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DNA Polymerase V and RecA Protein, a Minimal Mutasome

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Summary

A hallmark of the *Escherichia coli* SOS response is the large increase in mutations caused by translesion synthesis (TLS). TLS requires DNA polymerase V (UmuD′$_2$C) and RecA. Here, we show that pol V and RecA interact by two distinct mechanisms. First, pol V binds to RecA in the absence of DNA and ATP and second, through its UmuD′ subunit, requiring DNA and ATP without ATP hydrolysis. TLS occurs in the absence of a RecA nucleoprotein filament but is inhibited in its presence. Therefore, a RecA nucleoprotein filament is unlikely to be required for SOS mutagenesis. Pol V activity is severely diminished in the absence of RecA or in the presence of RecA1730, a mutant defective for pol V mutagenesis in vivo. Pol V activity is strongly enhanced with RecA mutants constitutive for mutagenesis in vivo, suggesting that RecA is an obligate accessory factor that activates pol V for SOS mutagenesis.

Introduction

The SOS regulon in *E. coli* is composed of more than 40 genes that enable the cell to cope with extensive UV- and chemical-induced DNA damage (Courcelle et al., 2001; Friedberg et al., 1995). The regulation of the SOS response pivots around the activities of RecA, which serves as an activator of the response, and LexA, which is a transcriptional repressor. DNA damage leads to the formation of single-stranded DNA (ssDNA) gaps at stalled or uncoupled replication forks (Friedberg et al., 1995) where RecA can assemble as a nucleoprotein filament, referred to as "activated" RecA (Kuzminov, 1999). The RecA filament functions as a coprotease that facilitates an autocatalytic cleavage of the LexA transcriptional repressor (Little, 1984; Luo et al., 2001). Derepression of genes in the SOS regulon then ensues (Walker, 1984).

The SOS response presumably evolved to help cells survive the deleterious consequences of DNA damage. However, one SOS pathway, translesion DNA synthesis (TLS), has its own deleterious effects in that it is often error-prone and leads to a dramatic increase in mutagenesis. The RecA coprotease plays a pivotal role in TLS, facilitating the activation of the UmuD mutagenesis protein via a LexA-like self-cleavage to form UmuD′ (Burckhardt et al., 1988; Nohmi et al., 1988; Shinagawa et al., 1988). UmuD′ binds UmuC tightly to form a stable UmuD′$_2$C complex (Bruck et al., 1996; Shen et al., 2003).

The molecular basis of damage-induced TLS involves the intrinsic DNA polymerase activity of UmuD′$_2$C (Reuven et al., 1999; Tang et al., 1998, 1999). Unlike the replicase pol III, pol V (UmuD′$_2$C) can facilitate the bypass of a variety of DNA adducts (Fujii et al., 2004; Tang et al., 2000) while making base substitution errors with a frequency of 10^{-3} to 10^{-4} on undamaged DNA (Tang et al., 2000). Pol V performs error prone TLS in vivo in concert with accessory proteins that include RecA protein, β sliding processivity clamp, and possibly single-stranded DNA binding protein (SSB) (Becherel et al., 2002; Echols and Goodman, 1990; Goodman, 2000; Lenne-Samuel et al., 2002). Pol V, with the same accessory proteins, copies damaged DNA templates in vitro (Reuven et al., 1998; Tang et al., 1998, 2000), causing errors that mirror in vivo mutations (Tang et al., 2000). Neither β clamp (Pham et al., 2002) nor SSB (Fujii et al., 2004; Pham et al., 2002) are essential for TLS in vitro, although their presence can enhance pol V bypass efficiency under specific assay constraints (Maor-Shoshani and Livneh, 2002; Pham et al., 2001; Tang et al., 1999). In contrast, pol V cannot catalyze TLS in the absence of RecA (Fujii et al., 2004; Pham et al., 2001; Pham et al., 2002). RecA stimulates pol V activity by more than 300-fold (Pham et al., 2001).

The characterization of certain missense *recA* mutants, in which RecA's role in SOS induction and cleavage of UmuD was retained, implied a direct role for RecA in TLS (Dutreix et al., 1989; Nohmi et al., 1988; Sweasy et al., 1990). Some *recA* mutants exhibited strikingly different abilities to promote SOS-dependent mutagenesis. Of particular interest are two RecA alleles, *recA1730* and *recA730*. When overproduced, *recA1730* (S117F) is functional for homologous recombination and LexA/UmuD cleavage but is unable to promote significant levels of pol V-dependent, damage-induced mutagenesis (Bailone et al., 1991; Dutreix et al., 1989). In contrast, *recA730* (E38K) is a mutant allele that exhibits constitutively high levels of pol V-dependent spontaneous mutagenesis (Sweasy et al., 1990; Witkin et al., 1982).

It was tacitly assumed for many years that the dramatic functional differences in the two mutant RecA proteins reflected their capacity to form a nucleoprotein filament that would help target pol V to DNA lesions (Dutreix et al., 1992; Frank et al., 1993). However, in a

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previous study (Pham et al., 2002) we demonstrated that RecA protein could stimulate pol V activity under conditions likely to preclude the formation of RecA nucleoprotein filaments. This suggested that RecA action in TLS might not involve the formation of a filament. Further, the RecA1730 protein appeared to promote pol V-mediated DNA synthesis up to, but not across, DNA lesions. This result suggested that RecA had two modes of action with pol V. We have since found that the RecA1730 protein used in the earlier study contained a minor contamination with pol III. As shown here, removal of the contamination yields RecA1730 that does not stimulate pol V under any conditions.

The goal of the present study is to define the role of RecA during SOS mutagenesis by examining pol V-RecA TLS and binding interactions in vitro. The result provides a new definition of a minimal mutasome (Echols, 1981) composed of pol V plus 2 RecA molecules; this appears to be the simplest protein complex enabling error-prone TLS.

Results

To explore the mechanisms governing TLS in *E. coli* and in particular the nature of pol V-RecA interactions during TLS, we have utilized wt RecA and three mutant RecA variants. These include two classical RecA mutants that have contrasting genetic phenotypes: RecA1730 (S117F), which is defective in pol V-dependent damage-induced mutagenesis, and RecA730 (E38K), which exhibits high levels of pol V-dependent spontaneous mutagenesis. Compared to wt RecA, RecA1730 and RecA730 bind to ssDNA with lower and higher affinities, respectively (Dutreix et al., 1992; Lavery and Kowalczykowski, 1992). We have extended these observations further by using a derivative of RecA730 lacking 17 C-terminal amino acids, RecA730-ΔC17 (Eggler et al., 2003), that exhibits an enhanced affinity for ssDNA. The templates employed were varied in length to encompass those long enough to support assembly of a RecA nucleoprotein filament downstream from the primer terminus as well as those too short to support RecA filament formation. The TLS experiments were performed in the presence of either ATP or its slowly hydrolysable analog ATPγS. Because RecA filaments are destabilized in the presence of ADP (Lee and Cox, 1990), an ATP regenerating system was used when ATP was present as a nucleotide cofactor.

Contrasting Effects of RecA1730 (S117F) and RecA730 (E38K) in Stimulating Pol V Activity

We measured RecA-dependent pol V synthesis taking place on a primer-template (p/t) DNA with a 6 nucleotide (nt) template overhang in the presence of either ATP (Figure 1A) or ATPγS (Figure 1B). Assembly of a typical RecA nucleotide filament limited to the region downstream of the primer terminus is almost certainly precluded on a 6 nt overhang because there is only enough room to bind two RecA monomers.

Wt RecA stimulates pol V synthesis on this DNA substrate in the presence of ATP or ATPγS (Figure 1). A larger enhancement in pol V stimulation occurs with ATPγS at low wt RecA levels compared to that observed with ATP (Figure 1). A small but detectable inhibition in DNA synthesis occurs at elevated levels of wt RecA in the presence of ATPγS (Figure 1B). As observed previously (Pham et al., 2002), the stimulation observed with wt RecA is significant but limited, with no more than ~35% of the available primers extended under any conditions.

