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Direct Observation of RecBCD Helicase as Single-Stranded DNA Translocases

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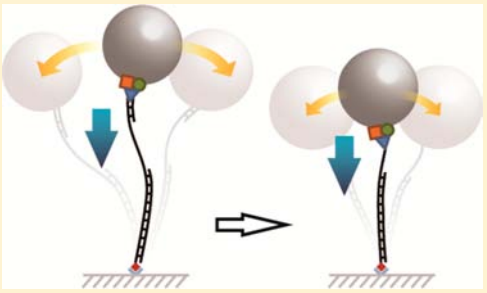
Supporting Information

ABSTRACT: The heterotrimeric *Escherichia coli* RecBCD enzyme comprises two helicase motors with different polarities: RecB (3′-to-5′) and RecD (5′-to-3′). This superfamily I helicase is responsible for initiating DNA double-strand-break (DSB) repair in the homologous recombination pathway. We used single-molecule tethered particle motion (TPM) experiments to visualize the RecBCD helicase translocation over long single-stranded (ss) DNA (>200 nt) with no apparent secondary structure. The bead-labeled RecBCD helicases were found to bind to the surface-immobilized blunt-end DNA, and translocate along the DNA substrates containing an ssDNA gap, resulting in a gradual decrease in the bead Brownian motion. Successful observation of RecBCD translocation over a long gap in either 3′-to-5′ or 5′-to-3′ ssDNA direction indicates that RecBCD helicase possesses ssDNA translocase activities in both polarities. Most RecBCD active tethers showed full translocation across the ssDNA to the dsDNA region, with about 19% of enzymes dissociated from the ss/dsDNA junction after translocating across the ssDNA region. In addition, we prepared DNA substrates containing two opposite polarities (5′-to-3′ and 3′-to-5′) of ssDNA regions intermitted by duplex DNA. RecBCD was able to translocate across both ssDNA regions in either ssDNA orientation orders, with less than 40% of tethers dissociating when entering into the second ssDNA region. These results suggest a model that RecBCD is able to switch between ssDNA translocases and rethread the other strand of ssDNA.



1. INTRODUCTION

DNA helicases are enzymes that unwind duplex DNA by coupling with the nucleoside triphosphate hydrolysis. By generating ssDNA strands, helicases initiate essential DNA metabolic processes of replication, recombination, and repair. In the process of generating ssDNA, it requires the enzyme to not only unwind duplex DNA but also translocate along the DNA substrate to achieve processive catalysis. In addition to duplex DNA unwinding, helicases have been implicated in displacing proteins from DNA substrates.[1−3] One of the recent proposed models for helicase motion is that helicases function as ssDNA translocases.[4,5] In this ssDNA translocase model, a helicase moves along ssDNA to separate two strands of DNA to achieve unwinding, to displace proteins bound to the DNA, and to generate ssDNA available for downstream biochemical reactions.

The *Escherichia coli* RecBCD is a processive helicase which initiates homologous recombinational repair. This superfamily 1 (SF1) heterotrimeric enzyme is composed of three subunits, RecB (3′-to-5′ helicase), RecC, and RecD (5′-to-3′ helicase), which enable the enzyme to move along DNA unidirectionally. In double-strand-break (DSB) repair, RecBCD initiates the process by recognizing the nearly blunt-end damaged DNA and then processes this DNA by unwinding DNA strands using its motors.[6,7] Here we used RecBCD helicase as a model system to investigate the ssDNA translocation mechanism of a helicase. We used single-molecule tethered particle motion (TPM) experiments[8] to directly observe the ssDNA translocation activities of RecBCD in real-time. We have constructed DNA substrates which contain a long ssDNA (∼200 nt) with no obvious secondary structure between duplex regions. As the bead-labeled enzyme translocates along the ssDNA substrate, the Brownian motion (BM) amplitude of the bead decreases as the DNA tether length reduces. We demonstrated that RecBCD helicase contains both 3′-to-5′ and 5′-to-3′ ssDNA translocase activities using single-molecule TPM methods.

