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Stimulation of the *Streptococcus pneumoniae* RecA protein-promoted three-strand exchange reaction by the competence-specific SsbB protein

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ABSTRACT

The effect of the transformational competence-specific *Streptococcus pneumoniae* single-stranded DNA binding protein, *Sp*SsbB, on the ATP-dependent three-strand exchange activity of the *Sp*RecA protein was investigated. Although *Sp*RecA exhibited only a trace level of strand exchange activity in the absence of *Sp*SsbB, an extensive strand exchange reaction was observed when *Sp*SsbB was added to the reaction solution after *Sp*RecA. A more limited strand exchange reaction was observed, however, when *Sp*SsbB was added to the reaction solution before *Sp*RecA. This dependence on the order of addition, together with additional DNA-dependent ATP hydrolysis experiments, indicated that the mechanism of stimulation may involve the postsynaptic binding of *Sp*SsbB to the displaced linear single-stranded DNA reaction product. When dATP was provided in place of ATP as the nucleotide cofactor (to suppress a potentially inhibitory effect of *Sp*SsbB on the interaction of *Sp*RecA with the circular ssDNA reaction substrate), the stimulatory effect of *Sp*SsbB on the strand exchange reaction was apparent regardless of the order in which it was added to the reaction solution. These findings suggest that *Sp*SsbB may be able to facilitate *Sp*RecA-promoted DNA recombination reactions during natural transformation in *S. pneumoniae*.

1. Introduction

The naturally transformable bacterium *Streptococcus pneumoniae* is able to take up DNA from its environment and incorporate it into its own chromosome [1]. This process serves as a general mutational mechanism that allows *S. pneumoniae* to change its genomic composition in response to environmental changes and stresses [2]. In the first step, an exogenous DNA molecule binds to the surface of the *S. pneumoniae* cell and one of the strands is degraded while the remaining complementary strand is transported into the cell interior. The internalized single-stranded DNA is then assimilated into a homologous region of the double-stranded *S. pneumoniae* chromosome [1]. Genetic studies indicate that the transformational recombination reaction is carried out by the *S. pneumoniae* RecA protein (*Sp*RecA), a DNA recombinase analogous to the well-characterized RecA protein from *Escherichia coli* (*Ec*RecA) [3,4].

The *Ec*RecA protein has single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA)-dependent ATP hydrolysis activities, and is able to promote a variety of ATP-dependent DNA pairing reactions that reflect its cellular recombination functions [5,6]. The most extensively studied reaction is the three-strand exchange reaction between a circular ssDNA and a homologous linear dsDNA [5–7]. In this reaction, *Ec*RecA first polymerizes onto the circular ssDNA to form a filament-like structure known as the presynaptic complex. The presynaptic complex then interacts with the linear dsDNA and new base pairing interactions are established between the circular ssDNA and the complementary strand of the linear dsDNA. The complementary linear strand is then transferred to the circular ssDNA to form a nicked circular dsDNA and a displaced linear ssDNA as the final reaction products. This reaction is stimulated by the *E. coli* single-stranded DNA binding protein (*Ec*SSB), which is generally included in assays as an accessory factor. During the presynaptic phase of the reaction, *Ec*SSB binds to the circular ssDNA substrate and removes regions of secondary structure which otherwise impede the binding of *Ec*RecA. *Ec*RecA then displaces *Ec*SSB from the circular ssDNA to form the presynaptic complex [8]. During the postsynaptic phase of the reaction, *Ec*SSB facilitates the formation of the fully-exchanged products by binding to the displaced strand that is generated when the circular ssDNA is paired with the linear dsDNA [9].

We previously used the ATP-dependent three-strand exchange reaction to characterize the strand exchange activity of the *Sp*RecA protein [10]. In our earlier study, the *S. pneumoniae* SsbA protein (*Sp*SsbA), a single-stranded DNA binding protein (SSB) analogous to the *Ec*SSB protein, was provided as an accessory factor [10,11]. We found that *Sp*RecA differed from *Ec*RecA in that presynaptic

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complex formation appeared to be inhibited (rather than enhanced) by the *Sp*SsbA protein. An extensive strand exchange reaction was observed when *Sp*SsbA was added to the reaction solution after *Sp*RecA had been allowed to interact with the circular ssDNA and linear dsDNA substrates, however, suggesting that *Sp*SsbA was able to facilitate the postsynaptic phase of the reaction [10].

