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Identification of the RecA Protein-loading Domain of RecBCD Enzyme

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Genetic recombination in *Escherichia coli* is stimulated by the recombination hotspot Chi (χ), a regulatory element that modifies the activities of the RecBCD enzyme and leads to loading of the DNA strand exchange protein, RecA, onto the χ-containing DNA strand. The RecBC enzyme, which lacks the RecD subunit, loads RecA protein constitutively, in a manner that is independent of χ. Using a truncated RecBC enzyme lacking the 30 kDa C-terminal domain of the RecB subunit, we show that this domain is necessary for RecA protein-loading. We propose that this domain harbors a site that interacts with RecA protein, recruiting it to single-stranded DNA during unwinding. This ability of a translocating enzyme to deliver material (RecA protein) to a specific target site (the χ sequence) parallels that of other cellular motor proteins.

Keywords: RecBCD enzyme; RecA protein; Chi sequence; recombination; helicase

Chi (χ) sites are *cis*-acting sequences (5′-GCTGGTGG-3′) that stimulate homologous recombination through the RecBCD-pathway of *Escherichia coli*. These sites play a critical role in processes such as conjugation, transduction, and the repair of double-stranded DNA (dsDNA) breaks that arise either during DNA replication (Kuzminov, 1995; Michel *et al*., 1997) or as a result of DNA damage (for a review, see Friedberg *et al*., (1995)). The RecBCD enzyme consists of three non-identical subunits, RecB, RecC and RecD. The holoenzyme is both a processive helicase, and an ATP-dependent nuclease. The enzyme initiates the process of homologous recombination by unwinding dsDNA, thus providing a suitable ssDNA substrate for the DNA strand-exchange protein, RecA (Dixon & Kowalczykowski, 1991; Roman & Kowalczykowski, 1989; Taylor & Smith, 1985). However, for RecBCD enzyme to be productive in recombination, the destructive nuclease activity must be carefully controlled.

χ exerts its regulatory effects by controlling two important aspects of RecBCD enzyme function (Figure 1): χ controls degradation of DNA by regulating the enzyme's asymmetric nuclease activities (Anderson & Kowalczykowski, 1997a; Dixon & Kowalczykowski, 1991, 1993), and it induces the loading of RecA protein onto ssDNA by RecBCD enzyme during unwinding (Anderson & Kowalczykowski, 1997b). This facilitated loading allows RecA protein to overcome a competitive disadvantage against single-stranded DNA binding (SSB) protein for binding to single-stranded DNA (ssDNA) (Kowalczykowski, 1991; Kowalczykowski *et al*., 1987; Kowalczykowski & Krupp, 1987).

RecBCD enzyme loads RecA protein asymmetrically, so that only the χ-containing strand of DNA (the ''top-strand'') becomes coated within a filament of RecA protein. Attenuation of RecBCD enzyme nuclease activity, coupled with the specific loading of RecA protein, leads to the preferential incorporation of this χ-containing ssDNA into pairing products that are formed with homologous supercoiled DNA (Anderson & Kowalczykowski, 1997b).

The RecBC enzyme (i.e. without the RecD subunit) is a proficient helicase, and is characterized by the virtual absence of dsDNA exonuclease activity. Recently RecBC enzyme was shown to mimic the behavior of χ-modified RecBCD enzyme, by loading RecA protein onto ssDNA during unwinding (Churchill *et al*., 1999). In contrast to the holoenzyme, however, RecA protein-loading by RecBC enzyme is constitutive, not

Abbreviations used: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; χ, 5′-GCTGGTGG-3′; SSB protein, single-stranded DNA-binding protein.