2. RESULTS

2.1. RecBCD Translocates along Long ssDNA Gap in Both Polarities. Purified biotinylated RecBCD enzymes attached with 220 nm streptavidin beads were found to successfully translocate along 907 bp fully dsDNA. At 30 μM ATP, the averaged translocation rate is 60 ± 15 bp/s (Brownian motion amplitude change: 5.6 ± 1.5 nm/s, N = 27, Figure S1 in Supporting Information [SI]), consistent with the previous report.[9] To directly test the ssDNA translocase activity of RecBCD helicases, we constructed the DNA substrates containing a long extended, secondary, structure-free ssDNA gap (details in Materials and Methods and SI). Control experiments using restriction enzyme digestion

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confirmed the existence of ssDNA gaps using the TPM setup (Figure S2 in SI).

The substrate 789 contains a 195 nt long ssDNA gap and is annealed from three ssDNA segments. This substrate is immobilized on the anti-digoxigenin-decorated coverglass through the 5′-labeled digoxigenin end (Figure 1B). Since RecBCD recognizes the blunt-end DNA substrates, the helicase enters through the 66 bp fully duplex DNA end and translocates along the ssDNA in the 3′-to-5′ polarity toward the surface. When bead-coated RecBCD molecules were introduced into the reaction chamber in the presence of ATP, the enzyme started translocating as it bound to the end of DNA substrate. Bead Brownian motion time trace showed smooth movement along the DNA substrate from the blunt entry end, translocating through the long ssDNA gap, to the ss/dsDNA junction, continuing onto fully dsDNA section until finishing unwinding/translocating all DNA substrate, and eventually dissociating from the surface (Figure 1B). No pauses were observed among all full translocation traces (N = 22), giving a similar pattern as in the case of translocating along fully duplex DNA. The successful translocation across long 3′-to-5′ ssDNA is direct evidence that RecBCD helicase possesses a 3′-to-5′ ssDNA translocase activity.[10] Considering that RecB is the 3′-to-5′ helicase,[10] it is reasonable to suggest that the 3′-to-5′ translocation is attributed to the ssDNA translocase activity of the RecB motor. Within experimental resolution, no apparent change in translocation speed was observed. Our proposal of RecB as a 3′-to-5′ ssDNA translocase is consistent with previous biochemical studies[11,12] that RecBC with only one ATPase motor can cross a 3′-to-5′ gap.

