

The stimulatory effect of CaCl₂, NaCl and NH₄NO₃ salts on the ssDNA-binding activity of RecA depends on nucleotide cofactor and buffer pH

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The single-stranded DNA binding activity of the *Escherichia coli* RecA protein is crucial for homologous recombination to occur. This and other biochemical activities of ssDNA binding proteins may be affected by various factors. In this study, we analyzed the effect of CaCl₂, NaCl and NH₄NO₃ salts in combination with the pH and nucleotide cofactor effect on the ssDNA-binding activity of RecA. The studies revealed that, in addition to the inhibitory effect, these salts exert also a stimulatory effect on RecA. These effects occur only under very strict conditions, and the presence or absence and the type of nucleotide cofactor play here a major role. It was observed that in contrast to ATP, ATP γ S prevented the inhibitory effect of NaCl and NH₄NO₃, even at very high salt concentration. These results indicate that ATP γ S most likely stabilizes the structure of RecA required for DNA binding, making it resistant to high salt concentrations. [BMB reports 2011; 44(5): 341-346]

INTRODUCTION

The RecA protein is found in virtually all bacteria (1, 2), and its homologues such as RadA and Rad51 are ubiquitous in archaea and eukaryotes, respectively (3-6). The RecA protein of *Escherichia coli* is a multifunctional protein that possesses the DNA exchange activity. The protein is essential for three distinct biological processes: (i) homologous DNA recombination (7, 8), (ii) induction of the DNA-damage SOS response (9, 10), and (iii) the error-prone replicative bypass of DNA lesions (SOS-induced mutagenesis) (11, 12). Not surprisingly, mutations of the *recA* gene have pleiotropic effects and affect not only recombination but also DNA repair, mutagenesis, and cell division (12). In order to be able to mediate this broad

spectrum of biological processes, RecA possesses a multitude of biochemical activities, including the homologous pairing and exchange of DNA strands, ATP- and DNA-dependent co-processing of effector proteins (e.g., LexA) as well as interactions with specialized DNA polymerases to facilitate error-prone DNA synthesis. All these activities are DNA-dependent, i.e., RecA must first bind to ssDNA before it can carry out its functions.

Salts exert various effects on living organisms at the whole body, cellular and molecular level. At the molecular level, various salts exhibit a stimulatory or inhibitory effect on the enzyme activity depending on the enzyme and on the salt concentration. Low NaCl or KCl concentrations are usually required for many enzymes to function properly, whereas high concentrations inhibit the enzyme activity. Other ions, divalent ions such as Mg²⁺ and Ca²⁺ in particular, are required for proper function of many enzymes, including those interacting with DNA, e.g., enzymes involved in transcription, DNA replication, repair and recombination (13). Also, the ssDNA-binding activity of RecA and its eukaryotic homologues was shown to require Mg²⁺ ions (14-16). Moreover, processes that involve genomes are affected by other salts. Monovalent ion salts were shown to increase the frequency of homologous recombination in plants (17-20). In bacteria, homologous recombination is also positively affected by salts (21-23). Interestingly, similar concentrations of KCl that stimulated homologous recombination in plants and bacteria showed a stimulatory effect on the activity of eukaryotic Rad51 (24, 25), but they had no effect or a slightly inhibitory effect on the activity of its bacterial homologue RecA (14). On the contrary, another K⁺ salt (potassium glutamate) was shown to promote DNA strand exchange by the *E. coli* RecA protein at low concentrations and inhibit this reaction at higher concentrations (26). However, studies on salt requirement for the RecA activity are very fragmentary. Here, we present broad-range studies on the effects of three major mono- and divalent ion salts on the ssDNA-binding activity of RecA tested under various reaction conditions including buffer pH and the presence/absence of nucleotide cofactor.