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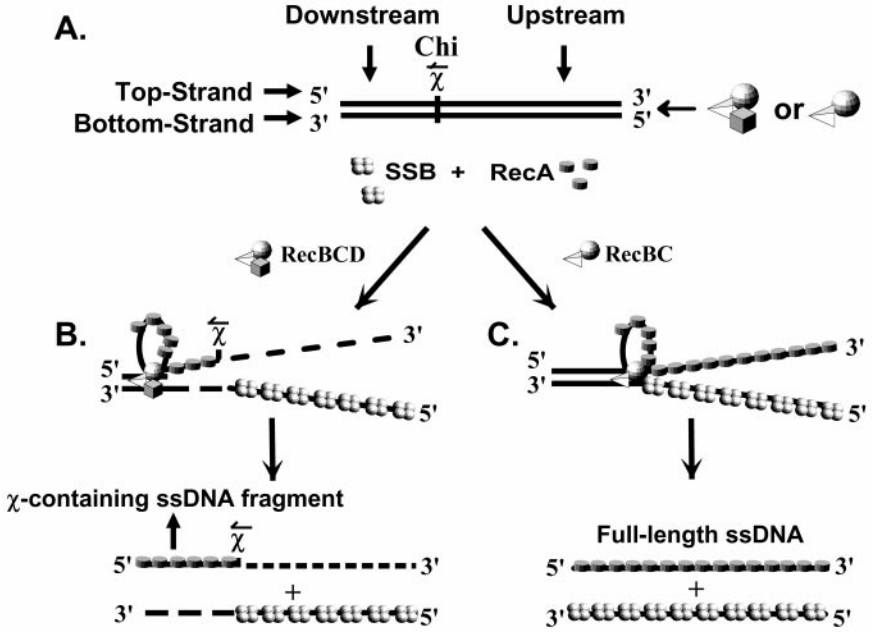

Figure 1. Loading of RecA protein onto ssDNA by RecBCD holoenzyme and RecBC enzyme. (a) The region of dsDNA between χ and the entry site of RecBCD enzyme is referred to as the ''upstream'' region, and the region between χ and the opposite end is termed the ''downstream'' region. RecBCD (or RecBC) enzyme enters the dsDNA at an end, and begins unwinding. (b) For RecBCD enzyme, χ-recognition results in a switch in the polarity of exonuclease degradation: 3′ → 5′ nuclease activity is attenuated, and 5′ → 3′ activity is activated. Two χ-specific fragments are produced. SSB protein outcompetes RecA protein for binding to the bottom-strand, upstream χ-containing fragment, but RecBCD enzyme facilitates the loading of RecA protein onto the top-strand, downstream χ-containing fragment. (c) RecBC enzyme is essentially devoid of dsDNA nuclease activity, and generates only full-length ssDNA from both strands. RecBC enzyme facilitates the loading of RecA protein onto the top-strand ssDNA, in a manner that is independent of the presence or absence of χ.

requiring activation by χ (Figure 1). Furthermore, RecBC enzyme loads RecA protein only onto the strand terminating 3′ at the enzyme's dsDNA entry site; this behavior is analogous to the asymmetric loading of RecA protein specifically onto the χ-containing strand by the holoenzyme (Figure 1).

Proteolysis experiments revealed that the RecB subunit consists of two discrete domains, a 100 kDa N-terminal domain, and a 30 kDa C-terminal domain (Yu *et al.*, 1998a). The N-terminal domain ($RecB_{1\text{-}929}$) can be reconstituted with RecC and RecD subunits to generate a processive helicase, but one that is devoid of detectable nuclease activity, indicating that the C-terminal domain is critical for nuclease activity (Yu *et al.*, 1998a, 1998b). $RecB_{1\text{-}929}$ protein can also be reconstituted with RecC protein, without the RecD subunit, to form the $RecB_{1\text{-}929}C$ enzyme. This dimeric enzyme with the C-terminal truncation in the RecB subunit is also a processive helicase (Yu *et al.*, 1998a).

The truncated $RecB_{1\text{-}929}C$ enzyme is a proficient helicase, but it does not promote RecA protein-mediated joint molecule formation

The early steps in homologous recombination can be reconstituted *in vitro*, where the unwinding of dsDNA by RecBCD enzyme is coupled to homologous pairing by RecA protein in the presence of SSB protein (Dixon & Kowalczykowski, 1991), referred to as ''coupled'' pairing reactions. In these reactions, ssDNA generated by RecBCD enzyme is paired with homologous supercoiled DNA to produce a structure known as a joint molecule. The preferential pairing of χ-containing ssDNA fragments by RecBCD enzyme, and of full-length ssDNA by RecBC enzyme, is due to the asymmetric loading of RecA protein onto ssDNA by these enzymes. Since the $RecB_{1\text{-}929}C$ enzyme is also a proficient helicase, we expected that it would similarly promote the efficient formation of joint molecules in coupled pairing reactions. We therefore were surprised to find using this method that $RecB_{1\text{-}929}C$ enzyme is unable to support efficient pairing (Figure 2).