In addition to *Sp*SsbA, which is a constitutively-expressed protein that may function in routine cellular functions in a manner analogous to that of the *Ec*SSB protein in *E. coli*, there is a second SSB protein in *S. pneumoniae*, *Sp*SsbB, whose expression is strongly induced when the cells become competent for natural transformation (there is no counterpart to the *Sp*SsbB protein in *E. coli*) [1,12,13]. It has recently been shown that *Sp*SsbB binds to the internalized exogenous single-stranded DNA and protects it from degradation by cellular nucleases [13,14]. In view of its central role in natural transformation, we have now investigated the effect of the *Sp*SsbB protein on the three-strand exchange activity of the *Sp*RecA protein.

2. Materials and methods

2.1. Materials

S. pneumoniae RecA protein [4] and *S. pneumoniae* SsbB protein [12] were prepared as described. All *Sp*RecA concentrations are expressed as total monomers, and all *Sp*SsbB concentrations are expressed as total tetramers. ATP, dATP, [γ-^{32}P]ATP, and [α-^{32}P]dATP were from Amersham Biosciences. Circular φX ssDNA (+strand) and circular φX dsDNA were from New England Biolabs. Linear φX dsDNA was prepared from circular φX dsDNA by *Pst*I digestion as described [7]. Single- and double-stranded φX DNA concentrations were determined by absorbance at 260 nm using the conversion factors 36 and 50 μg ml^{-1} A_{260}^{-1}, respectively. All DNA concentrations are expressed as total nucleotides.

2.2. Three-strand exchange assay

The reaction solutions contained 25 mM Tris acetate (pH 7.5), 10 mM magnesium acetate, 5% glycerol, 1 mM dithiothreitol, and the concentrations of circular φX ssDNA, linear φX dsDNA, *Sp*RecA, *Sp*SsbB, and ATP or dATP given in the relevant figure legends. At the indicated times, aliquots (20 μL) were removed from the reaction solutions and quenched with SDS (1% final concentration)/EDTA (15 mM final concentration). The quenched aliquots were analyzed by electrophoresis on a 0.8% agarose gel using a Tris acetate–EDTA buffer system. The substrates and products of the reactions were visualized by ethidium bromide staining [7].

2.3. ATP and dATP hydrolysis assay

The reaction solutions contained 25 mM Tris acetate (pH 7.5), 10 mM magnesium acetate, 5% glycerol, 1 mM dithiothreitol, and the concentrations of circular φX ssDNA or linear φX dsDNA, *Sp*RecA, *Sp*SsbB, and [γ-^{32}P]ATP/ATP or [α-^{32}P]dATP/dATP given in the relevant figure legends. The ATP and dATP hydrolysis reactions were monitored using a thin layer chromatography method as previously described [15].

3. Results

3.1. ATP-dependent three-strand exchange

The ATP-dependent three-strand exchange activity of the *Sp*RecA protein was examined in the absence and presence of *Sp*SsbB protein. In the three-strand exchange assay, a circular φX ssDNA (5386 nucleotides) and a homologous linear φX dsDNA (5386 base pairs) are recombined to form a nicked circular φX dsDNA and a linear φX ssDNA. The substrates and products of this reaction are readily monitored by agarose gel electrophoresis [7].

The ATP-dependent three-strand exchange reactions are shown in Fig. 1. *Sp*RecA exhibited only a trace level of strand exchange activity in the absence of *Sp*SsbB (Fig. 1A, *minus SsbB*). When *Sp*SsbB was added to the reaction solution after the *Sp*RecA, however, an extensive strand exchange reaction was observed in which most of the circular ssDNA substrate was converted into the nicked circular dsDNA product within 60 min (Fig. 1A, *SsbB last*). When *Sp*SsbB was added to the reaction solution before the *Sp*RecA, in contrast, only a small amount of nicked circular dsDNA product was formed, even after 120 min (Fig. 1A, *SsbB first*). These results showed that *Sp*SsbB was able to stimulate the ATP-dependent strand exchange reaction, but that the degree of stimulation was strongly dependent on the order in which the *Sp*SsbB was added to the reaction solution.