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RESULTS AND DISCUSSION

Cofactor and buffer requirements for the formation of RecA-ssDNA complexes

One of the RecA biochemical activities is binding and hydrolysis of NTPs in a DNA-dependent manner (11, 27-29). This biochemical activity is required for RecA to perform its multiple biological activities, including the ATP-dependent formation of homologously paired joint molecules and the exchange of complementary DNA strands where binding to ssDNA molecules is the first step of reaction (for review see Ref. 30). The ability of RecA to bind ssDNA was analyzed using RecA-buffer (pH 7.6) and MES-buffer (pH 6.2) as both buffers were previously shown to be nearly equally optimal for human Rad51 (15). The ssDNA-binding activity test revealed that RecA was

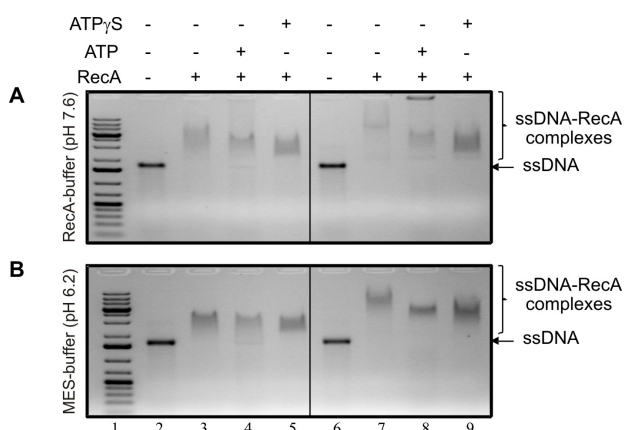


Fig. 1. Cofactor and buffer requirements for the ssDNA-binding activity of RecA. The ssDNA-binding activity of RecA was tested in RecA-buffer (A) or MES buffer (B) in the absence or presence of ATP or ATP γ S using 250 ng (4.7 μ M) of ϕ X174 single-stranded circular DNA as a substrate and 4 μ g (3.5 μ M; lanes 3-5) or 16 μ g (14.1 μ M; lanes 7-9) of RecA. Lanes 2 and 6: no RecA. Lane 1: DNA molecular size marker.

able to bind ssDNA both in the presence and in the absence of cofactors: ATP or its non-hydrolyzable analogue ATP γ S (Fig. 1). Noteworthy, sizes of the RecA-ssDNA complexes differed depending on the reaction conditions. In general, the size of the complex depends on the number of RecA molecules bound to a single DNA molecule, as it is dose-dependent (supplementary Fig. S1), and on the compactness of the complex. Complexes formed in the absence of nucleotide cofactor were always larger than those formed in the presence ATP γ S (Fig. 1A and B). When ATP was present in MES-buffer, the ssDNA-RecA complexes were slightly larger than those formed in the presence of ATP γ S (Fig. 1B). Assuming that equal amounts of RecA molecules were bound to DNA substrate, the observed differences in the size of the ssDNA-RecA complex are likely due to differences in the compactness of the complex induced by the presence or absence of nucleotide cofactor. Interestingly, the complexes formed in RecA-buffer containing ATP were of at least two types: medium- and large-sized complexes. The medium-sized complexes correspond to RecA-ssDNA filaments that represent the active form of RecA-ssDNA complexes (31), while the high molecular weight complexes correspond to the RecA-ssDNA network. This network formed upon aggregation of single-stranded DNA by RecA is an intermediate in homologous recombination, and it is then converted into RecA-ssDNA filaments. Next, the filaments co-aggregate with naked dsDNA followed by the formation of pre-synaptic complexes, homologous pairing and strand invasion (32). Similar forms (filaments and networks) have been also reported for human Rad51 (24). ssDNA not bound to RecA was often observed in these reactions (Fig. 1A, 2B-4B), indicating the release of DNA substrate upon ATP hydrolysis.

Although the larger medium-sized ssDNA-RecA complexes were formed in the presence of a higher amount of RecA (16 μ g), the protein used at the concentration of 3.5 μ M (4.0 μ g) was sufficient to form the complex with 250 ng (4.7 μ M) of ssDNA (Fig. 1). Therefore, these reaction conditions have been selected for analysis of the potential stimulatory and inhibitory effects of various salts on the ssDNA-binding activity of RecA.

