PsiB and ssDNA exists in a more sensitive and quantifiable experiment. Fluorescence anisotropy measurements showed that the native PsiB protein did not interact with a FITC-labeled 50 nt linear ssDNA oligomer even when PsiB is present at 10^3 excess relative to DNA molecules (Figure 6D). SSB was used as a positive control because of its high DNA-binding activity.

PsiB Interacts with the RecA Protein Free in Solution

One of the mechanisms proposed for PsiB is a direct interaction between PsiB and RecA when the latter protein is not bound to DNA (Bailone et al., 1988). We tested for this interaction in a fluorescence polarization experiment by incubating 60 nM labeled PsiB with increasing concentrations of the RecA protein. There

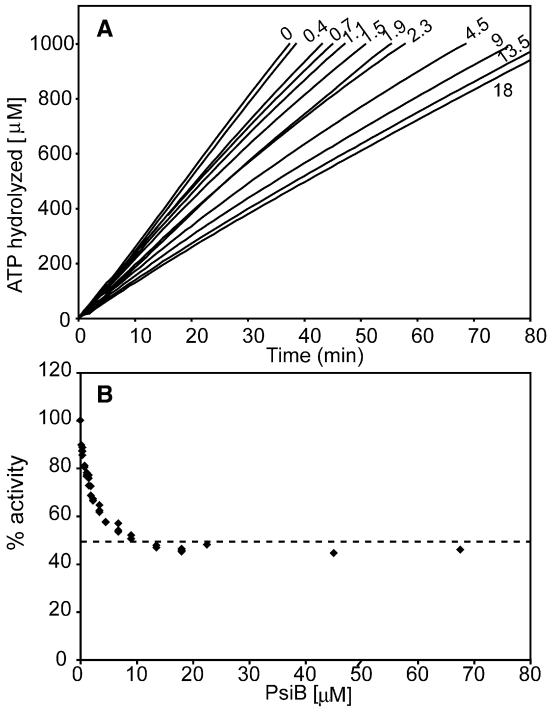

Figure 4. PsiB Partially Inhibits RecA ATP Hydrolysis on Poly(dT)

(A) Two separate experiments are combined to illustrate the saturation of the PsiB inhibition effect on RecA-mediated ATP hydrolysis on poly(dT) DNA. PsiB at the monomer concentrations shown in the figure was incubated with poly(dT) DNA (3 μM nt) for 10 min before the reaction was initiated by the addition of RecA protein (2 μM).

(B) RecA-mediated ATP hydrolysis as a function of PsiB monomer concentration. Percentage of activity was calculated as the rate of ATP hydrolysis at X μM PsiB divided by the rate of ATP hydrolysis in the control reaction for a particular experiment. The plot includes data from seven experiments performed on different days.

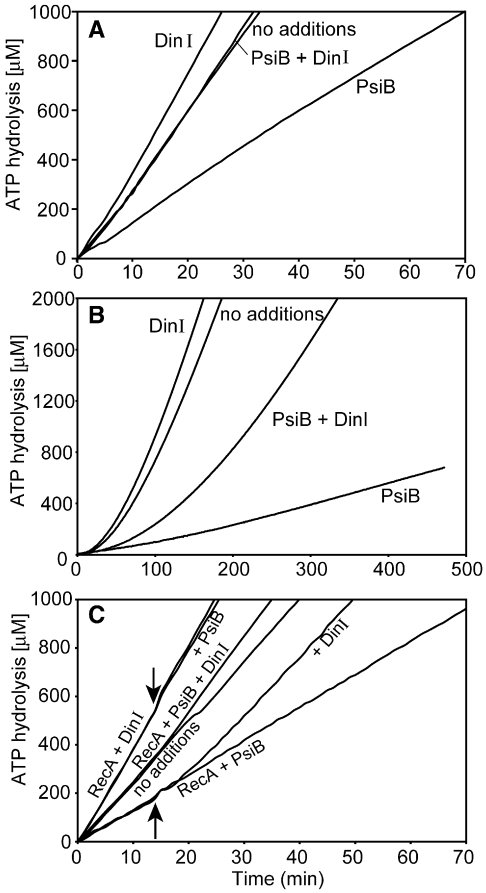

Figure 5. DinI Counteracts the PsiB Effect in the Absence, but Not in the Presence, of SSB

