

A Single Mutation, RecB_{D1080A}, Eliminates RecA Protein Loading but Not Chi Recognition by RecBCD Enzyme*

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Homologous recombination and double-stranded DNA break repair in *Escherichia coli* are initiated by the multifunctional RecBCD enzyme. After binding to a double-stranded DNA end, the RecBCD enzyme unwinds and degrades the DNA processively. This processing is regulated by the recombination hot spot, Chi (χ : 5'-GCTGGTGG-3'), which induces a switch in the polarity of DNA degradation and activates RecBCD enzyme to coordinate the loading of the DNA strand exchange protein, RecA, onto the single-stranded DNA products of unwinding. Recently, a single mutation in RecB, Asp-1080 \rightarrow Ala, was shown to create an enzyme (RecB_{D1080A}CD) that is a processive helicase but not a nuclease. Here we show that the RecB_{D1080A}CD enzyme is also unable to coordinate the loading of the RecA protein, regardless of whether χ sites are present in the DNA. However, the RecB_{D1080A}CD enzyme does respond to χ sites by inactivating in a χ -dependent manner. These data define a locus of the RecBCD enzyme that is essential not only for nuclease function but also for the coordination of RecA protein loading.

In *Escherichia coli*, the early steps of homologous recombination and dsDNA¹ break repair are catalyzed by the RecBCD enzyme and RecA protein (1). The RecBCD enzyme is heterotrimeric, being composed of the products from the *recB*, *recC*, and *recD* genes (for review see Ref. 2). The importance of these two enzymes is clearly illustrated by the behavior of their mutants; deletion of either the *recB* or *recC* gene reduces conjugal recombination proficiency by 100- to 1000-fold (3, 4), whereas a *recA* null mutation reduces it approximately 100,000-fold (5).

The central step in homologous recombination is catalyzed by RecA protein, which binds cooperatively to DNA and then promotes pairing and exchange between homologous DNA molecules (for review see Ref. 6). One requirement for this step, however, is that the donor DNA must possess a region of single-stranded character, because RecA protein binds poorly to dsDNA at physiological conditions. Initial binding of RecA protein is random, but subsequent cooperative binding extends

the RecA nucleoprotein filament in the 5' \rightarrow 3' direction (7). A result of this polar extension of the RecA nucleoprotein filament is that 3'-ssDNA ends are more likely to be coated with RecA protein than 5'-ssDNA ends and, therefore, are more recombinogenic (8). The processing of a dsDNA break into a form suitable for RecA protein binding is catalyzed by the RecBCD enzyme.

The RecBCD enzyme is both a helicase and a nuclease, and it initiates recombinational repair from dsDNA ends. After binding to an end, RecBCD enzyme processively unwinds and degrades the DNA, unwinding up to 30 kilobase pairs per binding event (9). Degradation of the DNA is asymmetric, with the 3'-terminal strand being degraded much more vigorously than the 5'-terminal strand (10, 11).

DNA processing by the RecBCD enzyme is regulated by an eight-base DNA element, Chi (χ ; 5'-GCTGGTGG-3') (12–15). χ was originally identified *in vivo* as a recombination hot spot that stimulates homologous recombination about 10-fold in its vicinity. *In vitro* analysis revealed that χ elicits this response by regulating a number of RecBCD enzyme activities. When a translocating RecBCD molecule recognizes a χ site, the enzyme pauses, and DNA degradation on the 3'-terminal strand is attenuated (10). The enzyme then continues unwinding the DNA but now degrades the 5'-terminal strand preferentially (16, 17) (Fig. 1). On DNA molecules longer than the processive translocation length, processing by RecBCD enzyme creates a long, 3' ssDNA overhang, which is both a common early intermediate in homologous recombination and an ideal substrate for RecA protein.

In addition to regulating the nuclease properties of the RecBCD enzyme, χ also induces the RecBCD enzyme to coordinate the loading of RecA protein onto the ssDNA downstream of χ (18). The initial binding of RecA protein to ssDNA is slow (19), and RecA protein cannot compete efficiently with other ssDNA-binding proteins (20, 21). Facilitation of RecA protein binding to ssDNA by RecBCD enzyme overcomes this inhibition (18). Reconstitution of *in vitro* recombination reactions using RecA protein, RecBCD enzyme and single-stranded DNA binding (SSB) protein established that these proteins catalyze efficient pairing between χ -containing dsDNA and homologous supercoiled DNA, forming a recombination intermediate known as a D-loop (10).

