Characterization of the ATPase Activity of RecG and RuvAB Proteins on Model Fork Structures Reveals Insight into Stalled DNA Replication Fork Repair

RecG and RuvAB are proposed to act at stalled DNA replication forks to facilitate replication restart. To clarify the roles of these proteins in fork regression, we used a coupled spectrophotometric ATPase assay to determine how these helicases act on two groups of model fork substrates: the first group mimics nascent stalled forks, whereas the second mimics regressed fork structures. The results show that RecG is active on the substrates in group 1, whereas these are poor substrates for RuvAB. In addition, in the presence of group 1 forks, the single-stranded DNA-binding protein (SSB) enhances the activity of RecG and enables it to compete with excess RuvA. In contrast, SSB inhibits the activity of RuvAB on these substrates. Results also show that the preferred regressed fork substrate for RuvAB is a Holliday junction, not a forked DNA. The active form of the enzyme on the Holliday junction contains a single RuvA tetramer. In contrast, although the enzyme is active on a regressed fork structure, RuvB loading by a single RuvA tetramer is impaired, and full activity requires the cooperative binding of two forked DNA substrate molecules. Collectively, the data support a model where RecG is responsible for stalled DNA replication fork regression. SSB ensures that if the nascent fork has single-stranded DNA character RuvAB is inhibited, whereas the activity of RecG is preferentially enhanced. Only once the fork has been regressed and the DNA is relaxed can RuvAB bind to a RecG-extruded Holliday junction.

Genome duplication is inherently accurate, is highly processive, and relies on the close interplay between the genetic recombination and DNA repair machinery (1–3). The need for this interplay arises because the replication machinery frequently encounters roadblocks that have the potential to stall or collapse a replication fork (4–6). The types of lesions that could disrupt replication include proteins bound to the DNA ahead of the replication fork, non-coding lesions in the template DNA, and either single or double strand breaks (3, 7, 8). Each of the different blocks could lead to a different type of damage to the DNA, and this is highlighted by the varied recombination and repair gene requirements for dealing with exposure to different types of DNA-damaging agents (8–11). Whatever its source, the block has to be removed or bypassed, and replication must be restarted.

In bacteria, stalled replication forks can be reversed (regressed) or directly restarted (8, 9, 11, 12). Although replication fork regression can in principle be spontaneous (13), it can also be catalyzed by a number of proteins (4, 14–16). Over the past several years, two branched DNA-specific molecular motors known as RecG and RuvAB have emerged as potential key players in the regression of stalled replication forks (4, 17).

Separate biochemical analyses of RecG and RuvAB demonstrated that each enzyme can bind to and process a variety of forked DNA substrates and recombination intermediates (14, 18–23). RecG is a 76-kDa monomeric enzyme that is capable of acting upon a variety of three- and four-way structures (17, 18, 24). Although it can act during branch migration to resolve Holliday junctions, its primary role may instead be to regress or reverse stalled replication forks (2, 18, 25–27). The RuvAB motor is composed of two non-identical subunits encoded by the ruvA and ruvB genes (28). The active branch migration complex is at least 535 kDa in size and consists of at least a tetramer of RuvA protein that binds one face of the Holliday junction and two homohexameric rings of RuvB that function as chemomechanical motors to drive branch migration (29–33). There are studies that suggest that the active complex contains a RuvA octamer with the Holliday junction sandwiched in between, but this remains an open question (34, 35).

The Holliday junction resolution complex forms when a RuvC dimer (responsible for Holliday junction cleavage at the crossover point) associates with RuvAB (23, 36, 37). Although RuvABC can function in replication fork processing, its pri-
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may role might instead be to perform the branch migration stage of recombination (38). Furthermore, it is clear that both RecG and RuvAB can act on stalled replication fork substrates, producing suitable substrates for RuvC cleavage (10, 39). However, there is an important difference as RuvAB cannot unwind forked DNA to form a Holliday junction, whereas RecG is able to do so (14, 40).

Although the above mentioned studies show that each of these proteins can in principle function in DNA repair, having the capacity to act on model forked DNA substrates, they do not delineate how each may access a nascent, stalled replication fork, leading to its resurrection. To understand this, we performed a careful kinetic analysis of each enzyme in the presence of two groups of forked DNA substrates: the first group mimics nascent stalled DNA replication forks, whereas the second group mimics regressed forks (see Table 1).

Results show that RecG exhibits significant activity on the substrates in group 1, whereas these are poor substrates for RuvAB. In these DNA substrates, either one or both arms are single-stranded DNA. In addition, the single-stranded DNA binding protein (SSB)2 enhances the activity of RecG in the presence of these DNA molecules while inhibiting RuvAB. Furthermore, under conditions of vast RuvA excess, a condition that results in inhibition of RecG activity, SSB is able to rescue RecG by restoring the level of activity back to that observed in the absence of RuvA. Results also show that for the substrates in group 2, namely those that resemble a regressed fork, both DNA helicases are the most catalytically efficient of all the substrates tested herein. For these substrates, all arms are duplex DNA. The high catalytic efficiency of RuvAB on a fork with duplex arms requires cooperative binding of two substrate DNA molecules, whereas the enzyme binds only a single Holliday junction to achieve the same level of activity. In addition, from protein titrations, the activity of RuvAB saturates at one RuvA tetramer per DNA molecule where the DNA is either a fork with duplex arms or a Holliday junction. However, saturation of activity requires 3-fold more RuvB with the fork substrate than with the Holliday junction. Collectively, these data suggest that the preferred substrate for RuvAB is a Holliday junction as suggested previously (41, 42).

In summary, our data are consistent with our previous work supporting an early role for RecG in fork regression and a later role for RuvAB in fork rescue (43). The work in this study strengthens this view and shows that at a nascent stalled DNA replication fork RecG, likely in concert with SSB, acts first to regress the fork. Concurrently, SSB inhibits access of RuvAB to a nascent stalled fork, which is a poor substrate for RuvAB. Fork processing by RecG leads to the formation of a fork with duplex arms that could be accessed by PriA, resulting in reloading of the replisome (44–47). Instead, if further processing of this structure occurs, it would be carried by RecG, resulting in the formation of a Holliday junction-like structure as shown recently (48). Only now would RuvAB(C) act, resulting in junction movement either toward or away from the site of the original fork stall or in cleavage of the fork (5, 6).

Experimental Procedures

Chemicals—Phosphoenolpyruvate, nicotinamide adenine dinucleotide (NADH), pyruvate kinase, and lactate dehydrogenase were from Sigma. ATP and Q-Sepharose Fast Flow, 16/10 heparin FF, and Mono S 5/50 GL columns were from GE Healthcare. Bio-Gel HTP hydroxylapatite was from Bio-Rad. Dithiothreitol (DTT) was from Acros Organics. BSA was purchased from New England Biolabs.