(A) PsiB inhibits RecA, but DinI restores RecA WT ATP hydrolysis phenotype even when PsiB is present. The PsiB (11 μM) and DinI (8.4 μM monomers) proteins were incubated with poly(dT) (3 μM nt) for 10 min, and the reactions were initiated by addition of RecA (2 μM). At these concentrations, the effects of DinI and PsiB were canceled out.

(B) DinI partially rescues RecA from PsiB inhibition on SSB-bound M13mp18 cssDNA but is unable to restore WT ATP hydrolysis rates. The conditions of the reaction were as in (A) except that SSB was included and the DNA substrate was longer cssDNA rather than lssDNA.

(C) The PsiB-RecA interaction is dynamic, and PsiB cannot inhibit a stabilized filament. The experimental conditions are as in (A), except in the reactions where only one of the modulators (DinI or PsiB) was incubated with the DNA before RecA addition. The second modulator was added after 15 min (indicated by the arrows on the figure), and the effect was monitored on the basis of the change of RecA ATP hydrolysis.

was a large change in anisotropy of the probe (Figure 6E), indicating a PsiB-RecA interaction. The anisotropy continued increasing as more RecA protomers were added but did not achieve saturation in the range of concentrations tested.

To show that the continued increase in anisotropy was due to a specific RecA-PsiB interaction and not to a contribution from the intrinsic fluorescence of RecA protein, we labeled BSA with FITC dye. The labeled BSA was diluted until its signal was of the same intensity as PsiB and was similarly titrated with increasing concentrations of RecA. No changes in the BSA signal were observed, indicating that the RecA protein was not contributing a background signal.

Because saturation of the anisotropy signal was not achieved, the dissociation constant could not be calculated. In addition, only 1 of 11 PsiB monomers in this assay was labeled, possibly suppressing a signal increase at lower RecA concentrations. The observed increase begins in the nanomolar range of RecA concentration, indicating that a dissociation constant in the low micromolar range is possible. Later testing of a more heavily labeled preparation of PsiB indicated that FITC labeling increases the RecA inhibition activity of PsiB (data not shown).

DISCUSSION

We conclude that the PsiB protein inhibits RecA filament formation on SSB-coated ssDNA. That inhibition is sufficient to prevent the RecA-catalyzed LexA autocleavage necessary to induce the SOS response. We propose that the inhibition occurs via a mechanism not previously observed for a RecA family recombinase—an interaction between the RecA and PsiB proteins in solution. This work provides biochemical evidence for a mechanism proposed by Devoret and colleagues in their studies two