The domains responsible for the individual activities of the RecBCD enzyme are just starting to be elucidated. The RecBC enzyme (without the RecD subunit) is a very processive helicase but has no significant nuclease activity (16, 22, 23). Recently, it was shown that the RecBC enzyme also coordinates the loading of RecA protein and that this loading is independent of χ . Instead, RecBC enzyme constitutively loads RecA protein onto the DNA strand that terminates 3' at the dsDNA end at which RecBC enzyme entered (24).

The fact that RecBC enzyme has little nuclease activity

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¹ The abbreviations used are: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; SSB, *E. coli* single-stranded DNA-binding protein; χ° , non- χ -containing; χ^{+} , χ -containing; ATP γ S, adenosine 5'-O-(thiotriphosphate).

indicates that the RecD subunit plays an essential role in DNA degradation. However, analysis of mutations in the RecB subunit establishes that the C-terminal domain of this enzyme is absolutely essential for all nuclease activities of the RecBCD enzyme (25, 26). Furthermore, fusion of the C-terminal domain of the RecB subunit with the DNA binding domain of T4 phage gene 32 protein creates a nonspecific nuclease, showing that the C terminus of RecB subunit is indeed a nuclease. Finally, a single point mutation in the putative Mg²⁺ binding site of the RecB subunit, Asp-1080 → Ala, creates a holoenzyme (RecB_{D1080A}CD) that behaves much like the RecBC enzyme; it is a processive helicase with no measurable nuclease activity.

Here we show that despite being an efficient helicase, the RecB_{D1080A}CD enzyme is unable to load RecA protein. This inability to load RecA protein is independent of whether the processed DNA contains χ sites. Although the RecB_{D1080A}CD enzyme does not load RecA protein in response to χ , it can still recognize χ : χ -containing DNA is processed at a slower rate than DNA without χ . These data provide the first insight into the domain that is responsible for either 1) transmitting the χ recognition event into the enzymatic alterations that are necessary for proper RecBCD enzyme function or 2) RecA protein loading.

EXPERIMENTAL PROCEDURES

Enzymes—RecBC enzyme was purified as described (24). RecBC enzyme concentration was determined using an extinction coefficient of $3.6 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ at 280 nm, derived by adding the respective extinction coefficients for the individual subunits (23). No contaminating protein bands were detected when 1 μg of protein was loaded on an SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. RecB_{D1080A}CD enzyme was a gift from D. A. Julin and M. Yu (University of Maryland) (26). *E. coli* SSB protein was isolated from strain RLM727 and purified according to LeBowitz (27). Protein concentration was determined using an extinction coefficient of 3.0×10^4 at 280 nm. RecA protein was purified using a procedure based on spermidine precipitation² (28). Protein concentration was determined using an extinction coefficient of $2.7 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ at 280 nm.

All restriction endonucleases and DNA-modifying enzymes were purchased from New England BioLabs. The enzymes were used according to Sambrook *et al.* (29) or as indicated by the vendor.

DNA Substrates—The plasmids pBR322 χ^o (wild type) and pBR322 χ^+ F225 (14) were prepared from strains S819 and S818, respectively, provided by G. R. Smith and A. F. Taylor. Plasmid pBR322 χ 3F,3H was created by ligation of the oligonucleotide linker 5'-ATCTAGACCACC-AGCCAGCGGTGCCACCAGCTCAGCATCGACCACCAGCTCGA-GTGCA-3' into the *Pst*I-*Ase*I site of pBR322 χ^o (Chi sites are shown in bold), followed by insertion of a linker 5'-GTCATTAATGCTGGTGG-GCGCAGACTCGCTGGTGGTTCACATGGCGGCTGGTGGCTGCAG-3' into the second (position 1480) *Pvu*MI site of the plasmid. All plasmid DNAs were purified by cesium chloride density gradient centrifugation (29). The molar concentration of the dsDNA in nucleotides was determined using an extinction coefficient of $6290 \text{ M}^{-1} \text{ cm}^{-1}$ at 260 nm. Plasmid DNA was linearized with *Nde*I and radioactively labeled at the 5' end by sequential reactions with shrimp alkaline phosphatase followed by T4 polynucleotide kinase and [γ -³²P]ATP (NEN Life Science Products) using methods given by the vendor or by Sambrook *et al.* (29).