Reagents—All solutions were prepared using Barnstead nanopure water. Stock solutions of phosphoenolpyruvate were prepared in 0.5 M Tris acetate (pH 7.5). ATP was dissolved as a concentrated stock in 0.5 M Tris-HCl (pH 7.5) with the concentration determined spectrophotometrically using an extinction coefficient of 1.54×10^5 M⁻¹ cm⁻¹. NADH was dissolved in 10 mM Tris acetate (pH 7.5), its concentration was determined using an extinction coefficient of 6.25×10^3 M⁻¹ cm⁻¹, and it was stored in small aliquots at −80 °C. DTT was dissolved as a 1 M stock in nanopure water and stored at −80 °C. All reaction buffers described below were assembled at 10 times reaction concentration and stored in 1-mL aliquots at −80 °C.

DNA Cofactors—M13 mp18 ssDNA was prepared as described (49). The concentration of DNA was determined spectrophotometrically using an extinction coefficient of 8.780 M⁻¹ cm⁻¹ (nucleotides). Purified DNA was stored in small aliquots at −80 °C.

Oligonucleotides used to construct model fork substrates were purchased from Integrated DNA Technologies (Coralville, IA). The sequences were adapted from those used previously (43, 49) and contain a mobile homologous core flanked by heterologous sequences. Oligonucleotides were purified using denaturing polyacrylamide gels. This was followed by gel filtration using NAP-25 columns (GE Healthcare) and ethanol precipitation. The concentration of each oligonucleotide was determined spectrophotometrically using the extinction coefficient provided by Integrated DNA Technologies.

Model fork substrates were prepared by annealing six oligonucleotides in various combinations: PB170 (5′-CTAGAGCGCTGCGGAATTCCTGTTGGATCTGATGTCTAGAGC-TAGGCCCTCAACTATGAAATCCTGCTGCA-3′), PB171 (5′-GGATTTTCATAGTGAGGCTCTAGACAGTC-3′), PB172 (5′-TGCTGTCAAGACTATCGATCTAGCTGCTGCAGC-3′), PB173 (5′-CGGCGTCGAGTCGATCTAGCTGATAGCTCTAGACAGTCTAGACTGATGGCTCTAGACAGTC-3′), PB345 (5′-GGATTTTCATAGTGAGGCTCTAGACAGTC-3′), and PB346 (5′-GAGATAGTATGACGTCAGCCAATTTCACCGGTTGCTGCTGCA-3′). Purified oligonucleotides (1–10 μM molecules each in different annealing experiments) were annealed in a total volume of 50 μL containing 10 mM Tris-HCl (pH 7.5) or 10 mM Tris-OAc (pH 7.5), 100 mM NaCl, and 10 mM MgOAc. Annealing involved incubation of the DNA-buffer mixture in thin walled PCR tubes at 100 °C for 5 min followed by an overnight cooling step to room temperature. Junctions were added directly to ATPase assays.

2 The abbreviations used are: SSB, single-stranded DNA-binding protein; ssDNA, single-stranded DNA; STMP, salt titration midpoint; HJ, Holliday junction.
without further purification. The extent of annealing was verified by non-denaturing PAGE using 5’-end-labeled oligonucleotides annealed under identical conditions (data not shown). Typically, >95% of the DNA present was found to be in the annealed substrate (data not shown). Fork 1 was formed by annealing PB170 and PB173; fork 2 was formed by annealing PB170, PB171, and PB173; fork 3 was formed by annealing PB170, PB172, and PB173; and fork 4 was formed by annealing PB170, PB171, PB172, and PB173; and the Holliday junction contained oligonucleotides PB170, PB173, PB345, and PB346 Table 1.

Proteins—RecG protein was purified as described previously (43, 49) with the following modifications. The first column was a 30-ml Q-Sepharose column equilibrated in Buffer A (20 mM Tris-HCl (pH 8.5), 1 mM EDTA, 1 mM DTT, and 10 mM NaCl). The protein was eluted using a linear gradient (10–1,000 mM NaCl) with RecG eluting between 250 and 360 mM NaCl. The pooled fractions were subjected to heparin FF and hydroxylapatite chromatography as described (49). Pooled fractions from the hydroxylapatite column were dialyzed overnight into S Buffer (1 M KPO4 (pH 6.8), 1 mM DTT, 1 mM EDTA, and 100 mM KCl). The protein was applied to a 1-ml Mono S column and eluted using a linear KCl gradient (100–700 mM) with RecG eluting at 350 mM KCl. The fractions containing RecG were pooled and dialyzed overnight against storage buffer (20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 100 mM NaCl, and 50% (v/v) glycerol). The protein concentration was determined spectrophotometrically using an extinction coefficient of 49,500 M⁻¹ cm⁻¹ (50). The modifications to the purification procedure yielded a 4-fold increase in specific activity relative to that used previously (43).

RuvA and RuvB proteins were purified as described previously (51). The concentration of RuvA was determined using an extinction coefficient of 5,550 M⁻¹ cm⁻¹ (52). For the RuvB purification, the DEAE Bio-Gel A column was replaced by a 100-ml Q-Sepharose column that was equilibrated with TEGD buffer (20 mM Tris acetic acid (pH 7.5), 1 mM EDTA, 10% (v/v) glycerol, and 1 mM DTT), and the protein was eluted with a 1-liter linear gradient from 0 to 500 mM potassium acetate. The concentration of RuvB protein was determined using an extinction coefficient of 16,400 M⁻¹ cm⁻¹ (52).

RuvB K68A ATPase-deficient mutant was constructed as follows. The wild type ruvB gene was subcloned from pEAW112 into pET28 in-frame with the C-terminal histidine tag using Infusion cloning (Clontech). Next, codon 68 was mutated using the QuickChange mutagenesis kit (Agilent Technologies). Mutagenesis was confirmed by DNA sequencing; only codon 68 was found to be altered.

Then pET-ruvB-K68A was transformed into Tuner cells. A 10-liter culture was grown to an OD₆₀₀ of 0.4, induced with 100 μM isopropyl 1-thio-β-D-galactopyranoside, and grown for an additional 4 h. Cells were harvested and lysed, and the resulting lysates were subjected to nickel column chromatography as described previously (54). Fractions containing RuvB K68A were pooled; dialyzed against a buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 1 mM DTT; and subjected to Mono Q chromatography as described previously for wild type (52). The concentration of RuvB K68A protein was determined using an extinction coefficient of 15,930 M⁻¹ cm⁻¹.