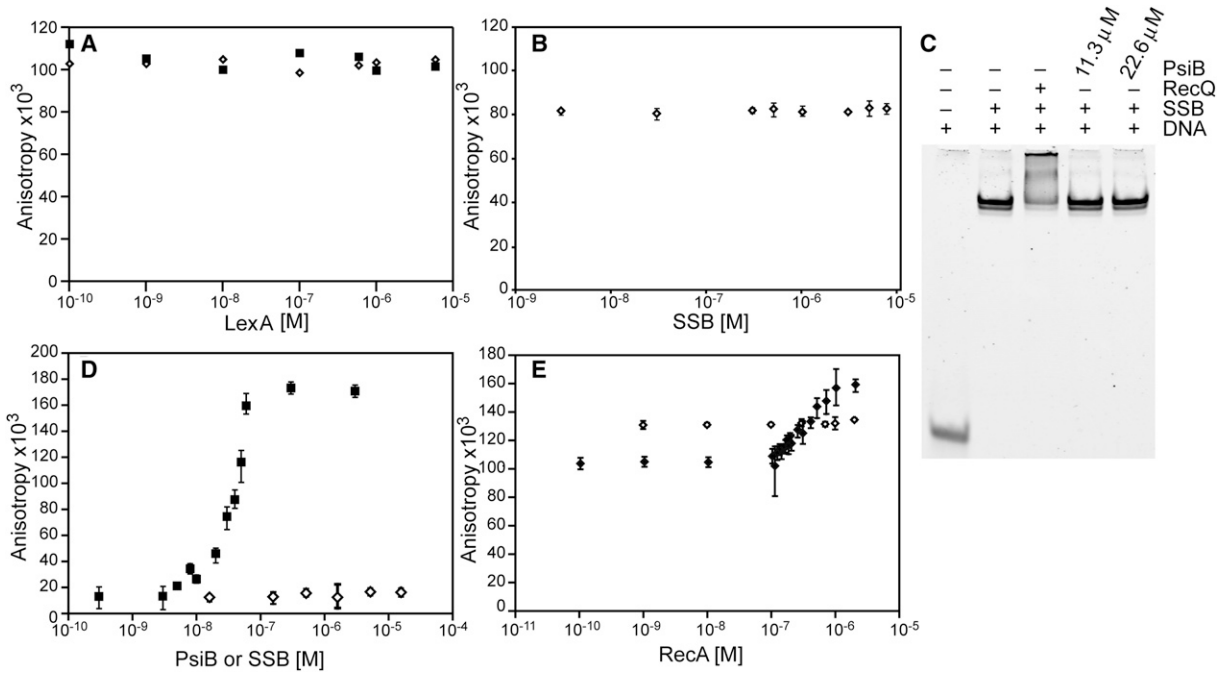

Figure 6. PsiB Interactions with LexA, SSB, DNA, and RecA

(A) PsiB does not interact with LexA. FITC-labeled PsiB at 60 nM was titrated with increasing concentrations of LexA. The anisotropy of the labeled PsiB was then measured.

(B) PsiB does not interact with SSB. The experiment was conducted as in (A).

(C) PsiB does not interact with SSB on ssDNA. SSB was preincubated with 10 μM ssDNA for 10 min at 37°C. Then either 0.25 μM RecQ or 11.3 μM or 22.6 μM PsiB monomers were added to the reaction and incubated for another 10 min at 37°C. The resulting complexes were analyzed on a 10% native polyacrylamide gel.

(D) PsiB does not interact with ssDNA. The fluorescence anisotropy of a labeled 50-nt oligonucleotide was followed after incubation with either PsiB (open symbols) or SSB (closed symbols) at the indicated concentrations.

(E) PsiB interacts with RecA free in solution. FITC-labeled PsiB (filled diamonds) was incubated with RecA over the concentration range of 0.1 nM to 2 μM. For a control, labeled FITC-BSA was diluted until its vertical and horizontal intensities were similar to the intensities emitted by the labeled PsiB. The BSA probe (open diamonds) was titrated with increasing concentrations of RecA. For panels (B–E), data are reported as the average of at least three determinations, with the error bars representing the minimum and maximum values. The points in panel (E) are based on three to nine determinations. In panel (A), two separate trials are shown.

decades ago (Bagdasarian et al., 1986; Bailone et al., 1988). The inhibition mediated by PsiB is much greater if the ssDNA to which RecA is to be bound is coated with SSB. The addition of PsiB to a RecA reaction reduces the rate and extent of RecA protein filament formation on SSB-coated ssDNA, as measured by both the indirect RecA-mediated ATPase assay (Figure 1A) and electron microscopy (Figures 1B and 1C). The PsiB protein interacts with free RecA protein (Figure 6E) but does not interact measurably with SSB, LexA, or DNA (Figures 6A–6D).