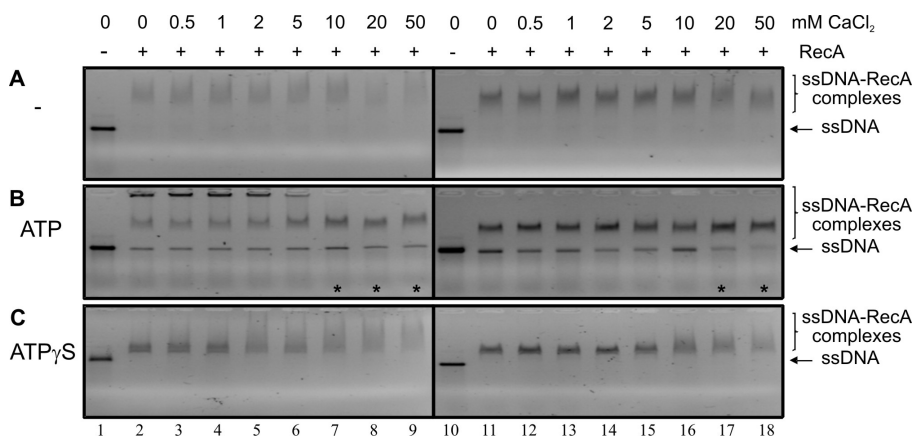


Fig. 2. The effect of CaCl₂ salt on the ssDNA-binding activity of RecA. The ssDNA-binding activity of RecA was tested in the absence of nucleotide cofactor (A) or in the presence of ATP (B) or ATP γ S (C), in RecA-buffer (left panels) or MES-buffer (right panels) using 250 ng (4.7 μ M) of ϕ X174 single-stranded circular DNA as a substrate, 4 μ g of RecA (3.5 μ M) and various amounts of CaCl₂ (the final concentrations are indicated on the top of the Figure; lanes: 2-9 and 11-18). Lanes 1 and 10: no RecA. The reactions showing induction of the ssDNA-binding activity of RecA are indicated by asterisks.

An increase in the intensity, molecular weight and/or sharpness of the medium-sized ssDNA-RecA bands would be an indicator of a stimulatory effect, whereas a decrease in the size and/or intensity of the RecA-ssDNA bands accompanied by the appearance of a free ssDNA substrate would indicate an inhibitory effect.

The effect of CaCl₂ on the ssDNA-binding activity of RecA

Ca²⁺ was previously shown to stimulate the DNA strand exchange activity of human Rad51 by modulating its ATPase activity (16). This is crucial for the formation of active hRad51-ATP-ssDNA filaments involved in DNA strand exchange during homologous recombination. Surprisingly, the effect of Ca²⁺ on the hRad51 activity was much stronger than that of Mg²⁺ (16). In case of bacterial and yeast homologues (RecA and γRad51), Mg²⁺ ions were required, and they were sufficient for most of the activities of the proteins (13, 16, 33 and 34). However, the stimulatory effect of Ca²⁺ on the activities of the RecA protein could not be excluded. Therefore, in this study, the influence of CaCl₂ salt on the ssDNA-binding activity

of RecA was analyzed. We observed that CaCl₂ exhibited no effect on the RecA activity in the absence of nucleotide cofactor and in the presence of ATPγS (Fig. 2A, C). Interestingly, the stimulation of the activity was observed for 10-50 mM and 20-50 mM CaCl₂ in RecA- and MES-buffer with ATP (Fig. 2B). This result indicates differences between RecA and hRad51 as various activities of the latter protein were stimulated by low Ca²⁺ concentrations and inhibited by high Ca²⁺ concentrations in a RecA-like-buffer containing ATP (16). However, our observations are in agreement with previous findings from studies on yeast Rad51 that showed the limited dependence of the protein activity on Ca²⁺ (16). This confirms the hypothesis suggested by Bugreev and Mazin that the mechanism of Ca²⁺-mediated regulation of Rad51 by modulation of its ATPase activity is evolutionarily recent (16). Our findings also contribute to integration of a complex picture of the effects that Ca²⁺ ions exert on various activities of RecA. Ca²⁺ was shown to inhibit the ATPase activity of RecA (35). Moreover, it did not stimulate the DNA strand-exchange activity of RecA (35), but depending on its concentration and the buffer pH,