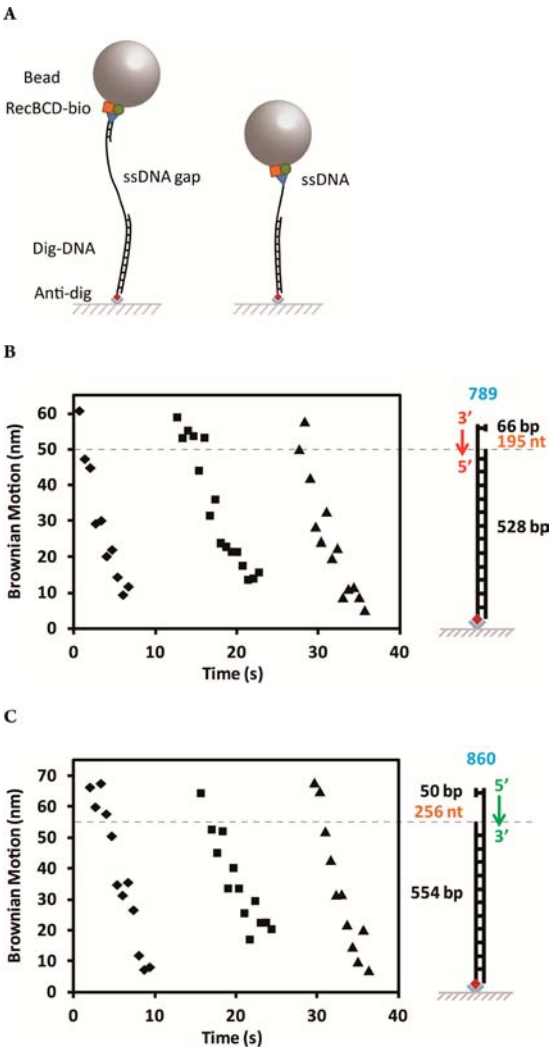

Figure 1. Observation of individual RecBCD helicases translocating along ssDNA using a tethered particle motion (TPM) experiment. (A) The DNA substrates contain a ∼200 nt long, unstructured ssDNA gap flanked by a double-stranded DNA end for RecBCD loading and were linked to the surface through a ∼500 bp dsDNA. Biotinylated RecBCD enzymes were attached to streptavidin beads visible under the optical microscope. Translocation occurred as the enzyme recognized the blunt-end DNA, and started unwinding. Brownian motion (BM) amplitude of the bead decreased as the enzyme translocated along DNA toward the surface. (B, C) Successful exemplary traces were observed as the enzyme translocated along the ssDNA region, across the ss/dsDNA junction, and to the surface end of the substrate (representative traces). Dashed lines referred to the BM amplitude at the ss/dsDNA junction following the ssDNA gap. Both DNA substrates containing either 3′-to-5′ ssDNA [(substrate 789, (B)] or 5′-to-3′ ssDNA [(substrate 860, (C)] showed successful enzyme translocation along a ssDNA region. Experiments were performed under the ATP concentration of 30 μM in the presence of the ATP regeneration system.

We also prepared the DNA substrate containing an ssDNA gap in the opposite direction to show that RecBCD will translocate in the 5′-to-3′ direction (substrate 860, Figure 1C). As shown in the exemplary BM time traces, RecBCD successfully translocated along this substrate and was capable of moving along the ∼500 bp dsDNA after the ss/dsDNA junction to the end of the surface-immobilized DNA (N = 33). Therefore, we concluded that RecBCD also possesses a 5′-to-3′ ssDNA translocase activity. Experiments including single-stranded DNA binding (SSB) proteins return with similar observation (Figure S10 in SI).

To verify the position of the ss/dsDNA junction at these substrates containing the ssDNA gap, we carried out a series of control experiments by putting a biotin label at the junction location (Figure S3A, SI). Control experiments showed the Brownian motion amplitude at the ss/dsDNA junction was 50.4 ± 3.6 nm for substrate 789 and 54.7 ± 5.3 nm for substrate 860 (dashed lines, Figure 1B and C). The Brownian motion for full-length substrates 789 and 860 are 55.2 ± 5.0 and 65.0 ± 8.0 nm, respectively. Brownian motion for the gapped substrates was found to be smaller than for duplex DNA with the same size, likely due to the fact that ssDNA is much more flexible (with a persistence length of <2 nm[13−15]) than that of dsDNA (persistence length ∼45−50 nm[16,17]). From the time traces, we could tell that the tether lengths had reduced as RecBCD translocated along the ssDNA region and across the junction. Translocation rates were determined by linear fitting of the Brownian time traces: substrate 789 (N = 22), 3′-to-5′ translocation, average rate = 5.0 ± 2.0 nm/s; substrate 860 (N = 33), 5′-to-3′ translocation, average rate = 4.7 ± 1.7 nm/s (Figure S4 in SI, at 30 μM ATP). The wide distribution of the histogram is consistent with previous studies and is likely due to the enzyme's heterogeneity.[18,19] The rates in either ssDNA polarities are indistinguishable and are similar to the duplex DNA unwinding rate, 5.6 ± 1.5 nm/s. This similarity in rates could be attributed to the limiting ATP concentration used here ($\ll K_M \approx 130$ μM for DNA unwinding[20]), as well as the experimental resolution. Nevertheless, we unambiguously observed the RecBCD direct translocation along the long ssDNA region (195−256 nt)

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that is much longer than the proposed large step size of the RecBC enzyme.[12] This ssDNA gap size is too long for the enzyme to step or jump over, so enzyme translocation along ssDNA is required.

2.2. RecBCD Translocation along Stretched ssDNA Substrates Using Flow TPM. Even though there is no apparent secondary structure in our prepared ssDNA gap region, we need to exclude the possibility that transient, nonspecific ssDNA loops could occur due to the flexibility of ssDNA which allows RecBCD to jump from one ss/dsDNA junction to another for all observed successful continuous translocation events. In order to rule out such looping possibility, a force was applied to the DNA substrates to remove potential, weak, transient interactions in the ssDNA region. If RecBCD were to jump across the ssDNA gap by these transient looping interactions, we would not see a continuous translocation but a pause or dissociation event at the first ds/ssDNA junction. The flow chamber for the flow TPM experiment was prepared as described previously.[21] Constant force was applied by a continuous laminar flow using a syringe pump. Experiments were performed under the flow of 0.5 mL/min (corresponds to 0.3 ± 0.05 pN in our experimental geometry). Higher force (1.5 pN) was also used, with similar behavior.