In suppressing the SOS response, the key effect of PsiB is the resulting inhibition of LexA autocleavage. Little LexA cleavage was seen for 90 min even when RecA monomers were in over four-fold excess relative to PsiB dimers (Figure 2). PsiB inhibits RecA-mediated SOS induction in vivo despite being present at lower concentrations in the cell (3,000 PsiB dimers versus 15,000 RecA monomers) (Bagdasarian et al., 1992; Stohl et al., 2003). The observed in vitro inhibition of RecA-facilitated autocatalytic LexA cleavage is thus sufficient to explain the suppression of the SOS response in vivo caused by PsiB during conjugation. The inhibition of LexA autocatalytic cleavage by PsiB is the strongest effect we observed for PsiB, a level of inhibition that seems to eclipse the inhibition of RecA filament formation. The protracted inhibition of LexA cleavage even after some RecA filaments are demonstrably established on the DNA may telegraph an additional mechanistic contribution of PsiB, such as a continued association of PsiB with the assembled RecA filament. If such a continued association occurs, it does not appear to affect ATP hydrolysis by the RecA filament, or DNA strand exchange. An inhibition of ATP hydrolysis is not needed, because ATP binding but not hydrolysis is required for RecA to form a nucleoprotein filament capable of stimulating LexA autocleavage (Gruenig et al., 2008; Little et al., 1980). This finding supports the observations in vivo that the main role of PsiB is to inhibit the induction of the SOS response. The results of our experiment also support the suggestion of Sandler and colleagues that there is a cellular threshold in the number or quality of RecA nucleoprotein filaments required for SOS induction (Centore et al., 2008; Gruenig et al., 2008). Thus, PsiB does not have to completely block RecA filamentation; it simply needs to prevent the attainment of the threshold level required for SOS induction. The molecular basis of the putative threshold has not been elucidated.