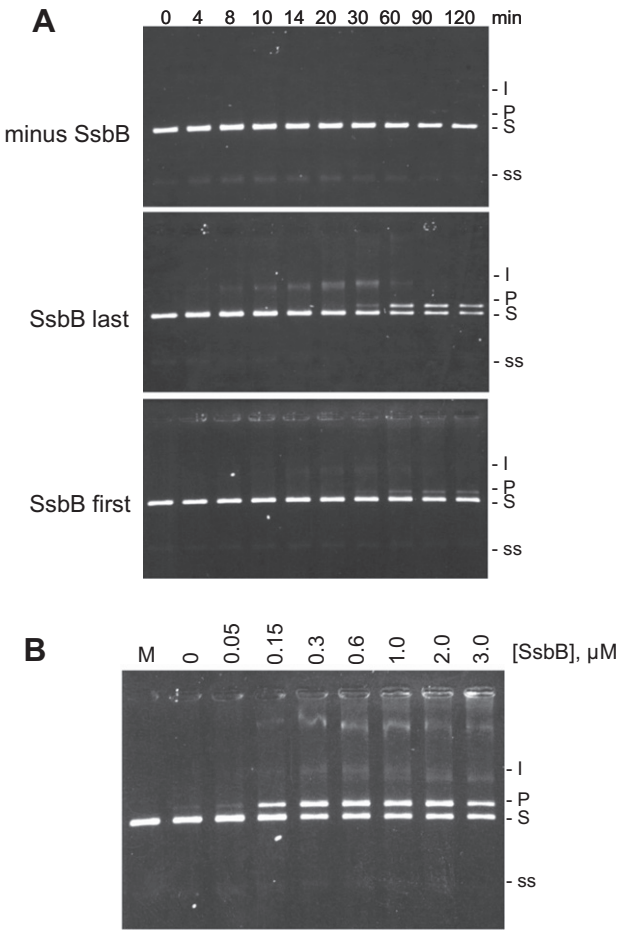

Fig. 1. Effect of *Sp*SsbB on *Sp*RecA-promoted ATP-dependent three-strand exchange. (A) Dependence on order of *Sp*SsbB addition. The reaction solutions contained 5 μM circular φX ssDNA, 15 μM linear φX dsDNA, 5 mM ATP, 6 μM *Sp*RecA, and no *Sp*SsbB (*minus SsbB*), or 0.3 μM *Sp*SsbB added 10 min after *Sp*RecA (*SsbB last*), or 0.3 μM *Sp*SsbB added 10 min before *Sp*RecA (*SsbB first*). The reactions were carried out at 37 °C for the indicated times and then analyzed by agarose gel electrophoresis. In these reactions, the circular φX ssDNA (5 μM total nucleotide) was limiting relative to the linear φX dsDNA (15 μM total nucleotide = 7.5 μM base pairs) and therefore the maximum amount of linear dsDNA that could be converted to nicked circular dsDNA product was 67%. (B) Dependence on *Sp*SsbB concentration. The reactions were carried out as described above (using the *SsbB last* order of addition) except that the concentration of *Sp*SsbB was varied from 0 to 3.0 μM. The 60 min time points are shown. Labels: *S*, linear φX dsDNA substrate; *I*, partially exchanged intermediates; *P*, nicked circular φX dsDNA product; *ss*, φX ssDNA substrate and product.

An additional set of reactions was carried out under the same conditions as those in Fig. 1A (using the *SsbB last* order of addition), except that the *Sp*SsbB concentration was varied from 0 to 3.0 μM. As shown in Fig. 1B, the efficiency of the strand exchange reaction increased with increasing *Sp*SsbB concentration until reaching a maximal level at 0.3 μM *Sp*SsbB (this concentration was used for the reactions shown in Fig. 1A). Previous studies have shown that this concentration of *Sp*SsbB would be sufficient to saturate the total amount of φX ssDNA (5 μM, circular substrate or linear product) that would be present in the strand exchange reaction solutions [16].

3.2. ATP hydrolysis

To explore the mechanistic basis for the pronounced dependence of the ATP-dependent strand exchange reaction on the order of *Sp*SsbB addition, the effect of *Sp*SsbB on the circular φX ssDNA and linear φX dsDNA-dependent ATP hydrolysis activities of the *Sp*RecA protein was examined. The reactions were carried out under the same conditions as the strand exchange reactions shown in Fig. 1A.