On the basis of the equipartition theorem, the tethered bead under constant force can be viewed as a damped pendulum,[22,23] and the mean potential energy of the bead perpendicular to the force direction is equal to the thermal energy, $1/2\ (F/l)\ \langle \Delta x^2 \rangle = 1/2\ k_B T$ (where F is the stretching force; l is the extension length; $\langle \Delta x^2 \rangle$ is the mean square displacement of the bead perpendicular to the force; k_B is the Boltzmann constant; T is the temperature in Kelvin). Here we set the force direction along the y-axis, so the bead motion on the x-axis is proportional to the tethered length. We can therefore monitor the bead's Brownian motion on the x-axis to determine the position of the enzyme on the substrate.

After anchoring the DNA samples in the chamber, RecBCD-beads were incubated with the substrate before applying force. The time traces showed that, in the presence of a stretching force, bead-labeled RecBCD showed continuous translocation along the anchored substrate 860 (Figure 2). 21 out of 26 time traces showed full translocation from the ssDNA region to the end to the end of dsDNA, while five traces showed bead dissociation from the substrate at the ss/dsDNA junction. Of all the traces, we did not observe any dissociation within the ssDNA region, which indicated that no jumping occurred during the ssDNA translocation. Similar patterns were also observed for substrate 789 (3′-to-5′ ssDNA orientation).

2.3. RecBCD Switched between Different DNA Strands. The ssDNA gap results showed that RecBCD is able to translocate along long ssDNA region, and move across the ss/dsDNA junction, with continuously translocation of the final duplex DNA in either ssDNA orientations. On the other hand, the RecBCD conformation after the ss/dsDNA junction is not known. Does RecBCD continuously translocate along dsDNA, engaging on only one strand of DNA? Or, will RecBCD re-engage both DNA strands, as in the case of unwinding duplex DNA? To address this question, we constructed DNA substrates containing two ssDNA regions with opposite orientations (Figure 3, and SI).

We started with the substrate 1655 (5′/3′) double-gapped DNA, which contains a 5′-to-3′ ssDNA region followed by a 3′-to-5′ ssDNA region in the direction of RecBCD translocation.

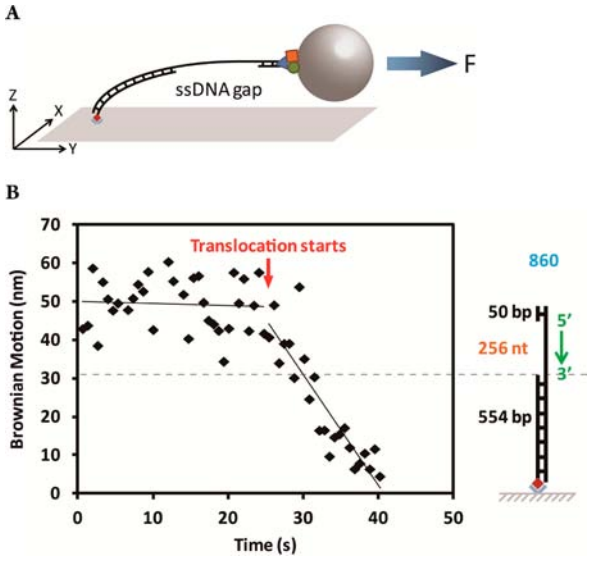

Figure 2. Observation of RecBCD translocating along ssDNA using a flow TPM experiment. (A) Gapped DNA substrate 860 was immobilized on the surface and incubated with RecBCD−bead complexes in the absence of ATP. A continuous buffer flow containing 30 μM ATP was applied along the y-axis (t = 0 s) to exert a constant stretching force (F = 0.3 ± 0.05 pN). Force was used to remove any potential, transient ssDNA interaction even though the sequence was designed to have no potential secondary structure. The bead Brownian motion perpendicular to the force direction was used to determine the enzyme position as it moved along the DNA substrate. (B) An exemplary time trace of Brownian motion during RecBCD translocation along a flow-stretched DNA substrate 860 (with 5′-to-3′ ssDNA region). Dashed lines referred to the BM amplitude at ss/dsDNA junction following the ssDNA gap. The successful trace showed continuous translocation across the ssDNA gap and dsDNA region.