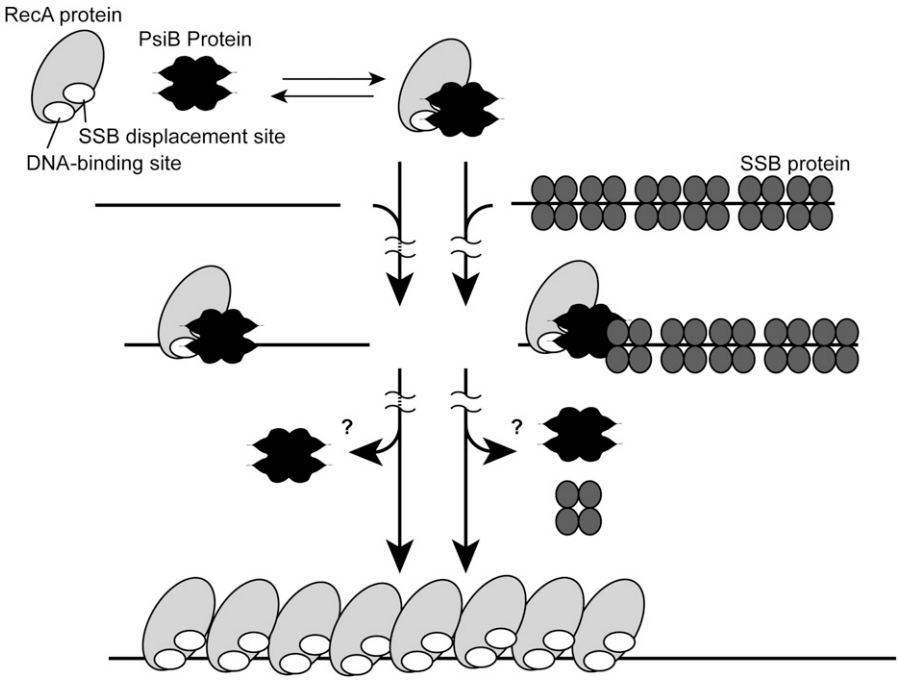

Figure 7. Proposed Mechanism of PsiB Activity

PsiB binding to free RecA in solution blocks the site on RecA, which is necessary for SSB displacement and partially blocks or allosterically distorts the DNA-binding site on RecA. When the single-stranded DNA is exposed (not bound by SSB), RecA can bind to it because the DNA-binding site is still partially available, whereas PsiB is bound in the SSB-displacement site (left panel). However, the affinity of PsiB-bound RecA for DNA decreases, which results in diminished DNA binding and ATP hydrolysis by RecA. When SSB is present, and RecA is bound by PsiB, RecA cannot displace SSB (right panel). The decreased affinity for DNA reduces RecA binding to any DNA not coated with SSB. The combination of the two inhibition effects of PsiB on RecA result in suppression of nucleation and filament extension of RecA on SSB-coated ssDNA.

In principle, PsiB could also inhibit LexA autocleavage either by mimicking LexA and competing for binding to the RecA nucleoprotein filament or by binding to LexA to prevent it from binding to RecA filaments on DNA. Fluorescence polarization experiments provide no evidence that PsiB interacts with LexA. Therefore, the PsiB mechanism is adequately described by invoking the demonstrated PsiB interaction with RecA.

A direct interaction between PsiB and RecA was proposed by Devoret and colleagues two decades ago (Bagdasarian et al., 1986; Bailone et al., 1988). The strength of the PsiB inhibition effect on SOS varies depending on which RecA phenotype is displayed by the strain harboring the *psiB* gene (Bagdasarian et al., 1986; Bagdasarian et al., 1980; Bailone et al., 1988). This result can now be rationalized in the context of a biochemically established PsiB-RecA interaction. The dose-dependent effect of PsiB on suppressing SOS (Bailone et al., 1988) can also be explained by the PsiB-RecA interaction.

The experiments incorporating the RecA filament-stabilizing protein DinI reveal a dynamic interaction between PsiB and RecA. As shown in Figure 5C, DinI can stabilize RecA on poly(dT) DNA in the absence of SSB even when RecA has been preincubated with PsiB in the reaction. In contrast, the interaction between DinI and RecA bound to DNA is stable, so PsiB cannot easily disrupt the preformed DinI-RecA filament complexes.

In Figure 7, we present a model for PsiB function that takes into account the evident importance of SSB in the inhibition patterns. We propose that RecA possesses an SSB displacement site in close proximity to or overlapping with the RecA DNA-binding site. We define an SSB displacement site to be a region that either directly interacts with SSB or that recognizes the DNA strand wrapped around SSB. The existence of such a RecA-SSB interaction site is implied by the capacity of C-terminal truncation mutants of RecA to bypass the need for mediator proteins in filament formation on SSB-coated ssDNA (Eggler et al., 2003). In effect, RecA can readily displace SSB from ssDNA; however, that capacity is suppressed by the RecA C terminus. The RecA C terminus itself acts as a kind of regulatory flap, occluding the SSB-displacement surface under most conditions. In our model, PsiB binds RecA primarily so as to cover the SSB-displacement surface, leading to the observed restricted access to DNA. This model is supported by the observation that PsiB inhibits filament formation onto SSB-coated ssDNA by a RecA mutant protein lacking the C terminus as much as it inhibits wild-type RecA, eliminating the advantage of C-terminal deletion (RecA ΔC17; V.P., unpublished data). The binding of PsiB in the SSB-displacement site must also partially occlude the RecA-binding site for DNA, thus decreasing RecA affinity for DNA. This would explain the partial inhibition of the RecA ATPase activity on poly(dT) DNA when SSB is absent. The SSB-displacement surface that we postulate on the RecA protein has not yet been structurally defined.

EXPERIMENTAL PROCEDURES

Chemicals were purchased from Fisher and Sigma, except for DTT, which was purchased from Research Organics, and IPTG, which was purchased from Gold Biotechnology. NdeI and BamHI and T4 ligase were purchased from New England BioLabs.