The circular ssDNA-dependent ATP hydrolysis reactions are shown in Fig. 2 (*ssDNA*). An initial rate of ATP hydrolysis of 31 μM min^{-1} was observed when *Sp*RecA was added to the circular ssDNA in the absence of *Sp*SsbB. This result demonstrated that *Sp*RecA was able to form an active complex on the circular ssDNA substrate under these reaction conditions (see Ref. [10] for further analysis). When *Sp*SsbB was added to the reaction solution before the *Sp*RecA, however, virtually no ATP hydrolysis was observed. Moreover, when *Sp*SsbB was added to an ongoing ATP hydrolysis reaction, the reaction was immediately terminated. These results showed that *Sp*SsbB has a strong inhibitory effect on the circular ssDNA-dependent ATP hydrolysis activity of the *Sp*RecA protein.

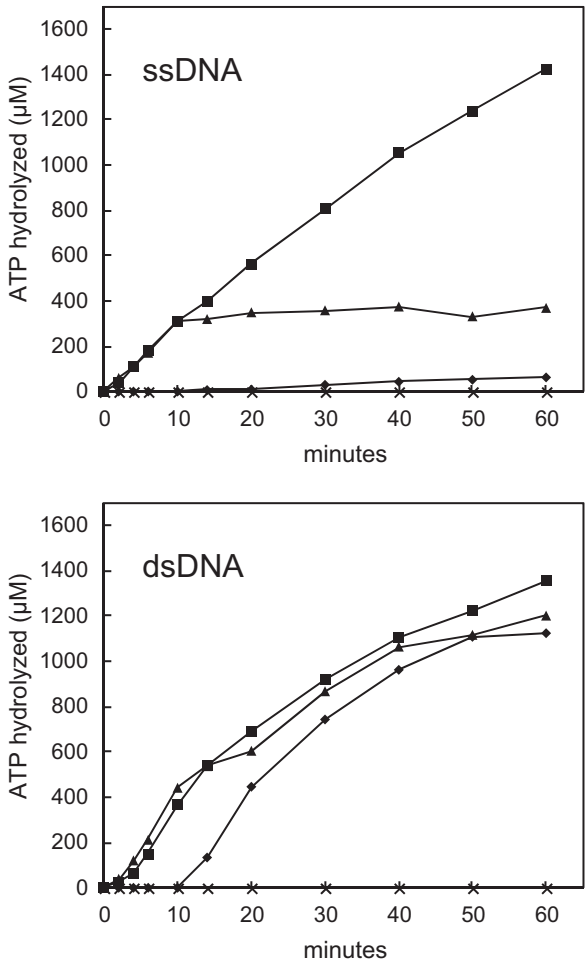

Fig. 2. Effect of *Sp*SsbB on *Sp*RecA-catalyzed ATP hydrolysis. The reaction solutions contained 5 μM circular φX ssDNA (*ssDNA*) or 15 μM linear φX dsDNA (*dsDNA*), 5 mM ATP, and 6 μM *Sp*RecA, with or without 0.3 μM *Sp*SsbB. The reactions were initiated by adding *Sp*RecA at 0 min with no *Sp*SsbB (■), by adding *Sp*SsbB at 0 min and *Sp*RecA at 10 min (♦), or by adding *Sp*RecA at 0 min and *Sp*SsbB at 10 min (▲). The reactions were carried out at 37 °C for the indicated times. *Sp*RecA exhibited no ATP hydrolysis activity under these reaction conditions when DNA was omitted from the reaction solution (×).

The linear dsDNA-dependent ATP hydrolysis reactions are shown in Fig. 2 (*dsDNA*). An initial rate of ATP hydrolysis of 40 μM min^{-1} was observed when *Sp*RecA was added to the linear dsDNA in the absence of *Sp*SsbB. This result was similar to that obtained with the circular ssDNA, and demonstrated that *Sp*RecA was also able to interact with the linear dsDNA under these conditions. In contrast to the results that were obtained with the circular ssDNA, however, the linear dsDNA-dependent ATP hydrolysis reaction was unchanged when *Sp*SsbB was added to the reaction solution, either before or after *Sp*RecA. These results were consistent with the expected inability of *Sp*SsbB to compete with *Sp*RecA for binding to the linear dsDNA (*Sp*SsbB has no detectable linear φX dsDNA binding activity under these reaction conditions; data not shown). Furthermore, these results showed that *Sp*SsbB does not have a general inhibitory effect on the activity of the *Sp*RecA protein and therefore indicated that the inhibition that was observed in the circular ssDNA-dependent ATP hydrolysis reaction was due to the binding of *Sp*SsbB to the circular ssDNA.