Figure 2 compares reactions with RecBC enzyme to reactions with $RecB_{1\text{-}929}C$ enzyme. Full-length ssDNA unwinding product is evident for both enzymes at two minutes. Unwinding by RecBC enzyme is followed by incorporation of ssDNA into homologous pairing structures (joint molecules): at 18 minutes, 22(±2) % of the total ssDNA produced has been incorporated (Figure 2(b)). Note that since RecBC enzyme loads RecA protein onto only one of the two strands of dsDNA during unwinding, the data for RecBC enzyme actually

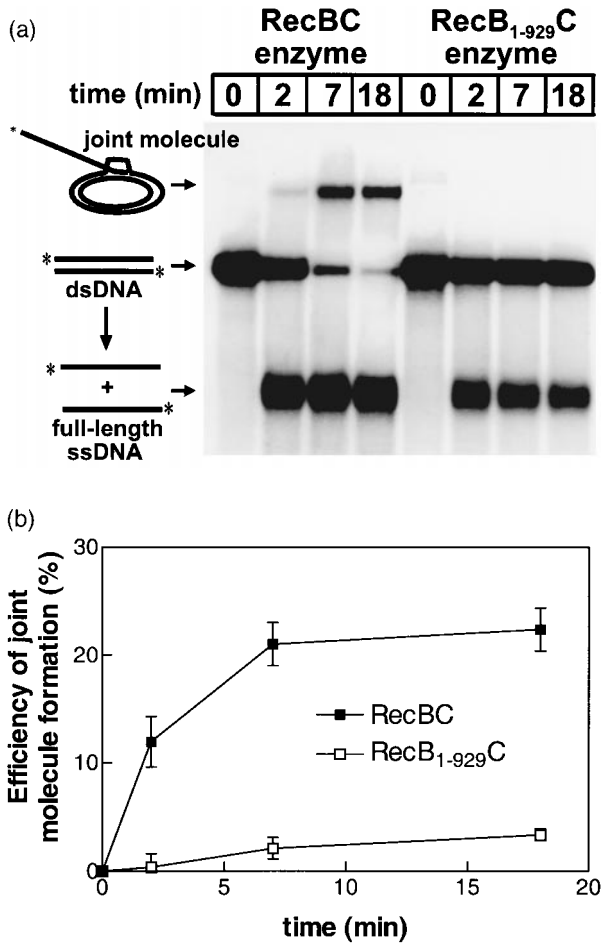

Figure 2. The truncated $RecB_{1\text{-}929}C$ enzyme does not promote joint molecule formation in RecABC coupled pairing reactions. The reactions contained either 2 nM RecBC enzyme or 17 nM reconstituted $RecB_{1\text{-}929}C$ enzyme. (a) Samples were taken at the indicated time points and analyzed by agarose gel electrophoresis. (b) Efficiency of joint molecule formation with either RecBC enzyme (filled squares), or $RecB_{1\text{-}929}C$ enzyme (open squares). Average values for duplicate reactions are shown. Pairing efficiency is defined as the percentage of full-length ssDNA unwinding product that was incorporated into joint molecules. The trace amount of pairing product formed in the reaction with $RecB_{1\text{-}929}C$ enzyme is not evident in the photographic copy, but is apparent in the original digital image. $RecB_{1\text{-}929}$ protein was provided by the laboratory of D. Julin (Yu *et al.*, 1998a). RecC protein was purified as described by Hickson *et al.* (1984). $RecB_{1\text{-}929}C$ enzyme was reconstituted by mixing the two subunits (300 nM $RecB_{1\text{-}929}$ protein and 400 nM RecC protein) in a buffer containing 10 mM potassium phosphate (pH 7), 20% (w/v) glycerol, 0.4 mg/ml BSA, 0.1 mM DTT and 0.1 mM EDTA, and incubating overnight at room temperature. The concentration of $RecB_{1\text{-}929}C$ enzyme is arbitrarily expressed as the concentration of the $RecB_{1\text{-}929}$ subunit throughout this article. RecBC, RecA, and SSB proteins were purified as previously described (Churchill *et al.*, 1999; Griffith & Shores, 1985; LeBowitz, 1985). The RecBC enzyme preparation was estimated to be 20% functional (Churchill *et al.*, 1999). The RecBC enzyme concentration is expressed as functional enzyme throughout. Standard reaction conditions consisted of 25 mM Tris acetate (pH 7.5), 8 mM magnesium acetate, 5 mM ATP, 40 μM (nucleotides) pBR322 dsDNA (χ^0) linearized with *Nde*I restriction enzyme, 20 μM RecA protein, 8 μM SSB, 1 mM DTT, and an ATP-regenerating system including 1 mM phosphoenolpyruvate and 4 units/ml pyruvate kinase. These conditions were the same as those used by Anderson & Kowalczykowski (1997b), except for a twofold higher concentration of SSB