Again, we have used control experiments to define the Brownian motion values at each region of the substrates (Figure S3B, SI). Full-length substrate 1655 has a BM of 83.2 ± 6.8 nm, and the second, third, and forth ss/dsDNA junctions have BMs of 58.3 ± 7.0, 40.9 ± 3.4, and 30.5 ± 2.9 nm, respectively. In the presence of RecBCD helicase, we observed either full translocation or translocation/dissociation of the enzyme. The time traces can be categorized into three different types: (a) full translocation, as in the case of full duplex DNA or single ssDNA gap substrates (fraction of 7/37); (b) full translocation with pause at BM ∼40 nm (fraction of 14/37); (c) translocation followed by enzyme dissociation at BM ∼40 nm (fraction of 14/37) (Figure 3A). For about 57% of the traces, we observed ((a) 19% and (b) 38%) that RecBCD carried out complete translocation from the beginning to the end of the DNA substrate, indicating RecBCD was able to translocate along first ∼300 bp dsDNA, 5′-to-3′ ssDNA, another ∼300 bp dsDNA, 3′-to-5′ ssDNA, and finally translocating to the end. The continuous translocation along two ssDNA gap regions directly showed that RecBCD can switch between ssDNA and dsDNA rapidly (as in type a). Traces of types b and c (76%) showed RecBCD pauses at the BM value ∼40 nm, which corresponds to the third junction (dashed line) at the end of the 5′-to-3′ DNA strand. Some of these pause events (∼38% of a total of 37 time traces) led to the dissociation of RecBCD from the DNA substrates. Pauses were identified by the peaks in the BM histograms (Figures S3B and S5 in SI).

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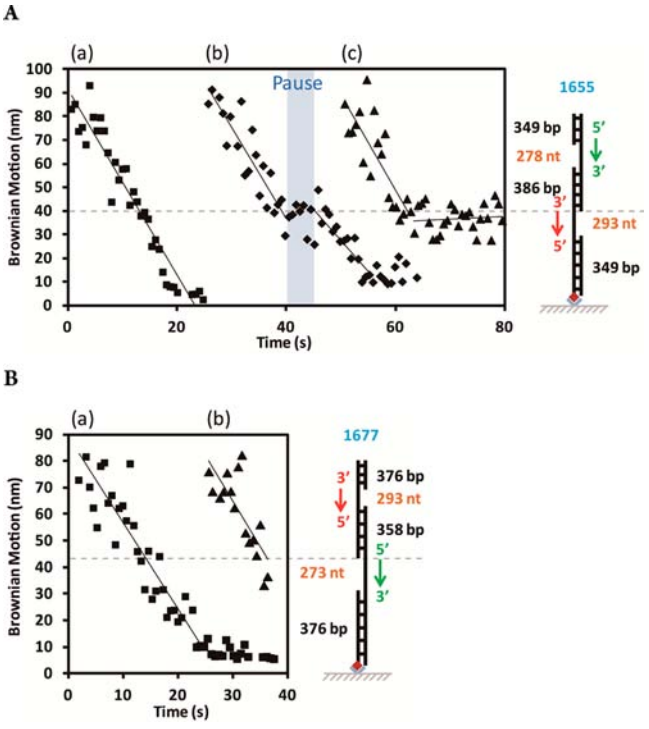

Figure 3. Time traces of individual RecBCD helicases translocating along DNA substrates containing two ssDNA gaps of opposite polarities. (A) RecBCD translocation along the double-gapped substrate 1655, with RecBCD first encountering a 5′-to-3′ ssDNA, followed by duplex DNA and then a 3′-to-5′ ssDNA region. Three translocation patterns were observed: (a) complete translocation with a single slope (7 traces out of 37, 19%), (b) complete translocation with a pause (14/37, 38%), (c) translocation followed by bead dissociation before the end of the DNA substrate (14/37, 38%). Two other traces cannot be categorized into these patterns. (B) RecBCD translocation along the double-gapped substrate 1677, with RecBCD first encountering a 3′-to-5′ ssDNA, following by duplex DNA and then a 5′-to-3′ ssDNA region. Two main patterns were observed: (a) complete translocation with a single slope (17/22, 77%) and (b) translocation with enzyme dissociated at the dashed line location (3/22, 14%). Two other traces cannot be categorized into these major patterns. Dashed lines referred to the BM amplitude at the ds/ssDNA junction shown.