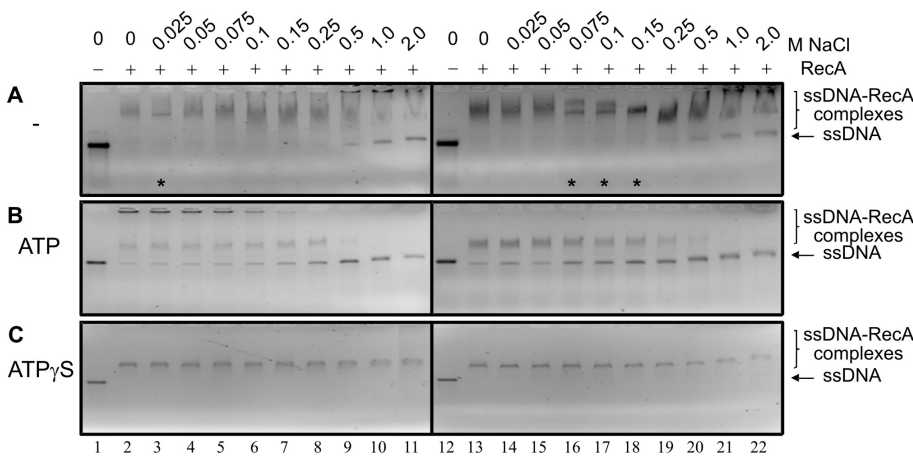


Fig. 3. The effect of NaCl salt on the ssDNA-binding activity of RecA. The ssDNA-binding activity of RecA was tested in the absence of nucleotide cofactor (A) or in the presence of ATP (B) or ATPγS (C), in RecA-buffer (left panels) or MES-buffer (right panels) using 250 ng (4.7 μM) of φX174 single-stranded circular DNA as a substrate, 4 μg of RecA (3.5 μM) and various amounts of NaCl (the final concentrations are indicated on the top of the Fig.; lanes 2-11 and 13-22). Lanes 1 and 12: no RecA. The reactions showing induction of the ssDNA-binding activity of RecA are indicated by asterisks.