SSB protein was purified from strain K12ΔH1Δtrp as described (55). The concentration of purified SSB protein was determined at 280 nm using ε = 30,000 M⁻¹ cm⁻¹. The site size of SSB protein was determined to be 10 nucleotides per monomer by monitoring the quenching of the intrinsic fluorescence of SSB that occurs on binding to ssDNA as described (56). Histidine-tagged SSBΔC8 was purified as described previously (54). The site size was determined to be 10 nucleotides per monomer. Gene 32 protein was purified as described previously (43). The site size was calculated to be seven nucleotides per monomer.

ATP Hydrolysis Assay—The hydrolysis of ATP was monitored using a coupled spectrophotometric assay carried out at 37 °C as described previously (43, 49). The standard reaction buffer for RecG contained 20 mM Tris acetate (pH 7.5), 1 mM DTT, 0.3 mM NADH, 7.5 mM phosphoenolpyruvate, 20 units/ml pyruvate kinase, 20 units/ml lactate dehydrogenase, 2 mM RecG, 1 mM ATP, and 10 mM magnesium acetate (but varied according to the DNA cofactor present). In early experiments, the reaction buffer for RuvAB was the same as for RecG except RecG was replaced by RuvAB (see below) and 2 mM DTT, 100 μg/ml BSA, and 6.3% (w/v) glycerol were used (51). In subsequent experiments, either BSA, glycerol, or both were omitted from reactions without noticeable rate effects.

Assays were performed in a reaction volume of 150 μl and initiated by the addition of enzyme following a 2-min preincubation at 37 °C of all other components. For assays with M13 ssDNA, the concentrations of RuvA and RuvB were held constant at 1.3 and 1 μM, respectively (2 RuvA tetramers:1 RuvB hexamer), and they were mixed together for 30 min on ice prior to addition (51). Using these concentrations, the concentration of RuvAB complex was calculated to be 167 nM. In contrast, in assays with model fork substrates, addition of preformed RuvAB complexes did not result in ATPase activity. To observe activity, proteins were added sequentially to reaction mixtures containing DNA and ATP as follows: RuvA first (650 nM monomer final) followed by a 5-min incubation then RuvB was added to initiate reactions (500 nM monomer final). The order of addition of SSB relative to either RecG or RuvAB is indicated in figure legends. The rate of ATP hydrolysis was calculated by multiplying the slope of a tangent drawn to linear portions of time courses by 159. In a typical reaction, close to 200 data points were used to draw a linear fit to the data to calculate reaction rates. To obtain kinetic parameters, data were analyzed using non-linear curve fitting in Prism v 5.04 (GraphPad Software, Inc.). DNA titration data were fit to the Hill equation (V = (Vₘₐₓ[DNA]ⁿ)/([S]₀[DNA]ⁿ + [DNA]ⁿ)) or the Michaelis-Menten equation (V = (Vₘₐₓ[DNA])/([Kₘ + [DNA]]) (57). In situations where the binding appeared cooperative (e.g. fork 4 and RuvAB), a comparison was done in Prism to determine which model more accurately described the data. Here models were compared using the comparison of fit function, and models were discriminated using both F-test and p values. In those instances where the Hill equation more accurately described the data, p values <0.0001 and high F-values were obtained (data not shown).
In salt titration experiments, the same RecG or RuvAB reaction buffers were used (see above). Once a steady-state rate of ATP hydrolysis was achieved, NaCl was added in 12.5 mM increments (1-μl volumes). This was repeated until all ATP hydrolysis ceased. The resulting hydrolysis rate in each steady-state region was calculated and expressed as a percentage of the steady-state rate in the absence of NaCl. The total volume used to calculate the final concentration of NaCl was adjusted after each addition to correct for the additions themselves. A line of best fit was drawn for data points between each addition to obtain the ATP hydrolysis rate after each salt increment. The average number of data points used to determine the reaction rate was 14. These rates were subsequently graphed to determine the concentration of NaCl resulting in a 50% reduction in the rate of ATP hydrolysis, which corresponds to the salt titration midpoint.

**Determination of the Stoichiometry of SSB Binding to Fork Structures**—The binding of SSB to fork substrates containing ssDNA tails was determined by monitoring the quenching of the intrinsic fluorescence of SSB that occurs on binding to ssDNA as described (56). In these assays, SSB was titrated with increasing amounts of annealed substrate until the maximum amount of intrinsic fluorescence was quenched. This corresponded to two SSB tetramers bound to fork 1 (two ssDNA tails) and one tetramer each bound to forks 2 and 3 (one single ssDNA tail each; data not shown).

### RESULTS

**Kinetic Analyses Reveal the Preferred Substrates for RecG and RuvAB**—In a previous study, we characterized the ATPase activity of RecG and RuvAB side by side on plasmid-sized DNA cofactors (43). This approach focused on the types of DNA that might exist in the vicinity of stalled replication forks. In this study, we focused our attention on DNA structures that may form at the fork itself. These model fork substrates, shown schematically in Table 1, are formed by annealing purified oligonucleotides, resulting in a fork with flayed ends (fork 1), forks with one single-stranded DNA arm (fork 2 in which the lagging strand is ssDNA or fork 3 in which the leading strand is ssDNA), a fork with two duplex arms (fork 4), and finally a Holliday junction. Forks 1–3, which contain one or more ssDNA arms, are assigned into group 1 and are thought to mimic nascent stalled replication fork structures. Fork 4 and the HJ, which contain duplex DNA arms, are assigned to group 2 as they are thought to mimic regressed fork structures. These junction DNAs contain a homologous core of 24 bp flanked by heterologous sequences so that each DNA helicase can mediate unwinding of the substrates (data not shown and Refs. 14, 17, and 18).

To further understand how RecG and RuvAB could act at a stalled replication fork, we characterized their ability to hydrolyze ATP in the presence of model fork substrates as a function of increasing DNA concentration. Experiments were done at a single magnesium ion concentration, a fixed amount of enzyme, and with 1 mM ATP, which is in excess of the $K_{m}^{ATP}$ for each enzyme (120 μM for RecG (49) and 154 μM for RuvAB (52)).