We also constructed a similar double-gapped ssDNA substrate 1677 (3′/5′), containing a 3′-to-5′ ssDNA region followed by a 5′-to-3′ ssDNA region in the direction of RecBCD translocation (Figure 3B). Substrate 1677 (3′/5′), has a full length BM of 81.9 ± 8.7 nm, and BM values of 65.9 ± 4.5, 44.6 ± 4.3, and 33.4 ± 3.3 nm at the second, third, and forth junction, respectively (Figure S3B, SI). Among all the time traces, two types of time traces were observed. (a) Full translocation (fraction of 17/22). (b) Translocation followed by enzyme dissociation at the third junction (fraction of 3/22) (Figure 3B). Different from substrate 1655 (5′/3′), most substrate 1677 (3′/5′) time traces (type a, 77%) showed no pause during the continuous translocation (Figure S5B, SI), as in the case of duplex DNA translocation, with an averaged translocation rate of 4.8 ± 1.9 nm/s. Also, only 14% of RecBCD dissociated at the third junction before continuous translocation to the end of the DNA substrates for this substrates (type b).

3. DISCUSSION

As proposed in the previous structural study,[6] RecC functions as a watershed to separate duplex DNA into two ssDNA strands and to thread ssDNA into the RecB and RecD translocases. The unwinding activity of the RecBCD helicase could be the consequence of each helicase motor translocating along ssDNA, withdrawing both strands from the pin. Previous studies have proposed that many SF1 helicases are able to translocate along ssDNA.[24−28] RecBC was also found to have ssDNA translocation activities in the ensemble-averaged experiments.[11,12] Here we directly monitored the ssDNA translocase activities of the wild-type RecBCD using single-molecule tethered particle motion experiments. By using long ssDNA gap (195−293 nts) substrates without secondary structure, we directly observed the ssDNA translocation activities of RecBCD enzymes.

In the substrates containing single ssDNA gaps, we have observed both the 5′-to-3′ and 3′-to-5′ ssDNA translocation events of wild-type RecBCD. As soon as the enzyme bound to the DNA (indicated by the bead appearance), the enzyme translocates along the DNA substrate with a uniform translocation rate, without significant rate differences between the duplex or ssDNA regions. Successful translocation across long ssDNA gap region of different orientations directly proves that the RecBCD enzyme contains 3′-to-5′ and 5′-to-3′ ssDNA translocase activities, and most likely attributed to its two helicase motors, RecB (3′-to-5′ helicase) and RecD (5′-to-3′ helicase).[10]

We also challenged the enzyme with substrates containing two ssDNA gaps of opposite orientations. For example, in the case of substrate 1655 (5′-to-3′ followed by 3′-to-5′, Figure 4A), as the enzyme translocates on the 5′-to-3′ ssDNA strand, likely using its RecD motor, and reaches the junction of ss/dsDNA, two scenarios could occur. The top 3′-to-5′ strand could either be rethreaded into the empty RecB motor, so that RecBCD continues unwinding the rest of the duplex strand using both motors (Figure 4A, iii a); or the enzyme could possibly fail to recruit the top strand, leading to enzyme dissociation (Figure 4A, iii b). RecBCD responds differently to the two double-gapped substrates 1655 (5′-to-3′/3′-to-5′) and 1677 (3′-to-5′/5′-to-3′), as shown in Figure 3. Most traces in both substrates showed completed translocation (~57% for substrate 1655 and ~77% for substrate 1677). However, we observed a higher fraction of RecBCD tethers continuously translocated along the full length of substrate 1677 without pause (~77%, compared to ~19% in substrate 1655, Figure 3); and about 14% of RecBCD tethers dissociated at the third ss/dsDNA junction, with no pause observed. Comparing both translocation events along double-gapped DNA substrates (a 38% dissociation along substrate 1655 versus 14% dissociation along substrate 1677), it seems that the 5′-terminating ssDNA is more likely to be recruited by RecBCD than the 3′-terminating ssDNA after the first gap. This observation is likely to be attributed to the 5′-to-3′ ssDNA translocation activities of RecD (5′-to-3′ motor) and the secondary translocase of RecBC.[4,11] The secondary translocase activity of RecBC has been proposed to aid RecBCD translocation along duplex DNA, and to prevent RecBCD from dissociating when encountering any short ssDNA gap ahead of the unwinding substrates. Therefore, since RecD and RecBC both provide 5′-to-3′ translocation,[4] the enzyme can have higher processivity to recruit 5′-terminating ssDNA, which lowers the possibility of