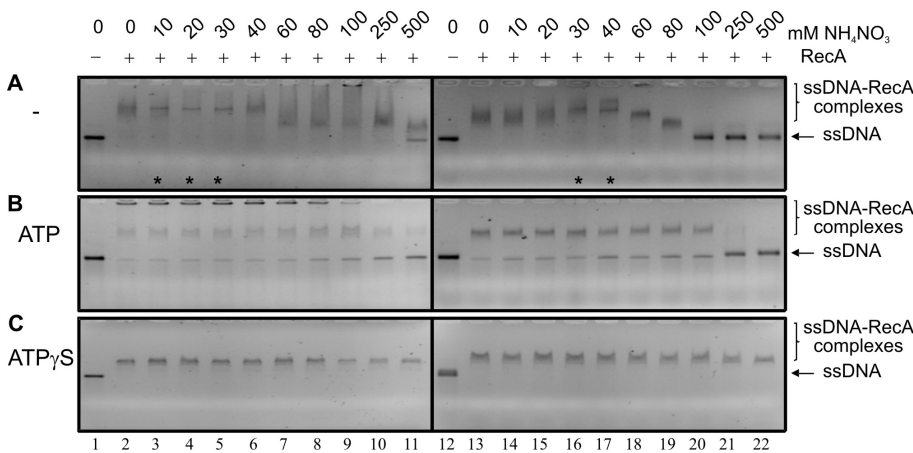


Fig. 4. The effect of NH₄NO₃ salt on the ssDNA-binding activity of RecA. The ssDNA-binding activity of RecA was tested in the absence of nucleotide cofactor (A) or in the presence of ATP (B) or ATPγS (C), in RecA-buffer (left panels) or MES-buffer (right panels) using 250 ng (4.7 μM) of φX174 single-stranded circular DNA as a substrate, 4 μg of RecA (3.5 μM) and various amounts of NH₄NO₃ (the final concentrations are indicated on the top of the Fig.; lanes 2-11 and 13-22). Lanes 1 and 12: no RecA. The reactions showing induction of the ssDNA-binding activity of RecA are indicated by asterisks.

Ca²⁺ was able to stimulate or inhibit the formation of ssDNA-RecA complexes (this study), an activity required for the first step of DNA strand exchange.

The effect of NaCl on the ssDNA-binding activity of RecA

Various analyses of the influence of a monovalent ion salt (NaCl) on the activities of RecA have been performed previously. However, these analyses were not only fragmentary concerning the reaction conditions but also limited in detection of subtle changes in the formation of ssDNA-protein complexes. Interestingly, all the former studies showed an inhibitory effect of NaCl on the activities of RecA. In the assays that mimic strand invasion during homologous recombination such as those where the formation of D-loops or joint molecules was catalyzed by the presence of RecA, NaCl at the concentration of 100 mM or 50-200 mM inhibited the activity of RecA in RecA-like buffer containing ATP (35, 36), although in 60 mM NaCl, RecA was still bound to ssDNA (36). Zaitsev and Kowalczykowski showed a dose-dependent (0-700 mM NaCl) dissociation of RecA-dsDNA complexes in MES-like buffer containing ATP (37). In addition, 300 mM NaCl was shown to inhibit the DNA-dependent ATPase activity of RecA (38). Finally, as it was quantified using an ethno-DNA fluorescence assay, ssDNA-RecA complexes in RecA-like buffer were shown to be destabilized by NaCl in a dose-dependent manner, although complexes formed in the presence of ATP showed higher stability as compared to those formed in buffer containing ADP or lacking a nucleotide cofactor (38, 39). The inhibitory effect of NaCl on the ssDNA-binding activity of RecA reported in this study (Fig. 3) not only confirmed the results of previous findings observed in RecA buffer that contained or lacked ATP but also supplemented them with new data which were obtained using RecA-buffer (pH 7.6) that contained ATP γ S and MES-buffer (pH 6.2) that was supplemented with either ATP or ATP γ S or using MES-buffer that lacked a nucleotide cofactor. It should be emphasized that a simple ssDNA binding assay employed in this study allowed us to show that NaCl might also positively affect RecA. The stimulatory effect of NaCl was observed at 25 mM concentration in RecA-buffer (pH 7.6) and at 75-150 mM concentration in MES-buffer (pH 6.2) (Fig. 3). Interestingly, this effect could be observed only in the absence of ATP/ATP γ S, thus confirming that not only hydrolysis but even binding of nucleoside triphosphate is not required for binding of RecA to ssDNA.

The effect of NH₄NO₃ on the ssDNA-binding activity of RecA

Finally, the influence of another monovalent ion salt (NH₄NO₃) on the ssDNA-binding activity of RecA was analyzed. So far, the effect of this salt has not been tested on the prokaryotic single-stranded DNA binding protein. However, ammonium nitrate along with other neutral salts containing the NH₄⁺ cation was shown to stimulate the DNA strand-exchange activity of human Rad51 (5, 25). Moreover, ammonium salts changed the preference of hRad51 binding to DNA by enhancing a higher