Reaction Conditions—The coupled RecA-RecBCD reactions were conducted in the presence of 25 mM Tris acetate (pH 7.5), 8 mM magnesium acetate, 5 mM ATP, 1 mM dithiothreitol, 1 mM phosphoenolpyruvate, 4 units/ml pyruvate kinase, 40 μM (nucleotides) 5' end-labeled *Nde*I-linearized dsDNA, 80 μM (nucleotides) supercoiled DNA, 20 μM RecA protein, 8 μM SSB protein, 2.25 nM total RecB_{D1080A}CD enzyme (corresponding to 0.25 RecB_{D1080A}CD enzyme molecules/linear dsDNA end), or 24.6 nM total RecBC enzyme (20% active, corresponding to 0.53 functional RecBC enzyme molecules/linear dsDNA end) (10, 18). RecB_{D1080A}CD and RecBC enzyme concentrations were chosen to provide approximately equal amounts of helicase units. Assays were performed at 37 °C.

Exonuclease protection assays were initiated by the addition of RecBC or RecB_{D1080A}CD enzyme after preincubation of all standard

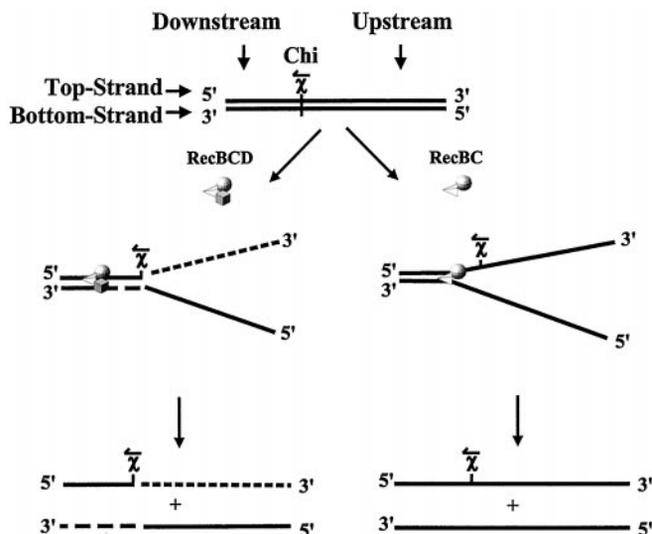


FIG. 1. Processing of dsDNA containing a χ site by the RecBCD enzyme. The arrow above the χ site indicates the direction that RecBC or RecBCD enzyme must travel in order to recognize χ . The region of dsDNA between χ and the entry site of the enzyme is the “upstream” region, and the region between χ and the opposite end is the “downstream” region. The strand of DNA that terminates 3' at the entry site for the enzyme is the top-strand; the opposite strand is the bottom-strand. RecBC enzyme has no nuclease activity; thus processing results in two full-length ssDNA products. RecBCD enzyme unwinds and degrades 3' → 5' upstream of χ . After χ , a switch in the polarity of exonuclease degradation to 5' → 3' leads to the production of both a bottom-strand, upstream χ -specific fragment and a top-strand, downstream χ -specific fragment. Adapted from Anderson and Kowalczykowski (18).

components except supercoiled DNA for 2 min. After 3 min, a mixture of poly(dT) and ATP γ S was added to a final concentration of 200 μM (nucleotides) and 5 mM, respectively. After 1 min of incubation, exonuclease I was added to a concentration of 100 units/ml (18, 24).

Unwinding reactions were performed in the presence of 25 mM Tris acetate (pH 7.5), 1 or 8 mM magnesium acetate, 5 mM ATP, 1 mM dithiothreitol, 1 mM phosphoenolpyruvate, 10 μM (nucleotides) linear (2.3 nM dsDNA ends) 5' end-labeled dsDNA, and 2 μM SSB protein. Reactions were preincubated for 2 min at 37 °C followed by addition of RecBCD (0.046 nM) or RecB_{D1080A}CD (0.23 nM) enzyme. Assays were performed at 37 °C.