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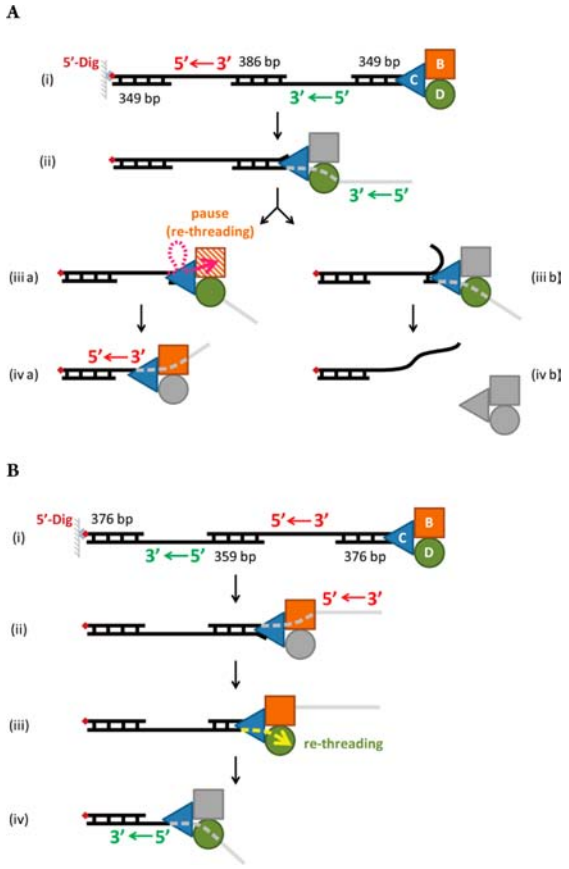

Figure 4. Working model of how RecBCD translocates along double-gapped DNA. (A) 5′-ssDNA to 3′-ssDNA. RecBCD started translocating along 5′-ssDNA using its 5′-to-3′ ssDNA translocase. When encountering ss/dsDNA junction (iii a and iii b), a pause was required for the 3′-ssDNA to be rethreaded into the 3′-to-5′ translocase channel. After the pause, the translocation switched to the 3′-to-5′ translocase to move along the 3′-ssDNA. (B) 3′-ssDNA to 5′-ssDNA. The 5′-to-3′ ssDNA translocase has a higher probability to rethread the 5′-ssDNA at the ss/dsDNA junction because no pause intermediate was observed. Starting from the 3′-ssDNA, the enzyme can easily recruit the 5′-ssDNA back to its 5′-to-3′ translocase channel and switch strands when it encounters the junction, and then uses the 5′-to-3′ translocase to translocate along 5′-ssDNA. Gray-colored regions indicate channels not binding DNA strands.

enzyme dissociation after a 5′-to-3′ gap. Under saturating ATP condition, the difference in the translocation rate of RecB and RecD motors could potentially contribute to the dissociation percentage as well.

We also observed pauses in the time traces for substrate 1655 (Figure 3A). In addition, the pause only occurs at the lower ds/ssDNA junction (BM ∼40 nm) but not at the upper ss/dsDNA junction (BM ∼60 nm). It might imply that the pause was caused by the 5′-to-3′ translocase waiting for the 3′-terminating ssDNA strand to be threaded back to the 3′-to-5′ channel. Since it has been shown that RecD is a faster motor than RecB,[10] it is also reasonable to speculate that when RecBCD switched from the 3′-terminated ssDNA end to the 5′-ssDNA, RecD would have to wait for RecB to reach the junction before dissociating from the 3′-end. Therefore, the pause could potentially reflect the time accounting for the rate difference between RecD and RecB. However, considering the limiting ATP concentration used in the experiments, no rate difference observed in the single-gapped 5′-to-3′ or 3′-to-5′ experiments, as well as the same translocation rate before and after the pause in the double-gapped experiments, pauses are unlikely caused by the translocation rate differences of the two motors. Considering the pause duration in average (5−20 s) is longer than the translocation time required for a 300 bp duplex DNA translocation (60 ± 15 bp/s, ∼5 s) (Figure S1, SI), the pause more likely represents the time required for the 3′-to-5′ ssDNA translocase recruiting ssDNA.