The results show that RecG exhibits the highest level of ATPase activity in the presence of the Holliday junction followed closely by fork 4. This is not simply a higher level of ATPase activity, but critically, RecG is more catalytically efficient in the presence of these DNA molecules than those with single strand character (Fig. 1, A and C, and Table 2). The high catalytic efficiency in the presence of fork 4 and the Holliday junction (1,196 ± 100 and 1,181 ± 100 min⁻¹·μM⁻¹, respectively) is not due to the enzyme binding more than one substrate DNA molecule, thereby enhancing its ATPase activity, as Hill coefficients of 1.3 ± 0.1 were obtained when data were approximated using the Hill equation. Although RecG turns over more ATP in the presence of the Holliday junction, the catalytic efficiencies in the presence of this substrate and fork 4 are within experimental error the same (Fig. 1C and Table 2). This is because the $K_{m}^{DNA, app}$ for fork 4 is 30% lower than that of the Holliday junction (Fig. 1D and Table 2).

When DNA cofactors with single-stranded DNA arms were analyzed, the catalytic efficiency of RecG decreases 1.6–4-fold relative to fork 4 (Fig. 1C and Table 2). Furthermore, within this first group of substrates, a strong preference for fork 3 is observed as evidenced by the highest catalytic efficiency, consistent with previous work (49). The high $k_{cat}/K_{m}^{DNA, app}$ value relative to forks 1 and 2 is due to increased turnover of ATP and a higher affinity for this DNA (Fig. 1, A and C, and Table 2). In contrast, when the lagging strand arm is ssDNA (fork 2), the catalytic efficiency of RecG decreases 2.5-fold relative to fork 3 (Fig. 1C). This is attributed to a modest decrease in ATP turnover from 2,455 ± 200 to 1,940 ± 100 min⁻¹ and a 2-fold increase in $K_{m}^{DNA, app}$ from 4.0 ± 0.4 to 7.8 ± 0.2 nM (Fig. 1C and Table 2). When both arms of the fork are single-stranded in character (fork 1), the catalytic efficiency of RecG improves slightly to 287 ± 40 min⁻¹·μM⁻¹, and this is attributed to the slight decrease in $K_{m}^{DNA, app}$ relative to fork 2. Finally, it is worth noting the contributions of duplex DNA in the arms of forks to RecG function. When both arms are ssDNA (fork 1), the $K_{m}^{DNA, app}$ is 5.3 ± 0.5 nM. In contrast, when both arms are double-stranded as in fork 4, RecG binds more tightly as evidenced by a 2-fold decrease in $K_{m}^{DNA, app}$ (Table 2). Surprisingly, when the leading strand arm is duplex (fork 2), this is a poor substrate for RecG as indicated by the highest $K_{m}^{DNA, app}$ of 7.8 ± 0.2 nM, which is 2-fold higher than that for fork 3.

In contrast to RecG, the catalytic efficiency of RuvAB is 3–7-fold higher on group 2 substrates than on those in group 1 (Fig. 1D and Table 2).
The high catalytic efficiency observed for fork 4 is surprising given the history of RuvAB in promoting branch migration (58) but is consistent with previous work (14, 59). However, the high level of activity observed in the presence of fork 4 is attributed to cooperative DNA binding as indicated by the sigmoidal curve for this substrate and a Hill coefficient for DNA binding of 2.3 [H11006]/H11006 [H11006]. Although RuvAB binds two fork 4 substrates to achieve full activity, this is not an optimal situation as indicated by the $K_{\text{m, app}}$ value of $58 [H11006]/H110063 [nM]$, which is 1.5-fold higher than that of the Holliday junction. Consequently, these data show that the optimal substrate for RuvAB is a Holliday junction.

As soon as ssDNA regions are present in the DNA substrate, RuvAB binds very poorly as evidenced by the 2.7–7-fold higher $K_{\text{m, app}}$ values for forks 2 and 3. This results in a significant reduction in catalytic efficiency of the enzyme. Consequently, these data suggest that these are not the optimal substrates for RuvAB.

**TABLE 2**

| DNA    | $V_{\text{max}}$ | $K_{\text{m, app}}$ or $S_{0.5 \text{ app}}$ | Hill coefficient | $k_{\text{cat}}$ | $k_{\text{cat}}/K_{\text{m, app}}$ |
|--------|------------------|----------------------------------------------|------------------|------------------|-------------------------------|
| Fork 1 | 3.0 ± 0.1        | 5.3 ± 0.5                                     | NR               | 1,520 ± 100      | 287 ± 40                      |
| Fork 2 | 3.9 ± 0.2        | 7.8 ± 0.2                                     | NR               | 1,940 ± 100      | 249 ± 20                      |
| Fork 3 | 4.9 ± 0.2        | 4.0 ± 0.4                                     | NR               | 2,455 ± 200      | 614 ± 100                     |
| Fork 4 | 6.0 ± 0.1        | 2.5 ± 0.2                                     | 1.3 ± 0.1        | 2,990 ± 100      | 1,196 ± 100                   |
| HJ     | 8.3 ± 0.2        | 3.5 ± 0.2                                     | 1.3 ± 0.1        | 4,135 ± 100      | 1,181 ± 100                   |

To determine whether the Hill or Michaelis-Menten equation more accurately described the data, a comparison was done using Prism. This analysis revealed that for fork 4 and the HJ, the Hill equation more accurately described the data, whereas for forks 1–3, the Michaelis-Menten equation was more accurate. The $K_{\text{m, app}}$ (or $S_{0.5 \text{ app}}$) for DNA is reported in $nM$ molecules.

NR, not relevant.

1, B and D, and Table 3). The high catalytic efficiency observed for fork 4 is surprising given the history of RuvAB in promoting branch migration (58) but is consistent with previous work (14, 59). However, the high level of activity observed in the presence of fork 4 is attributed to cooperative DNA binding as indicated by the sigmoidal curve for this substrate and a Hill coefficient for DNA binding of $2.3 ± 0.3$ (Fig. 1B and Table 3). Although RuvAB binds two fork 4 substrates to achieve full activity, this is not an optimal situation as indicated by the $K_{\text{m, app}}$ value of $58 ± 3 nM$, which is 1.5-fold higher than that of the Holliday junction. Consequently, these data show that the optimal substrate for RuvAB is a Holliday junction.

As soon as ssDNA regions are present in the DNA substrate, RuvAB binds very poorly as evidenced by the 2.7–7-fold higher $K_{\text{m, app}}$ values for forks 2 and 3. This results in a significant reduction in catalytic efficiency of the enzyme. Consequently, these data suggest that these are not the optimal substrates for RuvAB.

**SSB Plays a Key Role in Affecting the Activity of RecG on Model Fork Substrates**—Our previous work to understand how RecG functioned at a stalled fork demonstrated a role for the SSB in stabilizing RecG on ssDNA (43). This was demonstrated using both physical and functional interactions between SSB and RecG on M13 ssDNA. To determine whether SSB is important for the function of RecG on fork substrates, we again used the salt titration midpoint (STMP) of the ATPase activity of RecG as an indicator of stimulation of function. We reasoned that if SSB stabilized the interaction of RecG with fork DNA substrates then it could enhance the STMP at least 2-fold as we observed for M13 ssDNA (43, 49). This would be evidence of a similar, functional interaction between these two proteins.