Analysis of Reaction Products—Aliquots of the reaction mixture (20 μl) were taken at the indicated time points and added to 20 μl of stop buffer (0.125 M EDTA, 2.5% SDS, 10% Ficoll, 0.125% bromophenol blue, and 0.125% xylene cyanol) to halt the reaction and to deproteinize the sample. This was followed by the addition of 1.5 μl of 600 units/ml proteinase K and incubation at 37 °C for 5 min. Samples were electrophoresed in 1% agarose gels for approximately 15 h at 1.4 V/cm in TAE (40 mM Tris acetate (pH 8.0), 2 mM EDTA). The gels were dried onto DE-81 paper (Whatman) and analyzed on a Molecular Dynamics Storm 840 PhosphorImager using Image-Quant software.

RESULTS

RecB_{D1080A}CD Unwinds Linear DNA but Does Not Promote RecA-dependent Joint Molecule Formation

—Since RecBC enzyme is a helicase with no significant nuclease activity, processing of linear dsDNA by the RecBC enzyme produces two full-length ssDNA molecules. For convenience, we refer to the strand of DNA terminating 3' at the entry point of RecBC or RecBCD enzyme as the “top-strand” and the complementary strand of DNA as the “bottom-strand” (Fig. 1) (17). Recently it was shown that the RecBC enzyme coordinates the loading of RecA protein onto the top-strand of DNA during unwinding (24). This facilitated loading results in the efficient production of the RecA nucleoprotein complex, which mediates pairing between the top-strand DNA and a homologous supercoiled DNA to form joint molecules (24). Like RecBC enzyme, RecB_{D1080A}CD enzyme possesses helicase activity but no sig-

² S. C. Kowalczykowski, unpublished data.

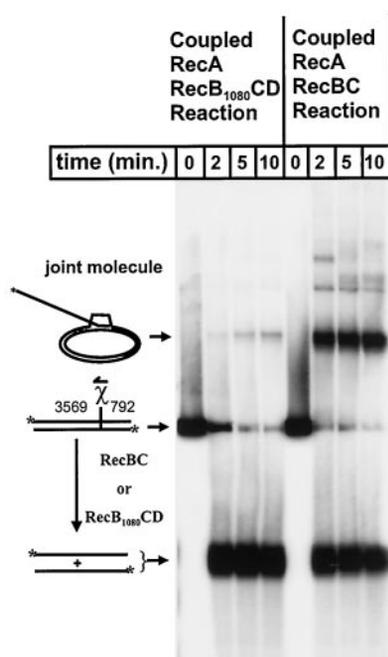


FIG. 2. The RecB_{D1080A}CD enzyme does not support RecA protein-dependent joint molecule formation in coupled RecA-RecB_{D1080A}CD pairing reactions. Coupled RecA-RecB_{D1080A}CD pairing reactions were performed as described (see “Experimental Procedures”). The location of the χ site is given in nucleotides. Reactions were started by the addition of either RecB_{D1080A}CD or RecBC enzymes. Full-length dsDNA, ssDNA, and the resultant joint molecule are indicated.

nificant nuclease activity (26). Furthermore, χ does not induce nuclease activity in either enzyme³ (26). Thus, processing of linear DNA by either RecB_{D1080A}CD or RecBC enzyme produces only full-length ssDNA.

In Fig. 2, we tested the ability of RecB_{D1080A}CD enzyme to process linear dsDNA into substrates suitable for joint molecule formation by RecA protein. Linear pBR322 χ^+ F (which contains a χ site) and homologous supercoiled DNA were incubated with RecA protein and SSB protein, and then either RecB_{D1080A}CD enzyme or RecBC enzyme was added to start the reaction. After 2 min, the dsDNA was almost completely unwound by both RecB_{D1080A}CD enzyme and RecBC enzyme (Fig. 2). However, only 1% of the ssDNA produced by RecB_{D1080A}CD enzyme was incorporated into a joint molecule. In contrast, 36% of the ssDNA produced by RecBC enzyme was incorporated into joint molecules after 2 min. In the next section, we show that this dramatic difference reflects an inability of RecB_{D1080A}CD enzyme to load RecA protein.