RecA1730 fails to stimulate pol V-mediated DNA synthesis with either ATP (Figure 1A) or ATPγS (Figure 1B). Although RecA1730 shows impaired ability to assemble as filaments on ssDNA (Dutreix et al., 1992), this may be a moot point because a 6 nt template overhang is too short to support conventional filament assembly. Almost no polymerase activity was observed with ATP or ATPγS, even when it was replaced with a 21 nt overhang (Figure 1C). Therefore, the absence of pol V stimulation by RecA1730 may plausibly account for the absence of SOS mutagenesis in *recA1730* strains in vivo (Bailone et al., 1991; Dutreix et al., 1989).

RecA730 stimulates pol V activity much more effectively than wt RecA in the presence of ATP. Although RecA730 and wt RecA stimulate pol V to a similar extent with ATPγS at low RecA concentrations, the mutant protein shows a much stronger inhibition of synthesis at elevated RecA levels (Figure 1B).

The RecA730-ΔC17 mutant protein displaces SSB protein from ssDNA even better than RecA730 (Eggler et al., 2003) and potentially binds ssDNA more tightly than either wt RecA or RecA730. In the presence of ATP, this mutant stimulates pol V more effectively than any of the other RecA variants (Figure 1A). The effects of the RecA730-ΔC17 mutant saturate at 250 nM, roughly equimolar with pol V, and primer utilization increases to greater than 80%. Thus, the relatively low levels of primer utilization seen here (Figure 1) and in previous experiments with wt RecA (Pham et al., 2002; Tang et al., 1999) do not reflect inactive pol V. Instead, wt RecA protein does not provide optimal stimulation of pol V. A particularly strong inhibition of pol V activity occurs with increasing concentrations of RecA730-ΔC17 in the presence of ATPγS (Figure 1B). In the presence of ATP, however, low levels of the RecA730-ΔC17 mutant (~50 nM) strongly stimulate pol V (Figure 1A). The RecA730-ΔC17 mutant thus both stimulates and inhibits pol V activity much more avidly than wt RecA or either of the single mutants. This result demonstrates that pol V can be completely activated with no other protein present besides a RecA variant. The minimal complex active in pol V-mediated DNA synthesis thus includes UmuC, UmuD′, and RecA protein.

Translesion and Normal Pol V-Catalyzed DNA Synthesis Depends on Template Overhang Length

Using the 6 nt overhang with ATPγS, stimulation of pol V activity occurs at relatively low RecA levels, yet pol V is inhibited at higher RecA levels (Figure 1B). To obtain a better understanding of these two seemingly opposing phenomena, we measured pol V-catalyzed synthesis on p/t DNA substrates with template overhangs varying in 3 nt length increments, corresponding to the footprint of a RecA monomer binding to ssDNA. RecA filaments occur more readily on longer regions of ssDNA because of the cooperative binding property of RecA (Kuzminov, 1999).

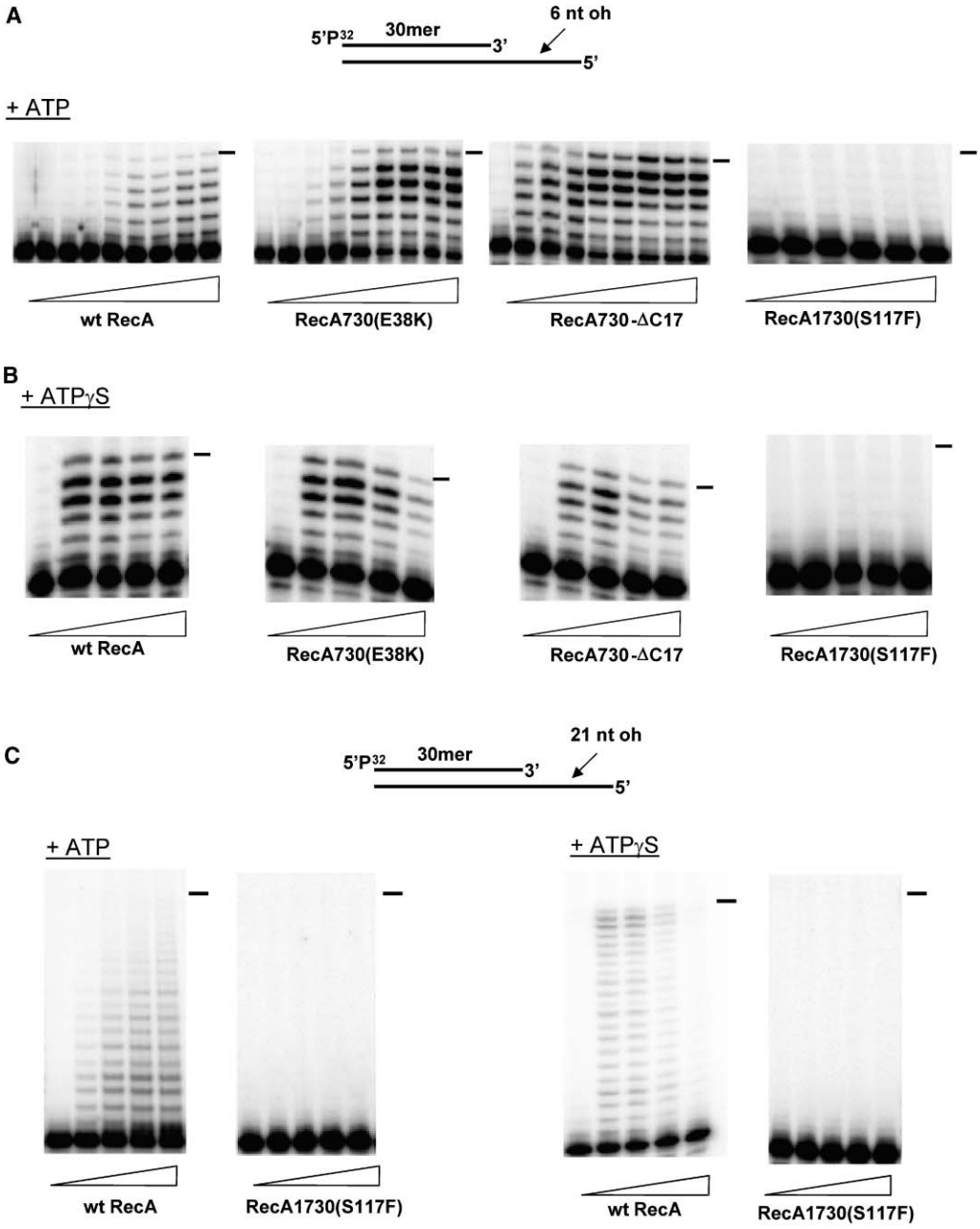

Figure 1. A Comparison of the Effects of Wt RecA and RecA Mutants on Pol V-Catalyzed DNA Synthesis

(A) Pol V-catalyzed synthesis is measured in the presence of continuously regenerated ATP. The protein concentrations used for wt RecA, RecA730 (E38K), and RecA730-ΔC17 were 0, 50, 100, 150, 250, 600, 1000, 2000, and 4000 nM, and the concentrations used for RecA1730 (S117F) were 0, 250, 600, 1000, 2000, and 4000 nM.

(B) Synthesis is measured in the presence of ATPγS. The concentrations used for wt and mutant RecA proteins were 0, 250, 600, 1000, and 2000 nM. The p/t DNA contains a 6 nt undamaged template overhang (oh) as shown in the sketch at the top.

(C) Synthesis is measured using a p/t DNA containing a 21 nt undamaged template oh with regenerated ATP (left gels) or ATPγS (right gels). Wt RecA and RecA1730 were present at 0, 250, 600, 1000, and 2000 nM. The dashed lines next to the gel images indicate the 5′ template ends.