The results in Fig. 2 suggested that *Sp*SsbB will have no effect on the interaction of *Sp*RecA with the linear dsDNA substrate, but may bind to the circular ssDNA substrate and interfere with the formation of a presynaptic *Sp*RecA-circular ssDNA complex, when it is added to a strand exchange reaction solution before *Sp*RecA. These findings could potentially account for the results in Fig. 1A, which showed that *Sp*SsbB stimulated the ATP-dependent strand exchange reaction most effectively when it was added to the reaction solution after *Sp*RecA had been allowed to interact with the reaction substrates.

3.3. dATP hydrolysis

To determine if the dependence of the ATP-dependent strand exchange reaction on the order of *Sp*SsbB addition was due to an inhibitory effect of *Sp*SsbB on the interaction of *Sp*RecA with the circular ssDNA substrate, an additional series of experiments was carried out in which dATP used in place of ATP as the nucleotide cofactor. It has been shown that RecA proteins bind more tightly to ssDNA in the presence of dATP than with ATP [17–19]. It was therefore anticipated that an inhibitory effect of *Sp*SsbB on the interaction of *Sp*RecA with the circular ssDNA substrate would be suppressed if dATP was provided as the nucleotide cofactor, and that this, in turn, would reduce or eliminate the dependence of the strand exchange reaction on the order of *Sp*SsbB addition.

The DNA-dependent dATP hydrolysis reactions that were carried out in the absence and presence of *Sp*SsbB are shown in Fig. 3. The initial rates of circular ssDNA-dependent dATP hydrolysis (44 μM min^{-1}) and linear dsDNA-dependent dATP hydrolysis (45 μM min^{-1}) that were observed in the absence of *Sp*SsbB were similar to those for the ATP hydrolysis reactions. Furthermore, the linear dsDNA-dependent dATP hydrolysis reaction was unaffected by the addition of *Sp*SsbB, again consistent with the expected inability of *Sp*SsbB to compete with *Sp*RecA for binding to the linear dsDNA. In contrast to the results that were obtained with ATP, however, the circular ssDNA-dependent dATP hydrolysis reaction was also unchanged when *Sp*SsbB was added to the reaction solution. These results showed that the potentially inhibitory effect

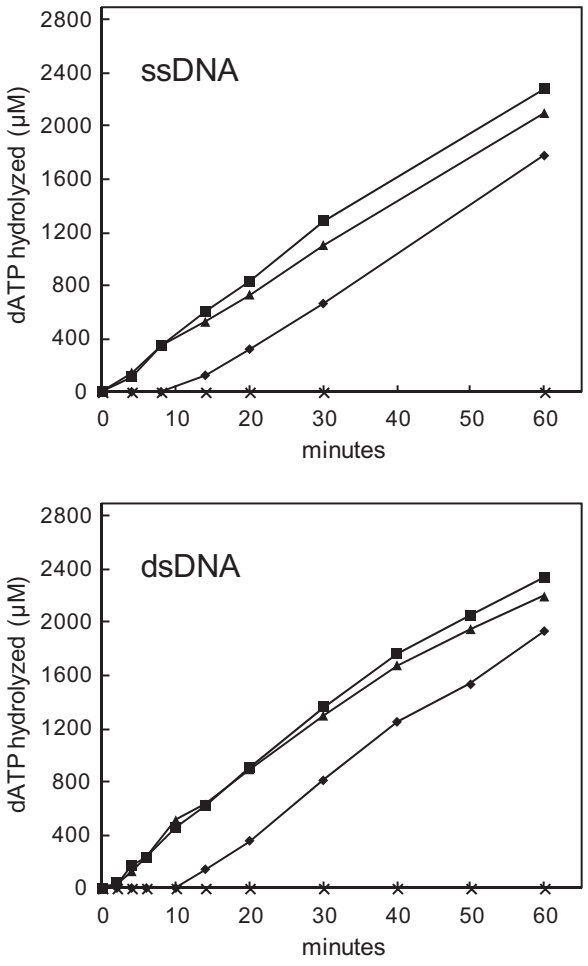

Fig. 3. Effect of *Sp*SsbB on *Sp*RecA-catalyzed dATP hydrolysis. The reaction solutions contained 5 μM circular φX ssDNA (*ssDNA*) or 15 μM linear φX dsDNA (*dsDNA*), 5 mM dATP, and 6 μM *Sp*RecA, with or without 0.3 μM *Sp*SsbB. The reactions were initiated by adding *Sp*RecA at 0 min with no *Sp*SsbB (■), by adding *Sp*SsbB at 0 min and *Sp*RecA at 10 min (♦), or by adding *Sp*RecA at 0 min and *Sp*SsbB at 10 min (▲). The reactions were carried out at 37 °C for the indicated times. *Sp*RecA exhibited no dATP hydrolysis activity under these reaction conditions when DNA was omitted from the reaction solution (×).