RecB_{D1080A}CD Enzyme Cannot Load RecA Protein, Regardless of χ —To test whether RecB_{D1080A}CD enzyme can load RecA protein onto the ssDNA products of unwinding, we examined the sensitivity of these unwinding products to exonuclease I degradation (18, 24). If the ssDNA products were coated with SSB protein, then they were sensitive to the 3' \rightarrow 5' exonuclease activity of exonuclease I (30). However, if instead RecA protein was loaded onto the ssDNA, then they were resistant to exonuclease I after stabilization with ATP γ S (18). In this assay, the linear dsDNA was 5' end-labeled and incubated with RecA protein and SSB protein. Either RecB_{D1080A}CD enzyme or RecBC enzyme was added and incubated for 3 min. Last, a mixture of excess poly(dT) and ATP γ S was added. The addition of the nonhydrolyzable ATP analog, ATP γ S, induced a high

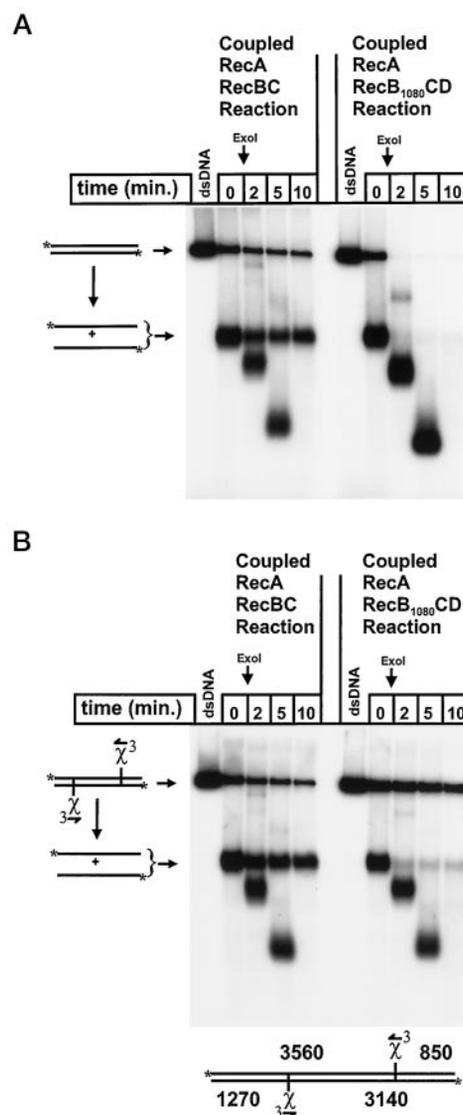


FIG. 3. The RecB_{D1080A}CD enzyme does not load RecA protein onto ssDNA, regardless of χ . Exonuclease I protection assays were performed as described (see “Experimental Procedures”). After preincubation of RecA and SSB proteins with the DNA, the reactions were started by the addition of RecB_{D1080A}CD or RecBC enzymes. The reactions were performed with either pBR322 χ^0 (A) or pBR322 χ^3 F,3H (B) DNA. The approximate location of the χ sites (B) in the nucleotides is indicated. The lane marked dsDNA represents the reaction before the addition of RecB_{D1080A}CD or RecBC enzymes. Zero time represents the time of initiation of unwinding but before the addition of exonuclease I; exonuclease I was added immediately after the 0 time point. Full-length dsDNA and ssDNA are indicated.

affinity DNA-binding state in RecA protein (31). This stabilized any bound RecA protein, whereas the addition of excess poly(dT) sequestered the free RecA and SSB protein. After another minute of incubation, the presence of RecA or SSB protein on the 3' ends of the ssDNA was detected by either protection or enhancement of exonuclease I degradation, respectively.

Because wild-type RecBCD enzyme requires activation by χ to load RecA protein, we compared the RecA-loading properties of RecB_{D1080A}CD enzyme with DNA either devoid of or containing χ (Fig. 3). RecA loading was examined using *Nde*I-linearized pBR322 χ^0 (which has no χ sites; Fig. 3A) and *Nde*I-linearized pBR322 χ^3 F,3H (which has 6 χ sites; Fig. 3B). When χ^0 DNA is unwound by RecB_{D1080A}CD enzyme in the presence of RecA and SSB proteins, only 2% of the resultant ssDNA was resistant to exonuclease I degradation (Fig. 3A, 10 min time

³ J. C. Churchill and S. C. Kowalczykowski, unpublished observations.