Pol V was used to copy DNA that had template overhangs of different lengths and contained an abasic lesion (X) (Figure 2, left gels) or undamaged DNA (Figure 2, right gels). DNA synthesis was measured as a function of the concentration of wt or mutant RecA in the presence of continuously regenerated ATP (Figures 2A and 2C) or ATPγS (Figures 2B and 2D). Clear differences are observed when comparing the effects of ATP with ATPγS. When copying a 6 nt overhang containing an abasic site in the presence of ATP and wt RecA, pol V-catalyzed synthesis is strongly stimulated, but TLS fails to occur (Figure 2A). In contrast, TLS does occur with wt RecA plus ATPγS (Figure 2B). When copying 9 and 12 nt overhangs, TLS is observed with wt RecA in

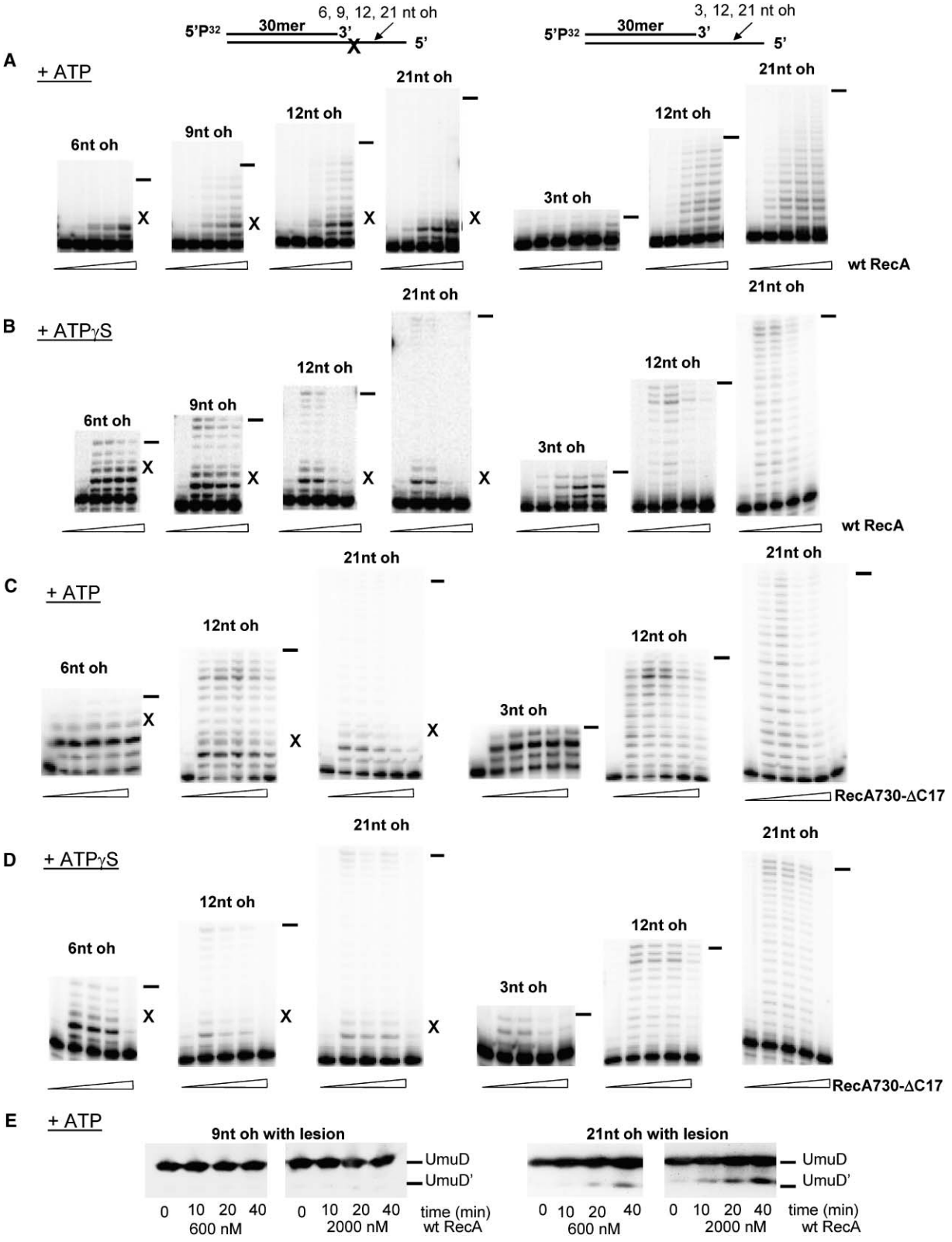

Figure 2. Pol V-Catalyzed DNA Synthesis and TLS Dependence on Template Overhang Length with Wt RecA and RecA730-ΔC17

Pol V-catalyzed TLS (left gels) and synthesis on undamaged DNA templates (right gels) were measured (A) in the presence of wt RecA with continuously regenerated ATP, (B) in the presence of wt RecA with ATPγS, (C) in the presence of RecA730-ΔC17 with continuously regenerated ATP, and (D) in the presence of RecA730-ΔC17 with ATPγS. RecA concentrations were 0, 250, 600, 1000, and 2000 nM. For the 3 nt normal template overhang with wt RecA plus regenerated ATP and for both normal and damaged template overhangs with RecA730-ΔC17 plus regenerated ATP, the highest protein concentration was 4000 nM. (E) RecA-filament-dependent UmuD to UmuD′ conversion is measured in the presence of continuously regenerated ATP and visualized with Western blotting. Reaction conditions were the same as for the primer-extension assays above. Damaged p/t DNA (20 nM) containing a template overhang was used for RecA filament formation at low (600 nM) and high (2000 nM) wt RecA concentrations.

the presence of ATP (Figure 2A). On the 21 nt template overhang, TLS is strongly inhibited as a function of increasing wt RecA and is abolished entirely at 4000 nM RecA (Figure 2A). In the presence of ATPγS, there is a marked inhibition of TLS at elevated levels of wt RecA (Figure 2B). For TLS in the presence of ATP, or for all reactions in the presence of ATPγS, the inhibition is enhanced when longer single-strand extensions are used (Figure 2).

Parallel experiments performed with the tight binding RecA730-ΔC17 mutant mirror the observations for wt RecA. However, stimulatory and inhibitory effects of the mutant RecA are stronger and occur at lower concentrations (Figure 2C and 2D). Whereas wt RecA stimulates pol V activity on undamaged DNA in the presence of ATP irrespective of RecA concentration (Figure 2A, right), the RecA730-ΔC17 mutant inhibits pol V activity at high levels, despite a stronger stimulation at low concentrations (Figure 2C, right). The tight binding RecA730-ΔC17 promotes measurable TLS in the presence of ATP on the 6 nt overhang, which had just 3 nt located downstream of the lesion (Figure 2C), whereas TLS does not occur with wt RecA under identical conditions (Figure 2A). The effects of wt RecA and RecA730-ΔC17 on pol V activity are virtually the same in the presence of ATPγS, on either damaged or undamaged DNA. Stimulation of pol V occurs at low RecA levels, and inhibition is observed at high RecA levels (Figures 2B and 2D). Notably, the RecA730-ΔC17 protein stimulates pol V more effectively with ATP than with ATPγS (Figures 2C and 2D), in contrast to what is seen with the wt RecA (Figures 2A and 2B).