Protein Purification

The *psiB* gene was cloned from *E. coli* strain JM101, which harbors an F′ conjugative plasmid. The gene was PCR-amplified, cloned into pET 21a vector, and transformed into the BL21 strain. Deviation from the published *psiB* gene sequence was at bases 409 and 410, where T and C, respectively, were inverted relative to the sequence posted at NCBI. The originally published sequence (Dutreix et al., 1988) agreed with the sequencing we observed. Harvested cells were lysed by sonication in 250 mM Tris-HCl (80% cation), 25% sucrose, and EDTA. PsiB protein was precipitated from the clarified cell lysate by the addition of ammonium sulfate to 43% saturation. The pellet was washed with 1 M ammonium sulfate twice and resuspended in 20 mM Tris, 80% cation, 1 mM EDTA, 10% glycerol (R buffer [pH 7.7]), dialyzed into R buffer, and then further dialyzed into 20 mM phosphate, 1 mM EDTA, and 10% glycerol (P buffer [pH 7.0]). The protein was purified successively on ceramic hydroxyapatite, Source S, and Source Q columns. The resulting PsiB was >94% pure by gel and nuclease free, except for the presence of a minor double-stranded DNA endonuclease. This contaminating activity relaxed less than 10% of 20 μM supercoiled plasmid DNA over a period of 2 hr under conditions typically used in our experiments. The analysis of the protein by MS gave a major peak at 15,878 Da (predicted Mr, 15,880 Da). The concentration of PsiB was determined using a native extinction coefficient $1.35 \times 10^4\ M^{-1}\ cm^{-1}$, determined by a published procedure (Edelhoch, 1967). A second preparation, which was found to be substantially nuclease free, was used for DNA strand exchange experiments and to repeat several of the reported experiments.

Wild-type RecA and SSB proteins from *E. coli* were purified with minor modifications as described elsewhere (Lusetti et al., 2003; Lohman and Overman, 1985). The RecA preparation included an additional size exclusion step. RecA concentration was determined as previously from the protein absorbance at 280 nm, corrected for scattering at 320 nm using extinction coefficient $2.23 \times 10^{-4}\ M^{-1}\ cm^{-1}$. The SSB preparation included a heparin and DEAE columns. The *E. coli* SSB protein used in fluorescence polarization was a kind gift of James Keck. SSB concentration was determined from the protein absorbance at 280 nm, corrected for scattering at 320 nm using the extinction coefficient $2.83 \times 10^{-4}\ M^{-1}\ cm^{-1}$. The *E. coli* DinI and LexA proteins were purified as described elsewhere (Gruenig et al., 2008; Lusetti et al., 2004a). Concentrations of the DinI and LexA proteins were determined using the extinction coefficients $1.44 \times 10^4\ M^{-1}\ cm^{-1}$ and $7300\ M^{-1}\ cm^{-1}$, respectively.

DNA Substrates

M13mp18 cssDNA and double-stranded DNA were purified as described elsewhere (Messing, 1983; Neuendorf and Cox, 1986). Poly(dT) DNA was obtained from Amersham, lot GG0076. Fluorescent oligomer den7 5′-GGC CTC GCG GTA GCT GAG CTC GGA GCG CAC GAT TCG CAC TGC TGA TGT TC-3′-fluoresceine was obtained from IDT.

ATPase Assay

The ATPase activity was followed using a coupled assay monitored in a Varian Cary 300 dual beam spectrophotometer, equipped with a temperature controller and a 12-position cell changer as described elsewhere (Lindsley and Cox, 1989; Morrical et al., 1986). The path length and the band pass were 1 cm or 0.5 cm and 2 nm, respectively. Regeneration of ATP from ADP in the presence of PEP was coupled to the oxidation of NADH, and the decrease in NADH concentration was followed at 380 nm. The extinction coefficient of NADH ($1.21\ mM^{-1}\ cm^{-1}$) at 380 nm was used to calculate the amount of ATP hydrolyzed.