point). In contrast, 48% of the ssDNA produced by RecBC enzyme is protected from exonuclease I. This pattern is unaffected by the presence of χ in the dsDNA; 6% of the ssDNA was protected when unwound by RecB_{D1080A}CD enzyme, and 44% of the ssDNA was protected when unwound by RecBC enzyme. These data show that, as expected, RecBC enzyme loads RecA protein only onto the top-strand, since approximately one-half of the ssDNA was protected from exonuclease I digestion (24). In contrast, the mutant RecB_{D1080A}CD enzyme was completely defective in RecA loading, as defined by this 3' end protection assay. Since the χ sites were not at the ends of the DNA tested, it was possible that RecB_{D1080A}CD enzyme might have started loading RecA protein only after it reached a χ site. Had this been the case, cooperative binding would have rapidly extended the RecA nucleoprotein filament to the 3' end of the top strand, resulting in exonuclease protection of the entire top-strand ssDNA molecule. Instead, no protection of either strand was observed. Alternatively, if RecA protein was loaded at χ , but extension of the nucleoprotein filament was inhibited for some unknown reason, exonuclease I degradation of the region of ssDNA upstream (3') of χ would have produced a ssDNA fragment of the same size as the top-strand, downstream χ -specific fragment (see Fig. 1) (17). No fragments of this size were observed (Fig. 3B). Thus, we conclude that the RecB_{D1080A}CD enzyme cannot load RecA protein regardless of the presence of χ .

RecB_{D1080A}CD Enzyme Recognizes χ —In Fig. 3, we showed that the RecB_{D1080A}CD enzyme is incapable of loading RecA protein, even when the DNA contains χ sites. One possible explanation for this failing is that χ recognition is required for activation of RecA loading, and the RecB_{D1080A}CD enzyme cannot recognize χ . The traditional way to examine χ recognition *in vitro* is to examine the production of χ -specific ssDNA fragments. However, since the RecB_{D1080A}CD enzyme lacks nuclease activity, it does not produce χ -specific fragments (26), (Fig. 3B). Nevertheless, in the absence of χ -specific fragment formation, it is still possible to detect χ recognition using the phenomenon of χ -dependent inactivation. Under conditions of low free Mg²⁺, wild-type RecBCD enzyme is inactivated after encountering a χ site while translocating through dsDNA (32). Therefore, the rate and extent for unwinding of χ -containing DNA by the RecB_{D1080A}CD enzyme was examined at low free Mg²⁺ concentrations. The concentration of free Mg²⁺ was controlled by varying the relative concentrations of Mg²⁺ and ATP (33). The unwinding of 5' end-labeled *Nde*I-linearized pBR322 χ^0 (which has no χ sites) was compared with unwinding of 5' end-labeled *Nde*I-linearized pBR322 χ 3F,3H (which has 6 χ sites). We observed that at all protein concentrations, the initial rates were approximately equal regardless of the presence of χ (Fig. 4A). However, the rate of unwinding of χ -containing DNA slowed later in the reaction. This difference was not as dramatic as that seen for wild-type RecBCD enzyme (Fig. 4B), which was completely inactivated after encountering χ sequences during unwinding.

Since the RecA-loading properties of the RecB_{D1080A}CD enzyme were tested at a "high" (8 mM) concentration of magnesium ion, the inactivation reactions were repeated in the presence of 8 mM magnesium (Fig. 5). As expected from previous work (32), wild-type RecBCD enzyme unwound both χ and non- χ -containing DNA completely, although the χ -containing DNA was unwound slightly slower. Surprisingly, the RecB_{D1080A}CD enzyme was completely inactivated by the processing of χ -containing DNA. This reaction was repeated at several different protein concentrations with the same results; unwinding of linear dsDNA containing χ inactivates the RecB_{D1080A}CD enzyme at high magnesium acetate concentrations (data not shown). These data show that the RecB_{D1080A}CD enzyme