Two independent measurements were made to verify that RecA filaments are absent on the 9 nt but present on the 21 nt overhang DNA substrates. First, we measured the autocatalytic cleavage of UmuD to form UmuD′ that was mediated by an activated RecA nucleoprotein filament (Burckhardt et al., 1988; Nohmi et al., 1988; Shinagawa et al., 1988). Cleavage was monitored through Western blotting with antibodies against UmuD/UmuD′ (Figure 2E). In the presence of the 9 nt overhang DNA substrate and regenerated ATP, no conversion of UmuD to UmuD′ was detected (Figure 2E, left), indicating the absence of a RecA filament. In contrast, the conversion UmuD → UmuD′ is clearly observed on the 21 nt overhang, and both the rate and extent of conversion are enhanced at higher wt RecA concentrations (Figure 2E, right). Second, we measured DNA-dependent ATP hydrolysis on the same 9 and 21 nt overhang substrates under conditions identical to those used to measure TLS and UmuD to UmuD′ conversion. Very little ATP hydrolysis occurred on the 9 nt overhang at wt RecA concentrations up to 4 μM, verifying the absence of RecA filament assembly, whereas significant hydrolysis was observed on the 21 nt overhang DNA: 1.8, 2.8, and 3.7 μM/min at levels of 1, 2, and 4 μM wt RecA, respectively. Based on the rate of hydrolysis at 4 μM wt RecA, we estimate that roughly 50% of the 21 nt overhang DNA is bound within RecA filaments.

Because no inhibition of TLS occurs at high wt RecA concentrations on the 9 nt overhang substrate (Figure 2A, 9 nt overhang), whereas a marked decrease in lesion bypass is observed on the 21 nt overhang (Figure 2A, 21 nt overhang), these data demonstrate that pol V-RecA-catalyzed TLS occurs in the absence of a RecA nucleoprotein filament but is inhibited in its presence.

Binding Affinities of Pol V, Wt, and Mutant RecA to a 6 nt DNA Template Overhang

We measured the affinities of mutant and wt RecA molecules to p/t DNA with a 6 nt template overhang and compared its binding to that with a ssDNA oligonucleotide (62-mer). Protein-DNA binding was observed as the change in steady-state rotational anisotropy (fluorescence depolarization) of a fluorescein dye molecule covalently attached near the 5′-end of a DNA strand (Figure 3). Because RecA730-ΔC17 exerts a stronger influence on pol V activity than either RecA730 (Figure 1) or RecA-ΔC17 (data not shown), the RecA730-ΔC17 mutant was used in the binding studies.

RecA730-ΔC17 binds to the 6 nt overhang p/t DNA in the presence of ATPγS with 4-fold greater affinity (apparent $K_d \sim 500$ nM) than wt RecA (apparent $K_d \sim 2200$ nM), whereas RecA1730 shows the weakest binding (apparent $K_d \sim 3000$ nM) (Figure 3A). RecA730-ΔC17 and wt RecA bind with a (2- and 5-fold) higher affinity to a 62 nt ssDNA, respectively, while there is only a small change in binding with RecA1730 (Figure 3A, inset). RecA730-ΔC17 and wt RecA bind to both the short overhang and long ssDNA even in the absence of a nucleotide cofactor, albeit with reduced affinities; RecA1730 shows no detectible binding to ssDNA in the absence of ATPγS (Figure 3B). There is thus a rough correlation between the capacity of the RecA variants to stimulate pol V and their binding to DNA. However, for a given RecA variant, DNA binding and pol V stimulation do not correlate well. For the RecA730-ΔC17 mutant, pol V stimulation saturates at RecA concentrations far below those needed to saturate the DNA binding reaction. For RecA1730, the limited DNA binding observed does not translate into any measurable activation of pol V.

In the absence of RecA, pol V binds equally well to the p/t DNA (apparent $K_d \sim 400 - 500$ nM) either with or without ATPγS (Figure 3C). Pol V binds to the short ssDNA overhang most likely through its positively charged UmuC subunit (Bruck et al., 1996). The negatively charged UmuD′ subunit shows no measurable binding to p/t DNA (Figure 3C).

Taken together, the DNA synthesis (Figures 1 and 2) and DNA binding (Figure 3) data crystallize two major observations. First, the inability of the RecA1730 mutant to stimulate pol V synthesis on damaged or undamaged DNA suggests that the role of RecA during TLS is to activate pol V, irrespective of either the presence or absence of a template lesion. Second, the assembly of RecA filaments can inhibit pol V-catalyzed TLS even under physiologically relevant, dynamic ATP conditions. The inhibition of pol V cannot be attributed solely to the presence of a filament blocking access of pol V to a 3′-primer end. Although pol V-catalyzed TLS is often inhibited (see data for a 6 nt overhang with ATPγS [Figure 2B] and a 21 nt overhang with ATP [Figure 2A]), synthesis prior to the lesion is not impeded and is actually stimulated. Thus, the assembly of a RecA nucleoprotein filament proximal to a DNA damage site is likely to hinder, perhaps even abrogate, pol V-catalyzed TLS and is therefore unlikely to serve as a requirement for TLS.

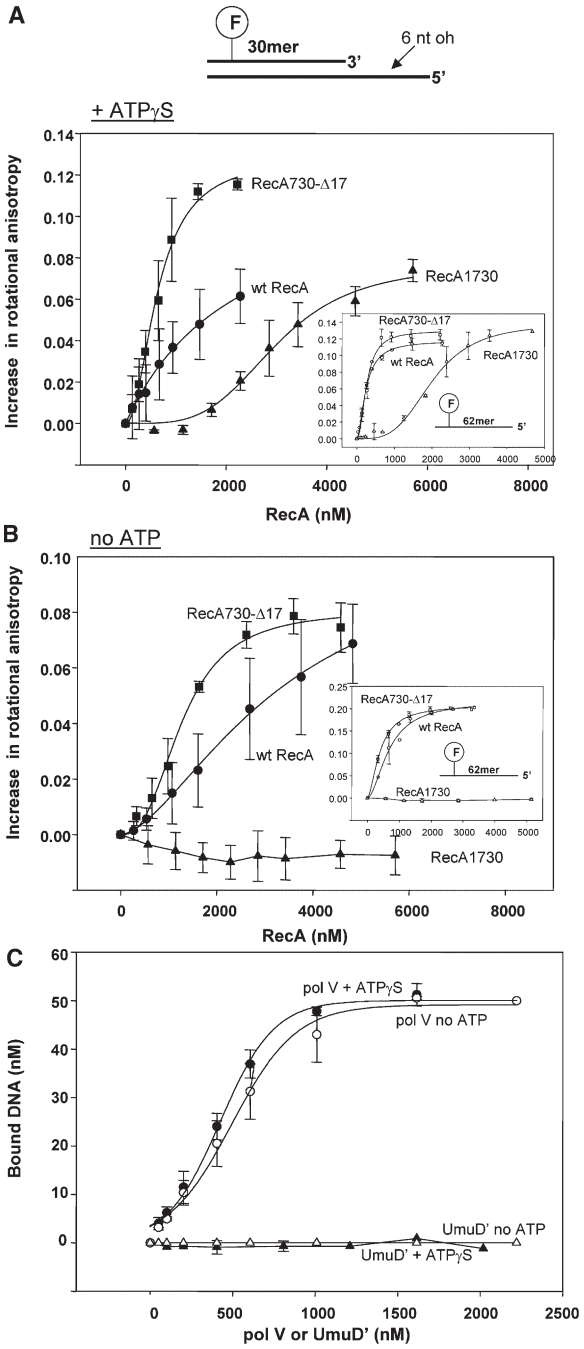

Figure 3. Binding of RecA, Pol V, and UmuD′ to DNA by Using Steady-State Rotational Anisotropy

The binding of RecA to DNA was measured as a function of protein concentration. The p/t DNA was composed of a fluorescein-labeled 30-mer primer strand annealed to a 36-mer template strand.

(A) RecA-DNA binding affinities measured in the presence of ATPγS were wt RecA (K_{app} = 2.2 μM) (●), RecA730-ΔC17 (K_{app} = 0.5 μM) (■), and RecA1730 (S117F) (K_{app} = 3 μM) (▲), and all were fit to a Hill equation. Inset, RecA binding affinities to ssDNA (62-mer) were measured with wt RecA (K_{app} = 0.4 μM) (○), RecA730-ΔC17 (K_{app} = 0.26 μM) (□), and RecA1730 (K_{app} = 2 μM) (Δ). Error bars represent 1 SEM.