The reactions were performed in 25 mM Tris-OAc (80% cation), 1 mM DTT, 3 mM potassium glutamate, 10 mM magnesium acetate, 5% glycerol (RecA buffer), an ATP regeneration system (10 u/ml pyruvate kinase and 3.5 mM PEP), a coupling system (1.5 mM NADH and 10 u/ml lactate dehydrogenase), and 3 μM DNA. The figure legends describe the DNA type used in the assays.

Where RecA protein modulators of function (i.e., PsiB and DinI) were present, the modulators were incubated with the master mix at 37°C for 10 min, and the reactions were started with the addition of RecA. The only exception is the PsiB/DinI challenge assay where the modulator proteins or their storage buffers are added to the reaction 15 min after the addition of 2 μM RecA. PsiB concentrations were varied in the assays and are pointed out in the figure legends.

Strand Exchange

Two orders of addition were used in this experiment. In one of the controls, RecA was allowed to nucleate on css M13mp18 DNA, and then ATP and SSB were added. Strand exchange was initiated by the addition of ldsDNA. In the experiment and second control for PsiB inhibition of RecA strand-exchange activity on SSB-coated DNA, the following order of addition was chosen. The reaction containing css M13mp18 DNA in RecA buffer, an ATP regeneration system (10 u/ml pyruvate kinase and 2.5 mM PEP), 3 μM ATP, and SSB was incubated for 10 min to allow SSB to bind to cssDNA. PsiB or compensatory PsiB storage buffer was added, and the reaction was further incubated for 10 min. RecA protein was added to the reaction and was allowed to bind to the SSB-coated cssDNA for 10 min. The reaction was initiated with the addition of homologous ldsDNA. Time point 0 represents the immediate withdrawal of a sample from the reaction after ldsDNA addition and mixing. The concentrations of the proteins and DNA used in the experiment are given in the figure legends.

Fluorescence Polarization

Pure PsiB protein was labeled by dialyzing it into 10 mM sodium bicarbonate (pH 9) and 10% glycerol and incubating with molar excess of fluoresceine isothiocyanide (FITC) (Sigma F7250) dissolved in DMSO overnight at 4°C (Lopper et al., 2007). The reaction was quenched with NH_4Cl (Petrushenko et al., 2002) to a final concentration of 50 mM, followed by dialysis into 20 mM Tris-Cl, 80% cation, 50 mM KCl, and 10% glycerol. The free dye was removed by centrifuging the labeled protein in microcon tubes three times and adding fresh storage buffer every time. The degree of labeling was determined by measuring the absorbance at 280 and 494 nm. The concentration of FITC was determined using extinction coefficient $68{,}000\ M^{-1}\ cm^{-1}$ at 494 nm, and the protein concentration was determined by the following formula: dilution factor × (A_{280} − 0.3 × A_{494})/PsiB extinction coefficient. The degree of labeling was one FITC label per 11 PsiB monomers. The low degree of labeling can be explained by the presence of only one lysine per monomer of PsiB. The protein was stored at −80°C. Testing of a subsequent and more highly labeled preparation indicated that FITC labeling may modestly increase PsiB activity. The measurements were done with Beacon 2000 (Invitrogen). The occurrence or absence of binding was determined by following the change in anisotropy of the labeled PsiB.

PsiB-LexA Interactions

LexA protein was diluted in 20 mM Tris, 80% cation (pH 7.5), 200 mM potassium glutamate, 50 mM KCl, 1 mM magnesium acetate, 1 mM ATP, 4% glycerol, 0.1 g/l BSA, and 1 mM DTT buffer. The dilutions were used to titrate 60 nM FITC-PsiB probe. The dilutions for each concentration were prepared twice.

PsiB-SSB Interactions

The SSB protein was dialyzed into 20 mM Tris-Cl, 80% cation, 50 mM KCl, 10% glycerol, and 1 mM DTT and was supplemented with BSA to a final concentration of 0.1 g/l. Dilutions were made into the same buffer and were used to titrate 60 nM labeled PsiB protein. The dilutions for each concentration were prepared three times. The intensity of the measurements was at least 10 times the intensity of the background blank.