The results show that in the absence of SSB the STMP for RecG in the presence of substrates with at least one ssDNA arm is 16, 24, and 12 mM for forks 1, 2, and 3, respectively (Fig. 2 and Table 4). The addition of SSB protein results in a 2-fold increase in the STMP of forks 1 and 3 to 30 and 21 mM, respectively (43).
Substrates and in the absence of SSB concluded that a single RecG processed each fork substrate. This is consistent with the crystal structure of the enzyme bound to a fork 3 substrate (24). In solution, the stoichiometry of RecG bound to SSB is 2 per tetramer (43). As SSB stabilizes RecG on fork substrates, we thought it important to determine whether this stabilization is due to loading of more than a single RecG on a fork or whether the stoichiometry is still one RecG per fork. To address this issue, RecG was titrated against a fixed concentration of DNA (100 nM) in the presence and absence of stoichiometric amounts of SSB (relative to DNA), and the rates of ATP hydrolysis were measured.

The results show that for fork 1 saturation of ATPase activity of RecG was observed at a ratio of one RecG per fork. This stoichiometry was unaffected by the presence of SSB. Furthermore, in the presence of SSB, the rate of ATP hydrolysis increased 1.5-fold at or above 100 nM RecG (Fig. 3A). For fork 2, the stoichiometry in the presence and absence of SSB is 0.8, and the presence of SSB did not result in an increase in RecG ATPase activity. Similar data were obtained for fork 3 except that the stoichiometry was 1 (data not shown). For forks 1–3, determination of the saturation point had to be carefully done because when the concentration of RecG exceeded that of DNA, inhibition of activity was observed (data not shown). Consequently concentrations of RecG ≥120 nM were not included in this analysis. In summary, the stoichiometry is one RecG per fork, independent of the presence of SSB.

As RecG exhibits significant ATPase activity in the presence of fork 4 and the Holliday junction, we wanted to determine whether this is due to a monomer or to multiple RecG proteins bound simultaneously. This is possible in principle as the arms are each sufficiently long to accommodate one helicase molecule each, and RecG exhibits significant ATPase activity on linear, duplex DNA substrates (49, 60). As before, we titrated RecG relative to DNA, and the data are shown in Fig. 3C. The results show that ATPase activity saturates at approximately one RecG per DNA for each substrate. Furthermore, activity on the Holliday junction is inhibited once protein is in excess over DNA, whereas little or no inhibition is observed for fork 4 at elevated concentrations of RecG.

**RuvAB Requires One RuvA Tetramer for Full Activity—RuvA exists as a tetramer in solution (22). This tetramer binds to Holliday junctions, loads RuvB in a magnesium ion-dependent manner, and in the presence of ATP catalyzes branch migration.**

### TABLE 3

**DNA kinetic parameters for RuvAB**

Assays were done as described under “Experimental Procedures,” contained 10 mM MgOAc and 1 mM ATP, and were initiated by the addition of RuvB following a 5-min incubation of RuvA with the DNA. The final concentration of RuvAB was calculated to be 835 nM.

| DNA | $V_{max}$ | $K_{m}^{app}$ or $S_{0.5}^{app}$ | Hill coefficient | $k_{cat}$ | $k_{cat}/K_{m}$ |
|-----|-----------|-------------------------------|-----------------|----------|----------------|
| Fork 1 | 7.0 ± 0.2 | 61 ± 6 | NR $^{a}$ | 84 ± 3 | 1.4 ± 0.2 |
| Fork 2 | 10.4 ± 0.5 | 107 ± 10 | 1.9 ± 0.2 | 124 ± 6 | 1.2 ± 0.2 $^{b}$ |
| Fork 3 | 14.5 ± 1.0 | 283 ± 40 | NR | 174 ± 20 | 0.6 ± 0.2 |
| Fork 4 | 21.6 ± 0.6 | 58 ± 3 | 2.3 ± 0.3 | 250 ± 7 | 4.5 ± 0.4 $^{d}$ |
| HJ | 12.5 ± 0.4 | 40 ± 3 | 1.3 ± 0.1 | 150 ± 4 | 3.8 ± 0.4 |

$^{a}$ To determine whether the Hill or Michaelis-Menten equations more accurately described the data, a comparison was done using Prism. This analysis revealed that for forks 1 and 3, the Michaelis-Menten equation more accurately described the data, whereas for forks 2 and 4 and the HJ, the Hill equation was more accurate. The $K_{m}^{app}$ (or $S_{0.5}^{app}$) for DNA is reported in nM molecules.

$^{b}$ NR, not relevant.

$^{c}$ As the Hill equation more accurately describes these data, it is more accurate to use $k_{cat}/S_{0.5}$ instead of $k_{cat}/K_{m}$ to describe catalytic efficiency.

$^{d}$ Assays with this DNA were done in the presence of 5 mM NaCl.

### FIGURE 2. SSB stabilizes RecG on model fork substrates.

ATPase assays were performed as described under “Experimental Procedures” and were initiated by the addition of 1.92 nM RecG. DNA was present at 100 nM molecules, and reactions contained 10 mM MgOAc and 1 mM ATP. In assays with SSB, it was added to the DNA to 250 nM tetramer final concentration prior to reaction initiation by RecG. For each substrate, assays were done in duplicate. The resulting rates were calculated as described under “Experimental Procedures” with the relative activity expressed as a percentage of the hydrolysis rate of RecG in the absence of added NaCl. Arrows indicate the salt titration midpoint for fork 2 in the absence (24 mM) and presence of SSB (103 mM). Only fork 2 is shown for clarity with the STMPs for the remaining substrates shown in Table 4. **Open symbols**, reactions with RecG only; **closed symbols**, reactions done in the presence of SSB. The error bars represent the mean and error.

### TABLE 4

**Effects of monovalent cations on the ATPase activities of RecG and RuvAB**

Data were derived from ATPase assays containing 100 nM DNA, 1 mM ATP, and 10 mM MgOAc. In assays where SSB was present, it was preincubated with DNA prior to reaction initiation with RecG or RuvAB. For fork 1, SSB was present at 200 nM tetramer, and for forks 2 and 3, it was present at 100 nM tetramer.

| DNA | RecG | RecG + SSB | RuvAB $^{a}$ |
|-----|------|------------|-------------|
| Fork 1 | 16 | 30 | 72 |
| Fork 2 | 24 | 104 | 66 |
| Fork 3 | 12 | 21 | 17 |
| Fork 4 | 42 | 52 | 132 |
| HJ | 35 | 63 | 79 |

$^{a}$ Calculation of the STMP is described under “Experimental Procedures” and in the legend to Fig. 2.