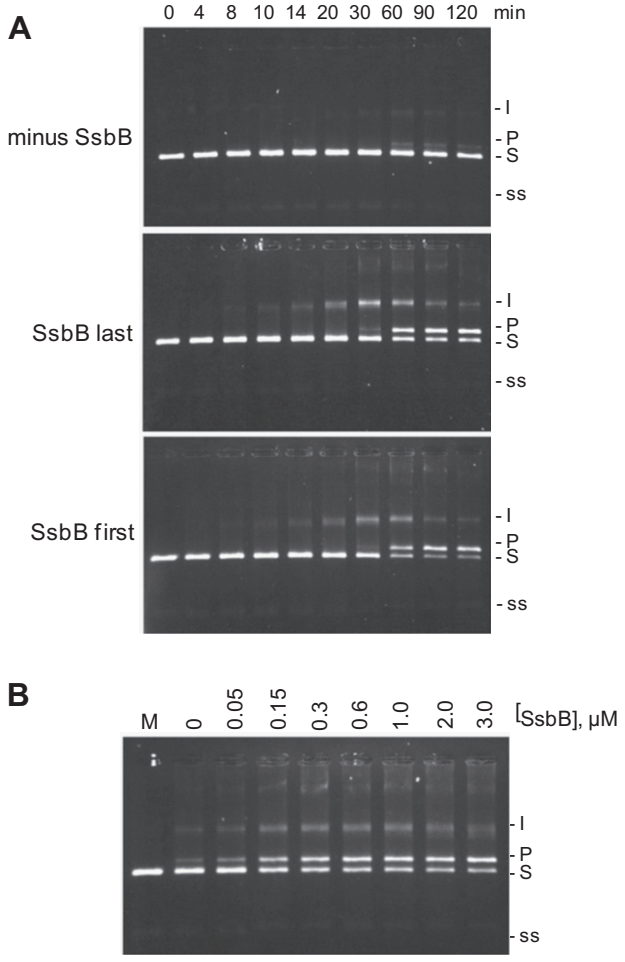

Fig. 4. Effect of *Sp*SsbB on *Sp*RecA-promoted dATP-dependent three-strand exchange. (A) Dependence on order of *Sp*SsbB addition. The reaction solutions contained 5 μM circular φX ssDNA, 15 μM linear φX dsDNA, 5 mM dATP, 6 μM *Sp*RecA, and no *Sp*SsbB (*minus SsbB*), or 0.3 μM *Sp*SsbB added 10 min after *Sp*RecA (*SsbB last*), or 0.3 μM *Sp*SsbB added 10 min before *Sp*RecA (*SsbB first*). The reactions were carried out at 37 °C for the indicated times and then analyzed by agarose gel electrophoresis. In these reactions, the circular φX ssDNA (5 μM total nucleotide) was limiting relative to the linear φX dsDNA (15 μM total nucleotide = 7.5 μM base pairs) and therefore the maximum amount of linear dsDNA that could be converted to nicked circular dsDNA product was 67%. (B) Dependence on *Sp*SsbB concentration. The reactions were carried out as described above (using the *SsbB last* order of addition) except that the concentration of *Sp*SsbB was varied from 0 to 3.0 μM. The 60 min time points are shown. Labels: *S*, linear φX dsDNA substrate; *I*, partially exchanged intermediates; *P*, nicked circular φX dsDNA product; *ss*, φX ssDNA substrate and product.

of *Sp*SsbB on the interaction of *Sp*RecA with the circular ssDNA could indeed be suppressed by providing dATP as the nucleotide cofactor.