PsiB-DNA Interactions

The PsiB protein was diluted into 20 mM Tris, 80% cation, 50 mM NaCl, 4% glycerol, 0.1 g/l BSA, and 1 mM DTT; 10 nM of den 7 DNA was titrated with increasing concentrations of PsiB protein. SSB protein was used as a positive control for a protein of similar molecular weight that binds DNA. The highest

dilution from the PsiB stock was from 1:10 (final concentration, 15.6 μM), which would have resulted in increase of glycerol concentration in the reaction from 4% to 4.6%. We assume that such increase will have a negligible effect on the potential interaction between PsiB and DNA at such high excess of protein.

PsiB-RecA Interactions

The RecA-PsiB FP experiments were performed as described for the PsiB-LexA FP experiments. The dilutions for each concentration were done three to nine times. The horizontal and vertical intensities of the sample were at least four times the intensities of the blank. In the control with labeled BSA and RecA, BSA was diluted until it had the same intensity as the labeled PsiB used in the reactions.

EMSA

The reactions were conducted in the same buffer as the ATPase assays. SSB (0.5 μM monomers) was incubated with den7 DNA for 10 min and then was incubated with PsiB (concentrations shown on figure) or RecQ (0.25 μM) for another 10 min. The complexes were analyzed on 10% native polyacrylamide gel. The position of the DNA on the gel was visualized using Typhoon 9410 scanner (Amersham Biosciences) with 488 nm excitation and 520 emission laser settings.

LexA Cleavage Assay

The assay was done generally as described elsewhere (Drees et al., 2004), albeit with a different order of addition. M13mp18 cssDNA was incubated with SSB and PsiB for 10 min at 37°C. RecA protein was added and allowed to equilibrate for 10 min. The reactions are initiated by the addition of LexA protein.

Electron Microscopy

Carbon films, mounted on 400-mesh electron microscopy grids, were first activated by a brief glow-discharge treatment (Grassucci et al., 2007). Activated grids were used immediately. Samples for electron microscopy were prepared in RecA buffer. The experiment consisted of a 10 min preincubation of 0.5 μM SSB and either 9 μM PsiB protein or the appropriate volume of compensatory storage buffer with 3 μM M13mp18 cssDNA in the presence of 3 μM ATP, and an ATP regeneration system containing 12 mM phosphocreatine and 10 units of creatine kinase. The reaction was then initiated with 2 μM RecA and was allowed to proceed for 100 min. All incubations were done at 37°C. The reaction mixture described above was diluted four times with RecA buffer, and 8 μl of the diluted sample was immediately adsorbed to the activated carbon film for 3 min. The grid was then touched to a drop of the above buffer followed by floating on a fresh drop of the same buffer for 1 min. The sample was stained by touching to a drop of 5% uranyl acetate followed by floating on a fresh drop of the same solution for 30 s. Finally, the grid was washed by touching to a drop of double distilled water followed by immersion in two 10-ml beakers of double distilled water. After the sample was dried, it was rotary-shadowed with platinum. This protocol was designed for visualization of complete reaction mixtures, and no attempt was made to remove unreacted material. Although this approach should yield results that give a true insight into reaction components, it does lead to samples with a high background of unreacted proteins.

To determine the proportion of the molecules observed that were either fully or partially coated by RecA protein or bound only by the SSB protein, at least eight separate regions of the grids from four different experiments were counted at an identical magnification for each sample. A molecule was considered discontinuous, or gapped, if it had a detectable region of SSB-coated DNA of any size or if there was a gap between two adjacent protein molecules. Imaging and photography were performed with a TECNAI G2 12 Twin Electron Microscope (FEI Co.) equipped with a GATAN 890 CCD camera. Digital images of the nucleoprotein filaments were taken at 15,000× magnification.

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