$^{b}$ The effects of SSB on the STMP of RuvAB were not tested as SSB inhibits the activity of this enzyme.

Surprisingly, the STMP for fork 2 increased 5-fold from 24 to 104 mM (Fig. 2 and Table 4). As fork 2 is the poorest fork substrate for RecG (Fig. 1), these data indicate a very specific and critical role for SSB in facilitating the action of RecG on this substrate.

**RecG Is Active as a Monomer on Model Fork Substrates**—Previous work done by Lloyd and co-workers (17) using model fork substrates and in the absence of SSB concluded that a single RecG processed each fork substrate. This is consistent with the crystal structure of the enzyme bound to a fork 3 substrate (24). In solution, the stoichiometry of RecG bound to SSB is 2 per tetramer (43). As SSB stabilizes RecG on fork substrates, we thought it important to determine whether this stabilization is due to loading of more than a single RecG on a fork or whether the stoichiometry is still one RecG per fork. To address this issue, RecG was titrated against a fixed concentration of DNA (100 nM) in the presence and absence of stoichiometric amounts of SSB (relative to DNA), and the rates of ATP hydrolysis were measured.

The results show that for fork 1 saturation of ATPase activity of RecG was observed at a ratio of one RecG per fork. This stoichiometry was unaffected by the presence of SSB. Furthermore, in the presence of SSB, the rate of ATP hydrolysis increased 1.5-fold at or above 100 nM RecG (Fig. 3A). For fork 2, the stoichiometry in the presence and absence of SSB is 0.8, and the presence of SSB did not result in an increase in RecG ATPase activity. Similar data were obtained for fork 3 except that the stoichiometry was 1 (data not shown). For forks 1–3, determination of the saturation point had to be carefully done because when the concentration of RecG exceeded that of DNA, inhibition of activity was observed (data not shown). Consequently concentrations of RecG ≥120 nM were not included in this analysis. In summary, the stoichiometry is one RecG per fork, independent of the presence of SSB.

As RecG exhibits significant ATPase activity in the presence of fork 4 and the Holliday junction, we wanted to determine whether this is due to a monomer or to multiple RecG proteins bound simultaneously. This is possible in principle as the arms are each sufficiently long to accommodate one helicase molecule each, and RecG exhibits significant ATPase activity on linear, duplex DNA substrates (49, 60). As before, we titrated RecG relative to DNA, and the data are shown in Fig. 3C. The results show that ATPase activity saturates at approximately one RecG per DNA for each substrate. Furthermore, activity on the Holliday junction is inhibited once protein is in excess over DNA, whereas little or no inhibition is observed for fork 4 at elevated concentrations of RecG.

**RuvAB Requires One RuvA Tetramer for Full Activity**—RuvA exists as a tetramer in solution (22). This tetramer binds to Holliday junctions, loads RuvB in a magnesium ion-dependent manner, and in the presence of ATP catalyzes branch migration.
Excess SSB Is Required to Inhibit RuvAB on Model Forks—

Previously, we suggested that RecG acts at a stalled fork prior to RuvAB and that SSB plays a key role for RecG perhaps at the loading stage (43). As we have been unable to detect a physical and functional interaction between SSB and RuvAB on plasmid-sized DNA substrates, we wanted to determine whether SSB would either inhibit RuvAB at a stalled fork or whether it might stimulate the ATPase activity in a manner similar to what we have observed for RecG. A stimulation is conceivable because previous work showed that RuvAB and SSB coordinate to bypass heterologous sequences during branch migration (65).

To determine the effects of SSB on RuvAB activity, forks 1–3 were bound to stoichiometric amounts of SSB (200 nM tetramer for fork 1 and 100 nM for forks 2 and 3), and the rate of RuvAB ATP hydrolysis was measured. When the rates were corrected for the DNA-independent rate in the presence of SSB, little to no effect of SSB on RuvAB was observed (Fig. 5A). Identical results were obtained when reactions were done with fork 4 and the Holliday junction. For fork 4, a small but noticeable stimulation was observed with both gp32 and SSBAC8 (a mutant lacking the last 8 residues critical for binding to RecG (43)). Furthermore, for forks 1 and 2, ATPase activity was inhibited 5-fold when SSBAC8 was added prior to RuvAB.

In vivo, SSB is present at 1–2,000 tetramers per cell (66), whereas RuvA and RuvB are present at 700 and 200 monomers per cell, respectively (38, 67). As stoichiometric amounts of wild type SSB did not inhibit RuvAB in vitro, we opted to titrate SSB relative to DNA in an attempt to more closely mimic the situation in vivo. Two separate SSB titrations were done: first in the presence of a stoichiometric amount of RuvAB relative to DNA and second with an excess of RuvAB. The results show no effects of SSB with fork 1 regardless of the concentration of RuvAB (Fig. 5, B and C). In contrast, on forks 2 and 3, the ATPase activity of RuvAB is inhibited by increasing amounts of SSB with 50% inhibition occurring at 200 nM tetramer with stoichiometric RuvAB, increasing to 380–400 nM when RuvAB is in excess (Fig. 5, B and C).

The inhibition observed may be due to a simple competition between SSB and RuvAB. Alternatively and in addition, some other mechanism may be in operation. To understand what this might be, we required a plasmid-sized ssDNA substrate. This is necessary as the maximum level of ATPase activity of RuvAB on forks 1–3 is very low (Fig. 1B). As a result, if SSB does affect RuvAB in any way, it may be difficult to clearly discern. Consequently, we selected M13 ssDNA as it is known to effectively stimulate the ATPase activity of RuvAB (43). We then compared the activity of the enzyme in the absence and presence of stoichiometric SSB relative to ssDNA (Fig. 6).

The results show that in the absence of SSB RuvAB exhibits significant ATPase activity on M13 ssDNA as observed previously (43, 51). The $V_{\text{max}}$ is 384 ± 12 μM/min, the $S_{0.5}$ for ssDNA, app, is 3 ±

Subsequent studies have suggested that instead two RuvA tetramers sandwich a single Holliday junction (63). This has been extended by studies suggesting that the active form of RuvA at a stalled replication fork is an octamer (34).

To determine whether RuvA is active as a tetramer or octamer, we performed RuvA titrations in the presence of either DNA subfork 4 or the Holliday junction and in the presence of a fixed amount of RuvB. The results show that for both DNA substrates activity saturates at one RuvA tetramer per DNA molecule (Fig. 4, A and B).