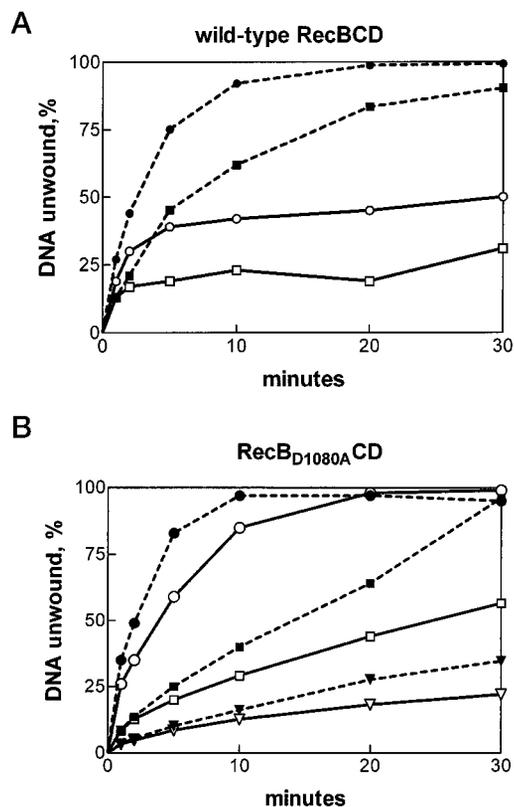


FIG. 4. The presence of χ sites slows unwinding by the RecB_{D1080A}CD enzyme at low magnesium ion concentrations. Unwinding reactions were performed at 1 mM Mg²⁺ as described (see "Experimental Procedures"). Reactions were started by the addition of RecB_{D1080A}CD (A) or RecBCD (B) enzymes. The percent unwound is calculated relative to the amount of dsDNA present before the addition of enzyme. A: ●, 150 pM χ^0 ; ■, 75 pM χ^0 ; ○, 150 pM χ^+ ; □, 75 pM χ^+ . B: ●, 660 pM χ^0 ; ■, 190 pM χ^0 ; ▼, 75 pM, χ^+ ; ○, 660 pM χ^+ ; □, 190 pM χ^+ ; ▽, 75 pM χ^+ .

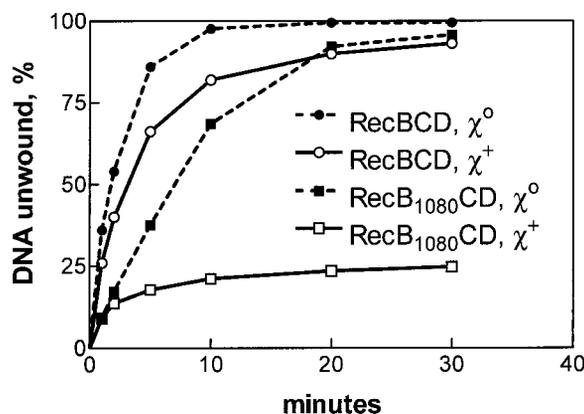


FIG. 5. RecB_{D1080A}CD enzyme is inactivated by χ at the high magnesium ion concentrations used for DNA pairing. Unwinding reactions were performed at 8 mM Mg²⁺ as described (see "Experimental Procedures"). The reactions were started by the addition of 0.23 nM RecB_{D1080A}CD or 0.046 nM RecBCD enzymes. The percent unwound is calculated relative to the amount of dsDNA present before the addition of enzyme.

can recognize χ , although this recognition leads to inactivation at high magnesium ion concentrations.

DISCUSSION

The mechanism by which RecA protein is loaded onto ssDNA by RecBCD enzyme remains unclear. However, our analysis of the RecB_{D1080A}CD enzyme reveals an important

role for the RecB subunit in the mediation of RecA protein loading. We show that unlike the RecBC enzyme, which is also a processive helicase with no significant nuclease activity, the RecB_{D1080A}CD enzyme cannot promote efficient D-loop formation in reconstituted recombination reactions (Fig. 2). Despite the absence of nuclease activity and the presence of helicase function, the RecB_{D1080A}CD enzyme does not stimulate joint molecule formation even if χ is present in the DNA. This inability to promote D-loop formation results from an inability of RecB_{D1080A}CD enzyme to load RecA protein onto the unwound ssDNA (Fig. 3). In contrast, RecBC protein promotes the loading of RecA protein onto about 50% of the resultant ssDNA, which is consistent with its ability to load RecA protein asymmetrically onto the top-strand of DNA during unwinding.