(B) RecA-DNA binding affinities to a p/t DNA were measured in the absence of nucleotide cofactor with wt RecA (K_{app} = 3.3 μM) (●), RecA730-ΔC17 (K_{app} = 1.27 μM) (■), and RecA1730 (S117F) (▲). Inset, the RecA binding affinities to ssDNA (62-mer) were measured with wt RecA (K_{app} = 0.68 μM) (○), RecA730-ΔC17 (K_{app} = 0.4 μM) (□), and RecA1730 (S117F) (Δ). Error bars represent 1 SEM.

(C) Pol V-DNA binding affinities to p/t DNA were K_{app} = 0.43 μM (●) in the presence of ATPγS and K_{app} = 0.5 μM (○) in the absence of a nucleotide cofactor. UmuD′ in the absence (Δ) or presence (▲) of ATPγS does not interact with p/t DNA. See Experimental Procedures for reaction conditions and binding affinity calculations.

RecA Binds to Pol V in the Absence of DNA or ATP

Wt RecA and RecA1730 were each labeled with fluorescein to investigate their interactions with pol V ($UmuD'_2C$) in solution in the absence of DNA. RecA ± ATPγS binds to pol V with an apparent K_d ~200 nM (Figure 4A). As a negative control, we used pol IV, which does not interact with RecA (Figure 4A). Based on the inability of $UmuD'_2$ to bind RecA (Figure 4A), we suggest that the UmuC subunit is responsible for interacting with RecA in the absence of DNA and ATP. However, the insolubility of free UmuC (Bruck et al., 1996; Woodgate et al., 1989) precludes a direct measurement of its interaction with RecA in the absence of $UmuD'_2$. Therefore, we cannot exclude the possibility that a conformational change in pol V enables binding to RecA via either one or both subunits.

RecA1730 was unable to stimulate pol V activity (Figure 1), and a simple explanation might be a defect in the ability of the two to interact. However, RecA1730 binds to pol V with about the same affinity as wild-type RecA (K_d ~150 to 250 nM) in the absence of DNA ± ATPγS (Figure 4B).

To confirm the DNA-independent interaction between pol V and RecA, we also conducted scanning force microscopy (SFM) studies (Figure 4C). RecA alone (panel I) as well as pol V alone (panel II) showed compounds with volumes roughly corresponding to their respective molecular weights of 38 kDa for RecA and 72 kDa for pol V. For both pol V and RecA, the total number of molecules examined was approximately 4000. RecA added to pol V (panel III) resulted in the formation of particles with a larger average volume than seen with either protein alone. Two distinct peaks are observed in the volume distribution of these data at 70 and 136 nm^3. It should be noted that neither pol V nor RecA is resolved as separate peaks. Thus, the peak at 72.0 nm most likely represents the overlapping distribution for each protein separately, whereas the new peak at about 136 nm^3 corresponds to a pol V-RecA complex. The remaining minor population of molecules falls in a broad distribution of larger sizes likely corresponding to aggregates also present in the pol V-alone distribution. The integrated area under the curve that has a peak at 136 nm^3 and corresponds to pol V-RecA complexes accounts for approximately 40% of all protein molecules observed via SFM.

RecA Facilitates the Binding of Pol V to DNA via the $UmuD'_2$ Subunit of Pol V

To address RecA-pol V interactions in the presence of DNA, we measured the change in rotational anisotropy of a fluorescein-labeled p/t DNA with a 6 nt overhang. Wild-type RecA, RecA1730, and RecA730-ΔC17 were used at concentrations in which each RecA alone had

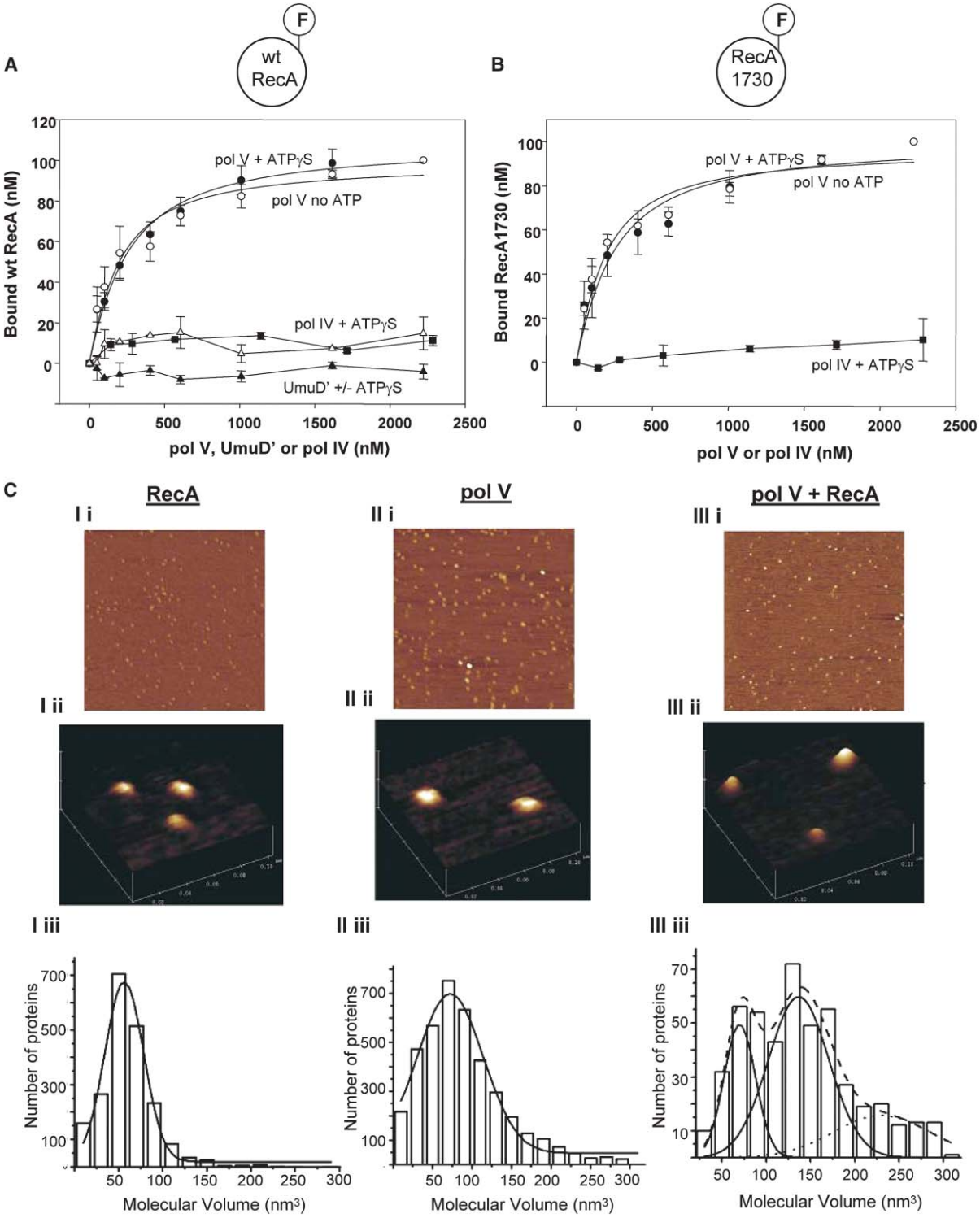

Figure 4. Binding of RecA to Pol V in the Absence of DNA

(A) Binding of wt RecA, labeled at its N-terminal with fluorescein, to pol V, UmuD′, or pol IV was measured as a change in steady-state rotational anisotropy. Binding affinities were determined for pol V plus ATPγS (●) (K_d = 197 nM) and pol V without nucleotide cofactor (O) (K_d = 147 nM). Pol IV (■) and UmuD′ (▲) do not bind to wt RecA either in the presence or absence ATPγS. Error bars represent 1 SEM.

(B) Binding of fluorescein-labeled RecA1730 to pol V or pol IV. Binding affinity for pol V plus ATPγS was [K_d = 195 nM] (●) and for pol V without nucleotide cofactor binding was (K_d = 142 nM) (O). Pol IV (■) does not bind to RecA1730. Error bars represent 1 SEM.