A similar set of experiments was done except we kept RuvA constant and varied RuvB. The results show that activity saturated at three to five RuvB monomers per RuvA tetramer in separate experiments (Fig. 4C). The low values may reflect the functional asymmetry in the RuvB hexamer (52). In contrast, saturation of activity in the presence of fork 4 required 2–3 times more RuvB or nine RuvB monomers per RuvA tetramer. As more RuvB is required to fully saturate activity on fork 4, this suggests that loading of RuvB by RuvA is impaired on a regressed fork substrate.
0.1 nM, and the Hill coefficient for DNA binding is 2.5 ± 0.3. In contrast, in the presence of SSB, the $V_{\text{max}}$ is reduced 2.4-fold to 159 ± 23 μmol/min, whereas the Hill coefficient (2.6 ± 1) remains unchanged, and the $S_{0.5,DNA,app}$ (4 ± 1 nM) is marginally increased. The reduction in $V_{\text{max}}$ induced in RuvAB by SSB results in a 3.4-fold reduction in catalytic efficiency of the enzyme down from 129 to 38 min$^{-1}$ nM. Therefore, in addition to impeding binding, SSB negatively impacts the catalytic efficiency of RuvAB on ssDNA possibly by altering the structure of the DNA to make it a poorer substrate.

Competition of RecG with RuvA Is Enhanced by SSB—The concentration of RecG is thought to be less than 10 copies per cell (68), and the basal levels of RuvA and RuvB are 700 and 200 copies per cell, respectively (38, 67). Therefore, it is reasonable to suggest that RuvAB could inhibit access of RecG to forks even though we have shown that RecG binds more tightly to junction DNAs (Fig. 1 and Tables 2 and 3). As both RecG and RuvAB exhibit ATPase activity in the presence of model forks, we first decided to test competition between these complexes as RecG versus RuvA only. We surmised that if RuvA is able to compete with RecG then it would inhibit the ATPase activity of the helicase.

Assays were done using 100 nM DNA, stoichiometric RuvA (100 nM tetramer), and 2 nM RecG. The results show that a modest inhibition of RecG is observed with 50 – 60% inhibition observed for fork 2 (Fig. 7A). As RuvA is present in vivo at significantly higher concentrations than RecG, we repeated these experiments using a RuvA titration with each amount of RuvA added at an ongoing assay initiated with RecG. These results show that RuvA alone can inhibit RecG, but this requires greater than a 5-fold excess relative to DNA to inhibit ATPase activity by 50% (Fig. 7B).

As we have shown that SSB enhances the ATPase activity of RecG on fork substrates, we wanted to assess whether SSB could rescue RecG inhibition mediated by an excess of the RuvA tetramer (Fig. 7C). To do this, separate ATPase assays with forks 1, 2, and 3 were initiated by addition of 2 nM RecG. Once the steady-state was achieved, 455 nM RuvA tetramer was added, establishing a new lower steady-state rate. Then SSB was added, and the new rate of ATP hydrolysis was recorded. The results show that 455 nM RuvA inhibited RecG 30–70% depending on the DNA substrate. Importantly, SSB is able to rescue RecG activity on forks 2 and 3. Surprisingly, rescue was most efficient when RecG and SSB were allowed to form a complex prior to being added to an ongoing reaction. Collectively, these data show that even under conditions of a 228-fold excess of RuvA SSB enables RecG to bind to a fork substrate and out-compete RuvA.

Finally, we wanted to determine whether RuvAB could compete with RecG. As RuvAB has significant ATPase activity, RuvB K68A was used instead. This mutant binds ATP and is defective for branch migration activity in vitro (69). In addition, it binds to RuvA, which facilitates its loading onto DNA. Furthermore, control ATPase assays reveal that it has no ATPase activity in the presence of RuvA and each of the model fork substrates (data not shown). To test whether RuvAB could inhibit the activity of RecG on model fork substrates, stoichiometric amounts of RuvA tetramer were bound to 100 nM substrate, and 2 min later, 1,200 nM RuvB was added. Following a 2 min period to permit loading by RuvA onto the DNA, 2 nM RecG was added to initiate reactions. Surprisingly, no inhibition of RecG was observed even in the presence of the Holliday junction, the preferred substrate for RuvAB (Fig. 7D).
DISCUSSION

The primary conclusion of this work is that RecG dominates the early stages of the processing of stalled DNA replication forks relative to RuvAB. This conclusion is derived from data showing that RecG exhibits significant activity on DNA substrates that mimic nascent stalled DNA replication forks and that activity is further enhanced by SSB protein. In contrast, the activity of RuvAB on these same substrates is low and is inhibited by an excess of SSB. In addition, our results show that RecG is more catalytically efficient on a regressed fork (that is a fork with two duplex arms), whereas RuvAB favors a Holliday junction. Collectively, these findings have important implications for the temporal sequence of events that occur at stalled DNA replication forks in vivo.

The primary conclusion is in agreement with our previous work showing that RecG acts at a stalled DNA replication fork first and that RuvAB acts later only once a Holliday junction-like structure has been produced. Furthermore and as we showed previously, our data are inconsistent with genetic data arguing for a critical role of RuvAB in the key, early stages of fork processing (4, 70). In our previous work on these helicases, we proposed a model with RecG acting first at a stalled replication fork, producing substrates for RuvAB that would contain an HJ-like structure and be relaxed in nature (43). Our kinetic data herein both support and extend this model. First, if the nascent stalled fork has regions of ssDNA, RecG or RecG and SSB bind, resulting in fork regression. The end product is a fork with two duplex arms. RecG remains bound to this substrate, further regressing the fork, leading to the extrusion of a Holliday junction-like structure. Only now would RecG dissociate, allowing RuvAB to bind. RuvAB could either continue to move the junction away from the site of the initial fork stall, or it could return the fork to its initial position. Further support for the handoff of RecG to RuvAB comes from work done by Lloyd and co-worker (10, 71, 72) who showed that RecG can process a fork 4-type substrate to create a Holliday junction, whereas RuvAB cannot.