3.4. dATP-dependent three-strand exchange

The three-strand exchange reactions that were carried out using dATP as the nucleotide cofactor are shown in Fig. 4. *Sp*RecA exhibited a low level of dATP-dependent strand exchange activity even in the absence of *Sp*SsbB (Fig. 4A, *minus SsbB*). A more extensive strand exchange reaction was observed, however, when *Sp*SsbB was added to the reaction solution after *Sp*RecA, with most of circular ssDNA substrate being converted into the nicked circular dsDNA product within 60 min (Fig. 4A, *SsbB last*). Moreover, in contrast to the results that were obtained with ATP, an equally extensive strand exchange reaction was observed when *Sp*SsbB was added to the reaction solution before *Sp*RecA (Fig. 4A, *SsbB first*). These results showed that when the potentially inhibitory effect of *Sp*SsbB on the interaction of *Sp*RecA with the circular ssDNA is suppressed by using dATP as the nucleotide cofactor, the stimulatory effect of *Sp*SsbB can be observed regardless of the order in which it is added to the strand exchange reaction solution.

A final set of reactions was carried out under the same conditions as those in Fig. 4A (using the *SsbB last* order of addition), except that the concentration of *Sp*SsbB was varied from 0 to 3.0 μM. As shown in Fig. 4B, the results were similar to those that were obtained for the ATP-dependent reaction (Fig. 1B), with optimal stimulation occurring at approximately 0.3 μM *Sp*SsbB (this concentration was used for the reactions shown in Fig. 4A). These results suggested that the dATP and ATP-dependent strand exchange reactions may be stimulated in a similar manner, when *Sp*SsbB is added to the reaction solution after *Sp*RecA.

4. Discussion

The ATP-dependent three-strand exchange reaction is strongly stimulated when *Sp*SsbB is added to the reaction solution after *Sp*RecA, whereas only a modest stimulation is observed when it

is added before *Sp*RecA. This pronounced dependence on the order of addition, together with the demonstrated inhibitory effect of *Sp*SsbB on the circular ssDNA-dependent ATP hydrolysis activity of *Sp*RecA, indicates that the mechanism of stimulation may involve a postsynaptic binding of *Sp*SsbB to the displaced linear ssDNA reaction product rather than a presynaptic binding to the circular ssDNA substrate. When the potentially inhibitory effect of *Sp*SsbB on the interaction of *Sp*RecA with the circular ssDNA substrate is suppressed by using dATP as the nucleotide cofactor, the stimulatory effect of *Sp*SsbB on the strand exchange reaction is apparent when it is added to the reaction solution either before or after *Sp*RecA. The dATP-dependent strand exchange reaction, like the ATP-dependent (*Sp*SsbB added last) reaction, proceeds optimally at *Sp*SsbB concentrations that would be sufficient to saturate the displaced linear ssDNA product in a postsynaptic stimulation mechanism [9].

The effects of the *Sp*SsbB protein on the strand exchange activity of the *Sp*RecA protein are similar to those that were described previously for the *Sp*SsbA protein [10]. This finding is consistent with previous results which showed that *Sp*SsbA and *Sp*SsbB have similar DNA binding properties under the solution conditions that were used for the strand exchange reactions [20], and suggests that *Sp*SsbA and *Sp*SsbB may have comparable effects on *Sp*RecA during natural transformation. However, although they have similar amino-terminal DNA binding domains, the carboxy-terminal domains of *Sp*SsbA and *Sp*SsbB differ significantly in length and composition [16], and studies of other bacterial SSB proteins have shown that the carboxy-terminal domains can serve as binding sites for other proteins involved in DNA metabolism [21]. It is therefore conceivable that *Sp*SsbA and *Sp*SsbB will interact with different subsets of proteins in the *S. pneumoniae* cell, and this may determine the extent to which *Sp*SsbA and *Sp*SsbB would be functionally interchangeable during natural transformation.

The dependence of the ATP-dependent three-strand exchange reaction on the order of *Sp*SsbB addition suggests that additional accessory proteins may be required in order for *Sp*RecA to initiate a transformational recombination reaction between the *Sp*SsbB-covered exogenous single-stranded DNA and the double-stranded *S. pneumoniae* chromosome. In this regard, it has been reported that the *S. pneumoniae* DprA protein is able to promote the binding of RecA protein to SSB-covered single-stranded DNA [22]. That study was carried out with the *Ec*RecA and *Ec*SSB proteins, however, and the relevance of the findings to the mechanism of transformational recombination in *S. pneumoniae* remains to be confirmed. In any case, our results suggest that once an *Sp*RecA-ssDNA complex has been formed and strand pairing between the single strand and a homologous region of the *S. pneumoniae* chromosome has been initiated, *Sp*SsbB would be able to facilitate the ensuing strand exchange reaction.

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