(C) SFM images of RecA, pol V, and pol V-RecA are shown. The scan field for I i, II i, and III i was 1 μm × 1 μm, and it was 100 nm × 100 nm for the three-dimensional projections in I ii, II ii, and III ii. In all SFM images, color indicates height from 0 to 1.5 nm, dark to bright. RecA is shown in I i and I ii, pol V is shown in II i and II ii, and pol V-RecA is shown in III i and III ii. The volume distributions for RecA (I iii), pol V (II iii), and pol V-RecA (III iii) are presented. The x axis is the molecular volume obtained from the SFM data whereas the y axis is the number of protein molecules in each peak. A Gaussian distribution was calculated for the data and is displayed as a solid black line. For III iii, the distinct peaks from the Gaussian function are displayed as solid black lines while the distribution for all peaks are displayed as dashed lines.

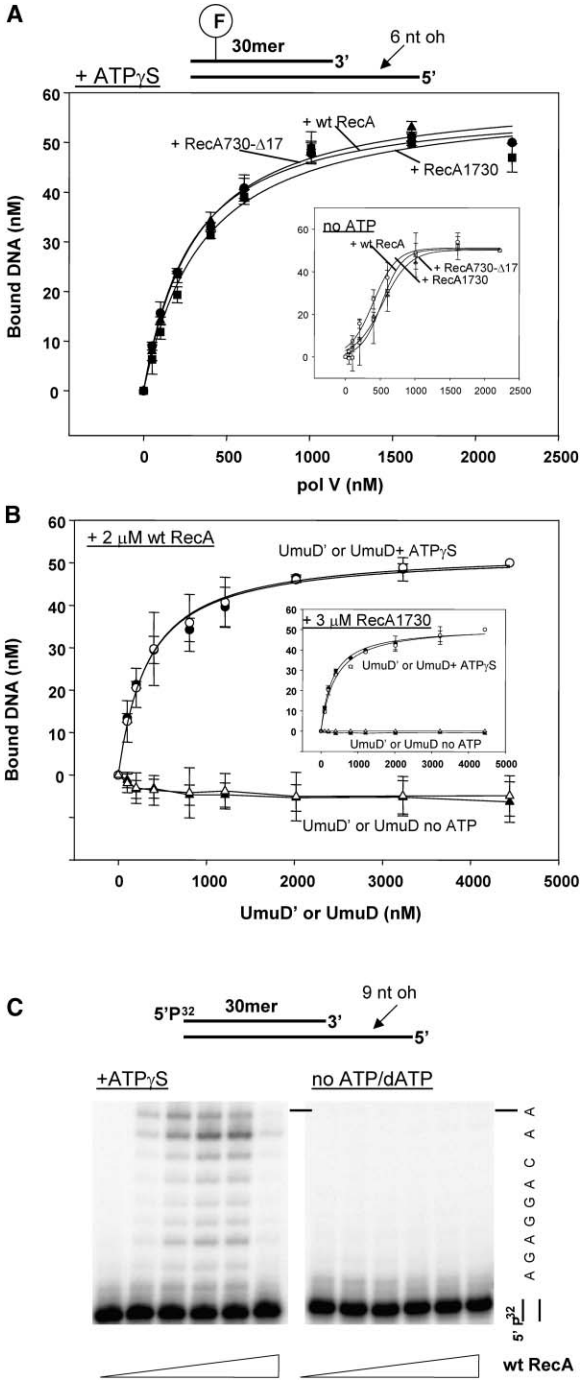

Figure 5. RecA Binds to Pol V in the Presence of DNA and ATP via the UmuD′ Subunit

(A) Binding of pol V to wt RecA, RecA1730, or RecA730-ΔC17 in the presence of fluorescein-labeled p/t DNA plus ATPγS. Pol V binding affinities were measured in the presence of 2 μM wt RecA (K_d = 245 nM) (●), 3 μM RecA1730 (K_d = 268 nM) (▲), and 1 μM RecA730-ΔC17 (K_d = 314 nM) (■). The different concentrations for various RecA were chosen to correct for their respective binding affinities and represent the concentration in which each RecA alone had about a 50% fraction of total DNA bound. Inset: the same pol V binding measurements were obtained in the absence of nucleotide cofactor and in the presence of 2 μM wt RecA (K_{app} = 0.53 μM) (○), 3 μM RecA1730 (K_{app} = 0.52 μM) (△), or 1 μM RecA730-ΔC17 (K_{app} = 0.42 μM) (□). Error bars represent 1 SEM.

(B) Binding of UmuD′ or UmuD to wt RecA in the presence of p/t DNA; UmuD′ (K_d = 300 nM) (●) and UmuD (K_d = 305 nM) (○) both bind to wt RecA (2 μM) in the presence of ATPγS. UmuD′ (▲) and UmuD (△) do not bind to wt RecA in the absence of nucleotide cofactor. Inset: binding of UmuD′ or UmuD to RecA1730 in the presence of p/t DNA; UmuD′ (K_d = 289 nM) (●) and UmuD (K_d = 366nM) (○) bind to RecA1730 (3 μM) in the presence of ATPγS while UmuD′ (▲) and UmuD (△) do not bind to RecA1730 in the absence of nucleotide cofactor. Error bars represent 1 SEM.

(C) Pol V-catalyzed DNA synthesis requires an ATP nucleotide cofactor. DNA synthesis was carried out using 5′-^{32}P-labeled 30-mer primer annealed to a template containing no dT in the overhang region. Wt RecA concentrations were 0, 250, 600, 1000, 2000, and 4000 nM.

about a 50% fraction of total DNA bound. The fluorescein-labeled p/t DNA was preincubated with RecA and ATPγS, and binding curves were obtained as a function of pol V concentration (Figure 5A). The pol V-DNA binding curves, which were sigmoidal in the absence of RecA (Figure 3C), fit instead to a rectangular hyperbola in the presence of RecA with about a 2-fold reduction in K_d ~245 nM (Figure 5A). The affinity of pol V to p/t DNA is the same regardless of which RecA was used in the experiment, as long as the RecA concentrations were adjusted to correct for their different DNA binding affinities (Figure 5A).

RecA-mediated stimulation in pol V binding to DNA requires a nucleotide cofactor, ATPγS. In the absence of ATPγS, the pol V binding curves revert to a sigmoidal form (Figure 5A inset) with virtually no change in apparent K_d compared to that in the absence of RecA (Figure 3C). To investigate which components of this multiple protein system are required to stimulate pol V binding to DNA, the binding of UmuD′ to DNA was measured in the presence of wt RecA (Figure 5B) and RecA1730 (Figure 5B inset). UmuD′ (Figure 5B) or pol V (Figure 5A) show a virtual coincidence in the RecA-stimulated binding to DNA, irrespective of which RecA is present, with a binding constant of about 200 nM. Whereas pol V interacts with p/t DNA in the absence of RecA or ATPγS, UmuD′ requires the presence of both RecA and nucleotide cofactor to allow it to bind to DNA (Figure 5B).

Combining the results of Figures 4 and 5, we have elucidated two distinct interactions between RecA and pol V. First, RecA binds to pol V in the absence of DNA and ATP, and then a second RecA binds to UmuD′ in a DNA and nucleotide cofactor-dependent manner. The very same interactions, with the same apparent affinities, are observed for RecA1730 (Figures 4B and 5A, and Figure 5B, inset). Thus, the measured RecA-pol V interactions seem to be necessary but are themselves insufficient for stimulation of pol V-catalyzed synthesis and TLS, because RecA1730 fails to stimulate pol V activity (Figure 1).