In the case of substrate 1655, if the 3′-to-5′ translocase is able to recruit the 3′-terminating ssDNA strand successfully right after RecBCD reaches the upper ss/dsDNA junction after the 5′-to-3′ ssDNA translocation, we should have observed no pause at all. Also, no pauses were observed in the single-gapped substrate 860 (Figure 1C). When RecBCD passed the 5′-to-3′ ssDNA region and enters the second ss/dsDNA junction in substrate 1655, either the 3′-ssDNA end was not correctly orientated to be threaded into the 3′-to-5′ translocase channel, or the 3′-to-5′ translocase alone cannot efficiently recruit 3′-ssDNA until the heterotrimeric enzyme encounters the lower third junction. Therefore, the pause duration must reflect the waiting time for the 3′-to-5′ translocase recruiting ssDNA. There is a possibility that the 3′-to-5′ translocase failed to recruit ssDNA in time, and the RecBCD complex dissociated (Figure 3A-c and Figure 4). Among the 28 paused traces (Figure 3A-b,c), we observed 14 tethers that dissociated. The fact that most tethers paused at the third junction in this substrate, and also half of the paused tethers dissociated supports our model that translocation along the 386 bp duplex region does not include the 3′-to-5′ translocase grabbing onto 3′-terminating ssDNA. As a consequence, the 5′-to-3′ ssDNA translocase activity during the 386 bp duplex region must be fully responsible for the unwinding of this duplex DNA region. This observation directly supports the model that unwinding is achieved by helicase translocating along ssDNA strand.

4. CONCLUSION

We directly visualized individual RecBCD helicase translocating along long, unstructured ssDNA substrates of different polarities. This proves that RecBCD functions as 3′-to-5′ and 5′-to-3′ ssDNA tranlocases. We also showed that RecBCD can successfully translocate along DNA substrates containing two ssDNA gaps of opposite polarities. It indicates that the enzyme switches between its 3′-to-5′ translocase and 5′-to-3′ translocase when encountering different polarities of ssDNA. These results provide supporting evidence that helicases unwind duplex DNA by translocating along ssDNA.

5. MATERIALS AND METHODS

5.1. DNA Substrate Preparation and Protein Purification. Preparations for the gapped substrates are described in details in SI (Figures S7−S9). Overexpression and purification of the biotinylated RecBCD enzyme were carried out as described previously.[21]

5.2. Single-Molecule TPM Experiments. Experimental conditions for slide and streptavidin beads (220 nm) preparations are the same as previously shown.[21,29,30] Time traces of bead-labeled RecBCD motions along DNA were recorded at 30 Hz using a Newvicon camera (DAGE-MTI). Bead centroid position at each frame is determined to nanometer precision by Gaussian fitting. The bead Brownian motion (BM) amplitude is defined as the standard deviation of bead center positions in 20 consecutive frames (0.67 s). Only tethers with symmetrical Brownian motion in x and y directions were analyzed to ensure single-molecule tethering. RecBCD translocation experiments were performed in 1× RecBCD reaction buffer (25 mM Tris-HCl, 1 mM magnesium acetate, 1 mM dithiothreitol, 80 mM sodium chloride,

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1 mg/mL BSA, pH 7.5) at 30 μM ATP in the presence of ATP regeneration system (20 units/mL pyruvate kinase (PK), 250 μM phosphoenolpyruvate (PEP)). Flow TPM experiment was done as previously described,[21] and a continuous laminar flow was achieved by using a syringe pump (KDS200; KD Scientific, Holliston, MA).

■ ASSOCIATED CONTENT

Supporting Information

Additional data and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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