protein affinity to ssDNA than dsDNA (25, 40). This is accompanied by analogous differences in the ssDNA-dependent and dsDNA-dependent stimulation of the hRad51 ATPase activity (25), although earlier reports suggested the reduction of the hRad51 ATPase activity in the presence of (NH₄)₂SO₄ (24). Recent work from our lab showed a positive effect of ammonium nitrate on the recombination rate as well as on the frequency and quality of transgene integration in tobacco (18) and wheat (41). It was speculated that this effect was in part associated with the ability of (NH₄)₂SO₄ to stimulate the activity of homologous recombination/repair proteins. The stimulatory effect of NH₄NO₃ on the ssDNA-binding activity of RecA was observed in this study under different reaction conditions than that in case of the DNA strand-exchange activity of hRad51. NH₄⁺ ions stimulated the activity of hRad51 when used at the 100-150 mM concentration in RecA-like buffer containing ATP, whereas the activity of RecA was stimulated by NH₄NO₃ at the concentration of 20-40 mM in RecA-buffer without nucleotide cofactor and at the 30-40 mM concentration in MES-buffer containing ATP (Fig. 4). These results show further differences between ssDNA-binding proteins originating from bacterial and higher eukaryotic organisms.

Interesting observations were made concerning the influence of nucleotide cofactors on the effect that CaCl₂, NaCl and NH₄NO₃ salts had on the ssDNA-binding activity of RecA. In most cases, the presence of ATP enhanced the inhibitory effect of salts (Fig. 2B, 3B and 4B). On the contrary, ATP γ S prevented the inhibition of the ssDNA-binding activity of RecA by salts (Fig. 2C, 3C and 4C). RecA was shown to bind to ssDNA in the absence of a nucleoside triphosphate (e.g., ATP); however, RecA-ssDNA complexes were stabilized by ATP γ S (Fig. 1) (14, 27). It was suggested that ATP γ S blocked dissociation of RecA from DNA, whereas ATP stimulated the release of RecA from ssDNA (14, 27). Not surprisingly, a small portion of the ssDNA substrate was not bound to RecA in the presence of ATP in RecA-buffer, whereas all DNA molecules were in complex with RecA when ATP was substituted by ATP γ S (Fig. 1). However, the differences observed in RecA binding to ssDNA in the presence of ATP and ATP γ S are likely not due to differences in hydrolyzability of these nucleoside triphosphates. Hydrolysis of ATP was shown not to be required for the dissociation of ssDNA-RecA complexes since it was also enhanced by ADP and certain nucleoside triphosphates that were not hydrolyzed by the RecA protein (14, 27).

In conclusion, we observed that the effect of all tested salts on the formation of the ssDNA-RecA complexes depended on the salt concentration, the pH of the reaction buffer and the presence/absence of nucleotide cofactor, except for CaCl₂, which effect was pH-independent and occurred only in the presence of ATP. Most importantly, we showed that these salts exert a stimulatory effect on the ssDNA-binding activity of RecA, although under strict conditions. This finding not only sheds more light on the nature of RecA-ssDNA interactions but also contributes to understanding how salts affect the rate of

DNA recombination.

MATERIALS AND METHODS

ssDNA binding assay

The single-stranded DNA binding activity of the RecA protein was tested as described previously for its human Rad51 homologue (42). The reaction mixtures (the final volume: 30 µl) contained either RecA-buffer (70 mM Tris-Cl pH 7.6, 10 mM MgCl₂ and 5 mM DTT) or MES-buffer (25 mM NaMES pH 6.2, 10 mM MgCl₂ and 5 mM DTT), 250 ng (the final concentration: 4.7 µM) of φX174 viron DNA (single-stranded circular DNA, New England Biolabs), the RecA protein (New England Biolabs) in the amounts indicated in the legends to Figures and were supplemented (when indicated) with ATP or ATPγS (Sigma; the final concentration: 2.0 mM). In the tests for the effect of salts on the ssDNA-binding activity of RecA, the reaction mixtures contained NaCl, CaCl₂ or NH₄NO₃ salts in the amounts indicated in Figures. The reactions were performed for 30 min at 37°C, and the reaction products were separated by electrophoresis in 0.8% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed at 60 V in 1x TAE buffer for 1.5-2.5 h at room temperature.

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