Stimulation of RecA-Mediated Pol V Synthesis Depends on ATP, but Not ATP Hydrolysis

We compared ATP hydrolysis rates (Table 1) for RecA1730 with wt RecA and RecA730-ΔC17 in the presence of p/t DNA used in the pol V synthesis assays with 6 and 21 nt template overhangs (Figures 1 and 2). As anticipated, there is a direct correlation between DNA-dependent ATPase activity and the capacity of RecA to bind to DNA (Table 1). Thus, RecA730-ΔC17 hydrolyzed

Table 1. ATPase Activity of RecA Mutants, Wt RecA, and/or pol V on DNA with Short Template DNA Overhangs

	p/t 6 nt oh[a] (%/min ATP→ADP)	p/t 21 nt oh[b] (%/min ATP→ADP)
Wt RecA	0.20	1.20
Pol V	0.01	0.03
Wt RecA plus pol V	0.20	1.20
RecA730-Δ17	1.10	2.20
RecA1730	0.02	0.20

Reactions contained ATP (1 mM), ATPα^{32}P (3000 Ci/mMol), RecA (1 μM), and/or pol V (240 nM) and a p/t DNA (20 nM) containing either a 6[a] or 21[b] nt template oh. ATP hydrolysis was measured as a function of time as described in the Experimental Procedures.

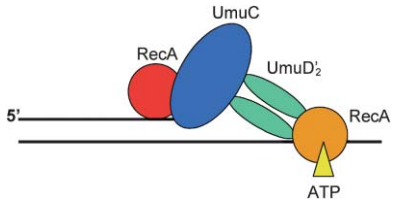

Figure 6. A Minimal Model Describing Pol V-Catalyzed TLS Involving Two RecA Binding Modes

Pol V binds to RecA most likely via its UmuC subunit in the absence of DNA and ATP and, in a separate reaction, binds to RecA via its UmuD′ subunit, requiring the presence of DNA and ATP. The two pol V-RecA binding reactions are the minimal requirements for TLS. Pol V-catalyzed TLS does not require RecA nucleoprotein filament assembly, β/γ processivity complex, or SSB.

ATP most avidly, whereas wt RecA ATP hydrolysis was about 2- to 5-fold slower for each DNA substrate. Hydrolysis by RecA1730 was 6- to 10-fold slower than wt RecA (Table 1). In each case, the ATP hydrolytic reactions probably reflect the total amount of RecA bound to DNA. The deficiency in the case of RecA1730 is probably a reflection of its relatively weak binding to the DNA cofactor. The presence of pol V has no measurable effect on ATP hydrolysis.

Since dATP is present as a pol V substrate in all of the previous DNA synthesis reactions (Figures 1 and 2), it was important to show that synthesis did not occur in the absence of ATP. No measurable pol V-catalyzed synthesis was observed in the absence of ATP or dATP using p/t DNA lacking T on the ssDNA overhang (Figure 5C, right). In contrast, synthesis in the absence of dATP occurs with ATPγS (Figure 5C, left). Thus, an active RecA species, which is a RecA plus ATP bound to ssDNA, is an essential requirement for stimulation of pol V synthesis. ATP hydrolysis is not required.

Discussion

In this study, we define the minimal mutasome (Echols, 1981), which is the simplest protein complex required to promote pol V-mediated DNA synthesis and error-prone TLS. We also define a model for the role of RecA in pol V function, in which this minimal complex has center stage. First, the minimal mutasome is composed of UmuD′$_2$C (pol V) plus two RecA protein molecules, where pol V is almost entirely dependent on RecA and the enhancement by RecA requires an ATP nucleotide cofactor. Second, RecA interacts with pol V in two distinct ways but is not dependent on the formation of a RecA filament. Third, the enigmatic role of RecA in TLS is not simply to target pol V to a stalled fork. Instead, RecA directly activates pol V, possibly as a subunit of the active pol V holoenzyme complex. The interactions between RecA and pol V are clearly important in characterizing this active complex, but the binding interactions are not by themselves sufficient for activation. These ideas are summarized in the model depicted in Figure 6, and we expand on the elements of this model below.

The Minimal Functional Pol V Complex Includes RecA and UmuD′$_2$C

In the case of SOS-induced mutagenesis in *E. coli*, in vivo data show that pol V, RecA, and a β/γ processivity complex are needed (Friedberg et al., 1995), and SSB may also be involved (Echols and Goodman, 1990; Goodman, 2002). On the other hand, biochemical data have revealed that pol V and RecA may be the only components needed to copy damaged template sites per se (Figure 2) (Pham et al., 2002). The primary function of the β clamp might be to coordinate exchange between replicative pol III and the three error-prone *E. coli* polymerases (Becherel et al., 2002; Goodman, 2002; Lenne-Samuel et al., 2002; Napolitano et al., 2000). Although β might facilitate TLS by tethering pol V to DNA, its presence is not required for TLS (Pham et al., 2002), whereas pol V cannot copy damaged DNA in the absence of RecA (Fujii et al., 2004; Pham et al., 2001, 2002; Reuven et al., 1998; Tang et al., 1998). The capacity of the RecA730-ΔC17 double mutant to promote the extension of at least 80% of the p/t DNA by pol V reveals the intrinsically robust capacity of RecA to activate pol V. The pol V activation function of wt RecA is suppressed, to some degree, by the RecA C terminus, as is the case for other RecA activities (Eggler et al., 2003; Lusetti et al., 2003). In contrast to the effects of RecA730-ΔC17, the RecA1730 protein appears deficient in activating pol V (Figure 1). This result underscores the central importance of RecA protein to pol V function.

There Are Two Distinct Types of Interaction between Nonfilamentous RecA and the UmuD′$_2$C Complex

RecA and pol V form a stably bound complex in the absence of DNA, with roughly similar affinities (150 − 200 nM) either with or without ATPγS (Figure 4A). The binding of pol V to RecA was measured in aqueous solution under conditions comparable to those used to measure DNA synthesis. We refer to the formation of a pol V-RecA complex in the absence of DNA and ATP as mode 1 binding. The formation of a complex between RecA and pol V in the absence of DNA was verified independently using SFM (Figure 4C). Although it seems likely that RecA interacts with UmuC during mode 1 binding based on the indirect evidence that UmuD′ does not bind to RecA in the absence of DNA (Figure 4A), we cannot verify this point directly because UmuC is insoluble in aqueous solution in the absence of UmuD′ (Bruck et al., 1996; Woodgate et al., 1989). Thus, it is possible that the RecA mode 1 binding also involves an interaction with the UmuD′ subunit following a conformational change in pol V.

A separate mode of binding between pol V and RecA

requires the presence of DNA and ATP (Figures 5A and 5B). The binding affinity of pol V to p/t DNA increases with wt RecA present (Figure 5A). The binding of either mutagenically active UmuD′ or inactive UmuD to p/t DNA in the presence of RecA and ATPγS (Figure 5B) is similar to the binding of pol V to DNA (Figure 5A). Thus, a second binding mode of pol V with RecA has been revealed, one that involves UmuD′ interacting with RecA and that requires DNA plus a nucleotide cofactor (ATPγS) (Figure 5B). Recall that UmuD′ does not bind to DNA in the absence of RecA (Figure 3C). Thus, the need to have a nucleotide cofactor for RecA-UmuD′ binding, i.e., mode 2 binding, presumably explains why pol V-catalyzed DNA synthesis fails to occur in the absence of ATP (or ATPγS) (Figure 5C).

Neither mode of binding RecA to pol V involves assembly of a RecA nucleoprotein filament. Optimal activation of pol V occurs at low RecA concentrations, especially with the RecA730-ΔC17 mutant in which optimal activation occurs with 1:1 RecA, pol V stoichiometry. Stimulation occurs with p/t substrates lacking sufficient single-stranded DNA downstream of the primer terminus for filaments to form, and conditions that promote the formation of persistent RecA filaments uniformly inhibit pol V function (Figure 2E). Conversely, TLS clearly occurs when nucleoprotein filaments are absent (Figure 2E). We suggest that RecA filaments are not required for pol V-mediated TLS, and indeed their presence can be detrimental.