Why is RuvAB unlikely to act prior to RecG at a fork? First, in the presence of substrates that mimic nascent stalled replication forks (group 1 substrates and forks 1–3), the catalytic efficiency of RuvAB is extremely low, and the ATPase activity of the enzyme is inhibited by an excess of SSB. As many of the proposed structures resulting from DNA replisome stalling contain ssDNA regions and therefore are likely to be bound by SSB, we suggest that these are not substrates for RuvAB in vivo. In contrast, forks with ssDNA tails are substrates for RecG (Fig. 1A). RecG binds with higher affinity to forks 1–3 than does RuvAB (Tables 2 and 3). Intriguingly, the activity of RecG on these substrates is enhanced by SSB protein in a substrate-de-
ependent manner. Fork 2 has a 3′-OH group on the nascent leading strand at the fork and is therefore an optimal substrate for PriA (47). In contrast, this substrate is not optimal for RecG and consequently requires a stabilizing factor, SSB, whose presence results in a 5-fold increase in the STMP (Fig. 2). This stabilization occurs even though fork 2 contains a 5′-ssDNA tail that is not the preferred DNA polarity for SSB binding (73). Therefore, these data suggest that if PriA were not present in the vicinity of a fork then RecG alone could bind albeit poorly. In contrast, stable binding would take place with the help of SSB, thereby ensuring that fork regression would occur. In contrast, the results in the presence of fork 3 are different as only a 2-fold stimulation of the STMP by SSB is observed. In reactions initiated by a RecG + SSB mixture, the two proteins were incubated on ice for 30 min prior to addition. D, RuvAB does not inhibit RecG on model fork substrates. In these assays, 100 nM RuvA (in tetramer) was added first to the reaction mixture containing 100 nM junction DNA; 2 min later, 1,200 nM RuvB K68A (monomer) was added followed 2 min later by 2 nM RecG. In reactions where only RecG was present, it was added to reactions following a 2-min preincubation of all components. These experiments were done on the same day using the same tubes of substrates and proteins to eliminate potential complications from variations between preparations. The error bars represent the mean and error.

Second, the importance of SSB is further evident in RuvA-RecG competition experiments. In these assays, we show that a 228-fold excess of RuvA over RecG inhibits the activity of the helicase (Fig. 7). Critically, addition of SSB obviates inhibition by RuvA with a greater level of competition provided by a RecG-SSB complex. Therefore, in vivo where an excess of RuvA relative to RecG exists, the enhanced ability of RecG afforded by SSB to out-compete the Ruv proteins means RecG processes nascent stalled DNA replication forks and that RuvAB does not.

Third, full ATPase activity of RuvAB requires DNA substrates that mimic regressed forks, namely DNA substrates with duplex arms. Only fork 4 and the Holliday junction are able to fully support the ATPase activity of this enzyme. However, full activity with fork 4 requires that two substrate DNAs bind simultaneously (Table 3). Two fork 4 molecules may be bound to a single RuvAB in head-to-tail, head-to-head, or head-to-arm configurations (supplemental Fig. 1). Although this is feasible in vitro, in vivo this would correlate with a single RuvAB having to bind two forks simultaneously, a scenario that is perhaps unlikely. In contrast, only a single Holliday junction is bound by RuvAB, resulting in maximal ATPase activity. This correlates well with the widely held view that RuvAB acts at the late stages of recombination where it binds to the Holliday junction and with our model that proposes that RuvAB acts in the later stages of fork rescue only once a Holliday junction-like structure has been produced (41, 42). Furthermore, we show that the active form of RuvAB on either a regressed fork (substrate 4) or a Holliday junction contains a single RuvA tetramer. This is in sharp contrast to the work of others suggesting that the active form is instead an octamer (34, 61). These experiments used glutaraldehyde cross-linking and low concentrations of DNA to

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show octamer formation in vitro and to support the model that a RuvA octamer is critical to fork regression in vivo (34, 61). In contrast, in the experiments herein, 100 nM fork 4 or Holliday junction substrate DNA was titrated with RuvA in the presence of a fixed concentration of RuvB. In both cases, activity saturated at one RuvA tetramer per junction (Fig. 4). These experiments, perhaps the most straightforward to interpret as compared with the previous studies, show that a single RuvA tetramer binds to a Holliday junction and loads RuvB, leading to junction migration. In addition, 3–4-fold more RuvB was required for full activity on fork 4 compared with the Holliday junction. This suggests that loading of RuvB on forked DNA substrates is impaired. Therefore, we conclude that the preferred substrate for RuvAB is not a fork but is instead a Holliday junction.

In principle, both RecG and RuvAB could be present at a stalled DNA replication fork and could compete with one another for DNA binding. To understand which enzyme could be favored to bind these DNA substrates, we compared the apparent affinity of the two helicases for each DNA substrate. The results show that RecG binds 11–46-fold more tightly than RuvAB (Fig. 1 and Tables 2 and 3). Furthermore, the apparent affinities for fork DNA substrates exhibited by RecG are comparable with what we observed previously with plasmid-sized DNAs (43). In addition, when a side-by-side catalytic efficiency comparison is done, it is clear that RecG is 209–1,007-fold more catalytically efficient in the presence of model fork substrates than RuvAB. Collectively, these data suggest that in vivo RecG would act preferentially at a stalled replication fork. Surprisingly, the trend in catalytic efficiency for these five substrates is similar for both RecG and RuvAB (Fig. 1C). Catalytic efficiency is lowest when one or both arms are single-stranded DNA and increases when all arms are duplex DNA. This trend may be partly responsible for the apparent substrate overlap suggested to occur in vivo (18).

The in vivo concentrations of RecG and the basal levels of RuvA and RuvB are thought to be 10, 700, and 200 per cell, respectively (38, 67, 68). This would suggest that under normal growth conditions or under conditions where SOS is induced, RuvAB could be favored to act at a stalled replication fork. However, this view fails to take into account the SSB protein, which is present at 1–2,000 tetramers per cell (64). The critical role of SSB in dictating whether RecG or RuvAB is responsible for the early stages of stalled DNA replication fork processing is 2-fold. First, when present in excess over substrates with ssDNA arms, SSB inhibits the activity of RuvAB presumably by preventing binding of the enzyme to the DNA. Even if RuvAB were to gain access to a substrate with ssDNA character, SSB would act against this enzyme using a mechanism that alters the single-stranded DNA substrate, leading to a reduction in catalytic efficiency. Second, SSB stimulates the activity of RecG on these same fork DNAs either by stabilizing helicase binding or by enabling binding to occur. Details of the mechanism of stimulation of RecG by SSB will be published elsewhere. Third, even if RuvA were to bind to a fork first, SSB would enhance the ability of RecG to displace RuvA as shown in our rescue experiments in Fig. 7. Finally, even if the RuvAB complex were to form on a fork, it does not represent a barrier to RecG. Consequently, these data indicate that RecG is responsible for fork regression. They further support the model that RuvAB acts later in the repair process once a substrate that is relaxed in character and contains a Holliday junction-like structure has been generated by RecG as suggested by a recent single molecule study (43, 48).

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