Since χ recognition is required for RecA loading by wild-type RecBCD enzyme, one possible explanation for the inability of the RecB_{D1080A}CD enzyme to load RecA protein is that it simply cannot recognize χ . To test this possibility, we examined whether RecB_{D1080A}CD enzyme, like RecBCD enzyme, inactivates in response to χ . Using the χ -dependent inactivation assay, we established that unwinding by RecB_{D1080A}CD enzyme is slowed in the presence of χ (Fig. 4). This effect is not as dramatic as that seen with wild-type RecBCD enzyme, which is completely inactivated by χ . Unexpectedly, the RecB_{D1080A}CD enzyme is inactivated by χ in the presence of high Mg²⁺ (Fig. 5). In contrast, processing of DNA by wild-type RecBCD enzyme was only slightly slower when the DNA contained χ sites. These results establish that RecB_{D1080A}CD enzyme can indeed recognize χ , although its response to χ is somewhat different than for the wild-type enzyme.

Inactivation of RecBCD enzyme in response to χ at low concentrations of Mg²⁺ does not cause the enzyme to dissociate from the DNA at χ (32). Rather, RecBCD enzyme continues to process the DNA until it reaches the end of the DNA molecule; however, this χ -modified enzyme is unable to re-initiate unwinding on another DNA molecule. Examination of RecB_{D1080A}CD enzyme products after exonuclease treatment (Fig. 3) suggests that RecB_{D1080A}CD enzyme does not dissociate at χ either. If RecB_{D1080A}CD enzyme had dissociated at χ , a Y-shaped, partially unwound dsDNA structure would have formed. The subsequent degradation by exonuclease I would have resected the 3'-terminating ssDNA strand, thereby producing dsDNA with a 5' overhanging end. DNA of this type would be resistant to both RecBCD enzyme and exonuclease I (17), and hence, stable, but no intermediates of this type were observed. Thus, in this sense, the inactivation of RecB_{D1080A}CD enzyme observed at high Mg²⁺ is similar to that observed by RecBCD enzyme at low Mg²⁺; recognition of χ does not appear to cause dissociation of the RecB_{D1080A}CD enzyme from the DNA. Rather, χ -induced inactivation prevents the enzyme from starting a new round of DNA processing.

As previously mentioned, Asp-1080 in RecB protein was originally characterized as an essential component of the nuclease domain (26). Comparison with other nucleases suggested that it is required for binding of Mg²⁺ in the active site. Weaker binding of Mg²⁺ by RecB_{D1080A}CD enzyme can explain the χ -dependent inactivation observed at an unexpectedly high concentration of Mg²⁺ (Fig. 5). More importantly, however, our data illuminate a previously unknown additional role for this domain by establishing that it is also required for RecA protein loading. Even though RecB_{D1080A}CD enzyme can recognize χ by at least one measure and can continue unwinding past the χ site, it is unable to load RecA protein onto the resultant ssDNA. We believe there are two molecular interpretations of this data. 1) Asp-1080 in RecB is required to transmit the χ -recognition

signal to whatever element is responsible for RecA protein loading, or 2) Asp-1080 is an essential component of the RecA protein-loading machinery. Consistent with our observation that the C-terminal domain of the RecB subunit is important for RecA protein loading, analysis of the truncated RecB₁₋₉₂₉C enzyme, which has 30 kDa deleted from the C terminus, shows that it is also an efficient helicase that cannot load RecA protein.⁴ In addition, recent characterization of the RecB(Thr807Ile)CD enzyme (also known as RecB²¹⁰⁹CD enzyme) shows that it too is unable to load RecA protein.⁵ Thus, it is clear that the RecB subunit plays a central role in the loading of RecA protein onto ssDNA.

It remains unclear what role RecD subunit plays in RecA protein loading. RecBC enzyme loads RecA protein constitutively, whereas RecB_{D1080A}CD enzyme cannot load RecA protein at all. One interpretation of these results is that the RecD subunit represses the RecA protein-loading activities of the RecBCD enzyme, and only following its inactivation is RecA protein-loading manifest. However, this simple interpretation is complicated by the observation that RecB₁₋₉₂₉C enzyme cannot load RecA protein either. This shows that mutations in the RecB subunit can directly affect the RecA protein-loading properties of the enzyme. It will be interesting to test the RecA protein-loading properties of RecB_{D1080A}C enzyme to further clarify the controlling role of the RecD subunit. The exact nature of the facilitated loading of RecA protein and its relationship to the nuclease activity of the RecB subunit remains to be determined.

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