RecA Protein Activates Pol V Function

The proposed role for RecA in TLS is genetically separate from its roles during homologous recombination, SOS induction, and UmuD cleavage (Dutreix et al., 1989; Sweasy et al., 1990). We have shown that its role in TLS involves a direct activation of pol V. If RecA does not function as a filament in this capacity, then it is logical to hypothesize that RecA is functioning as an integral subunit of the active pol V complex. A simple binding interaction of RecA with UmuD′$_2$C is insufficient for this RecA function. RecA1730 appears to interact with pol V with all the facility of the wt RecA yet does not activate pol V. The wt and RecA730-ΔC17 proteins thus do something that RecA1730 cannot do, perhaps triggering a conformational change in pol V required for optimal activity.

In summary, the model of Figure 6 provides a construct within which to explore the function of pol V. Based on the two distinct interactions we have characterized between pol V and RecA, the parsimonious interpretation is that there are two RecA monomers involved in the activated pol V complex. However, additional studies will be needed to define the precise molecular composition of active pol V. Our model introduces salient new issues involving regulation of RecA activities and the mechanism of RecA-mediated activation of pol V.

Experimental Procedures

Enzymes and Buffers

ATP and ultrapure dNTPs were purchased from Amersham Pharmacia Biotech. Adenosine 5′[γ-thio]triphosphate (ATPγS) was purchased from Roche. Creatine Phosphokinase from rabbit muscle and phosphocreatine disodium salt were purchased from Sigma-Aldrich. A Fluorescein-EX Protein Labeling Kit was purchased from Molecular Probes. ATP-γ^{32}P (4500 Ci/mmol) for 5′-primer labeling and ATP-α^{32}P (3000 Ci/mmol) for ATP hydrolysis assays were purchased from ICN Biomedicals. Fluorescein dT for synthesis of fluorescein-labeled oligomers and dSpacer for synthesis of templates containing an abasic (tetrahydrofuran) lesion were purchased from Glen Research.

All assays were conducted at 37°C. The standard reaction buffer (R buffer) contained 20 mM Tris (pH 7.5), 8 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM EDTA, 25 mM sodium glutamate, and 4% (vol/vol) glycerol. Pol V (Bruck et al., 1996; Tang et al., 2000), pol IV (Kobayashi et al., 2002), $UmuD_2$ and $UmuD'_2$ (Frank et al., 1993; McDonald et al., 1998; Woodgate et al., 1989), wt RecA (Cox et al., 1981), RecA730(E38K), RecAC17, and RecA730-ΔC17 (Eggler et al., 2003; Lusetti et al., 2003) were purified as previously described. RecA1730(S117F) was overproduced in *E. coli* RW628 (*ΔumuDC595::cat, srl300:Tn10 ΔrecA306*) strain and was purified by a modification of a procedure previously described (Cox et al., 1981). After phosphocellulose chromatography (P-11, Whatman), fractions having the lowest levels of contaminating nuclease and DNA polymerase activities were combined. These were further fractionated (another P-11 column plus gel filtration on Superdex-75 HiLoad 26/60 gel; Amersham Biosciences). Fractions were assayed for polymerase, nuclease, and RecA-promoted strand-exchange activities. Only fractions with no detectable polymerase, <0.5% nuclease, and robust strand-exchange activity were combined.

Pol V Primer Extension Assays

Reaction mixtures (10 μl) contained standard reaction buffer and 20 nM p/t DNA constructed by annealing a 5′-^{32}P-labeled 30-mer primer to ssDNA template strands either with or without a synthetic abasic lesion. The template strand varied in length, as illustrated in the figures. The reaction mixtures were preincubated for 3 min with RecA and ATPγS (0.5 mM) or RecA, ATP (1 mM), phosphocreatine (3 mM), and creatine phosphokinase (1 unit/reaction), as indicated in the figures. The reactions were initiated by addition of pol V (240 nM) and dNTP substrates (0.5 mM) and terminated after 10 min by adding 20 μl of 20 mM EDTA in 95% formamide. The extended DNA products were separated on a 12% polyacrylamide denaturing gel. Gel band intensities were measured by phosphorimaging with IMAGEQUANT software (Molecular Dynamics).

UmuD to UmuD′ Conversion Assay

Standard reaction mixtures (50 μl) with 20 nM of unlabeled p/t DNA containing 9 or 21 nt template overhangs with a lesion were preincubated for 5 min at 37°C with RecA, ATP (1 mM), phosphocreatine (3 mM), and creatine phosphokinase (1unit/reaction). After the addition of 20 μg UmuD, a 10 μl aliquot was removed at time points 0, 10, 20, and 40 min and put into SDS-loading dye. The products were separated on a 12% SDS-protein gel and visualized with Western blotting by using antibodies against UmuD/UmuD′.

Steady-State Rotational Anisotropy Measurements

Protein-DNA and protein-protein binding measurements were performed by measuring changes in steady-state fluorescence depolarization (rotational anisotropy) (Lakowicz, 1983) essentially as described previously (Bertram et al., 2004). A fluorescein probe (F) was used to label either a 62-mer ssDNA strand or a 30-mer primer. F-wt RecA and F-RecA1730 (S117F) was N terminally labeled (primary amine) by using a Fluorescein-EX Protein Labeling Kit (Molecular Probes). The fluorescence depolarization measurements were performed at an excitation wavelength of 495 nM and an emission wavelength of 520 nM. All experiments were repeated two to four times. Reaction mixtures (70 μl) contained standard reaction buffer, 50 nM of the DNA substrate or 100 nM labeled protein, and 1 mM ATPγS when present as indicated in figures. The G-factor was determined, and the rotational anisotropy was calculated (Lakowicz, 1983). The anisotropy of free DNA was subtracted from each plot. Plots were fit to sigmoid or to Hill equations by using Sigmaplot software for apparent K_d. K_d values were determined as described previously (Bertram et al., 2004).

ATP-Hydrolysis Assays

Reaction mixtures (50 μl) contained standard reaction buffer, 1 mM ATP, 0.3 μM ATPα^{32}P, and 20 nM of unlabeled p/t DNA containing 6 or 21 nt template overhang regions. The reactions were initiated by adding 1 μM wt RecA, 1 μM RecA1730 (S117F), 1 μM RecA730-ΔC17, 1 μM RecAE96D, 240 nM pol V, or 1 μM wt RecA plus 240 nM pol V. Aliquots (5 μl) were removed from the reactions at t = 0, 1, 2, 10, and 30 min. The reactions were terminated by addition of 5 μl 90% formic acid. The reaction products were separated by using thin layer chromatography. The conversion of ATP to ADP plus inorganic phosphate was detected on a PhosphorImager and quantitated with IMAGEQUANT software (Molecular Dynamics). ATP hydrolysis was also measured by using a pyruvate-kinase/lactate dehydrogenase system as described previously (Drees et al., 2004).

SFM Measurements of Pol V-RecA Binding

For the imaging of pol V and RecA, each protein (7.1 μM) was diluted in a 2:1 (v/v) mixture of deposition buffer (10 mM HEPES [pH 7.5] and 10 mM $MgCl_2$) and pol V dilution buffer (20 mM HEPES [pH 7.5], 5 mM dithiothreitol, 1mM EDTA, 100 mM sodium glutamate, and 4% glycerol). For the imaging of pol V-RecA complexes, the proteins were mixed in a 1:1 molar ratio (3.5 μM) and left to equilibrate on ice for 10 min. The reactions were then diluted in a 2:1 mixture (v/v) of pol V dilution buffer and deposition buffer. To obtain the proper surface coverage on mica, the reactions were diluted as described (Ratcliff and Erie, 2001). For all reactions, the diluted protein solutions were added to freshly cleaved mica, left for 30–60 s, washed in HPLC-grade water (Sigma-Aldrich), and dried in air. SFM imaging was conducted on a Digital Instruments Nanoscope IV in non-contact tapping mode at a scan frequency of 2 kHz and a scan size of 1 μM. Volume analysis of the data was carried out as described (Ratcliff and Erie, 2001).

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