protein in the experiments described. Reactions were initiated by the addition of reconstituted $RecB_{1\text{-}929}C$ enzyme, or RecBC enzyme. All reactions were performed at 37 °C. For these joint molecule formation assays, supercoiled pBR322 (χ^0) plasmid DNA (80 μM nucleotides) was also included as the homologous pairing partner. After deproteinization, samples were loaded onto 1% (w/v) agarose gels and electrophoresed for ten hours at 2.2 V/cm in TAE.

reflect a 44% pairing efficiency for top-strand DNA (Churchill *et al.*, 1999). By contrast, pairing efficiency in the reaction with $RecB_{1\text{-}929}C$ enzyme is only 3(±1)%. This low level of pairing is similar to levels observed in uncoupled reactions containing RecA protein and SSB protein in the complete absence of helicase, where the ssDNA substrate was produced by heat-denaturation of dsDNA (Churchill *et al.*, 1999). Thus, although RecA protein is at a competitive disadvantage relative to SSB protein for binding to ssDNA (unless a helicase is present that actively loads RecA protein), a residual amount of pairing is still detectable. We therefore conclude that $RecB_{1\text{-}929}C$ enzyme cannot load RecA protein onto ssDNA.

It is also evident from Figure 2(a) that $RecB_{1\text{-}929}C$ enzyme is unable to unwind all of the available dsDNA under these reaction conditions. Although the initial rate of unwinding for both enzymes is the same for two minutes, further unwinding by $RecB_{1\text{-}929}C$ enzyme subsequently ceases, with only about 50% of the dsDNA unwound. The reduced extent of unwinding by $RecB_{1\text{-}929}C$ enzyme does not affect our comparison of this enzyme's pairing efficiency with that of RecBC enzyme, since pairing efficiency (Figure 2(b)) is normalized relative to the amount of ssDNA produced.

We believe that the inability of $RecB_{1\text{-}929}C$ enzyme to unwind all of the DNA in our experiments reflects an inactivation of the mutant enzyme when unwinding DNA under these specific conditions. In agreement with this conclusion, Yu *et al.* (1998a) showed that this enzyme unwound only a fraction of the total dsDNA under conditions (10 mM Mg^{2+}/5 mM ATP) that were similar to ours (8 mM Mg^{2+}/5 mM ATP). Although the dsDNA used by Yu *et al.* (1998a) contains χ sequences, whereas our substrate does not, it is known that this enzyme does not respond to χ. Our data would suggest that the inactivation of the enzyme under these conditions is independent of χ.

The truncated $RecB_{1\text{-}929}C$ enzyme cannot load RecA protein onto ssDNA

The surprising result that $RecB_{1\text{-}929}C$ enzyme does not promote efficient pairing of ssDNA in coupled pairing reactions prompted us to evaluate the ability of the truncated $RecB_{1\text{-}929}C$ mutant to load RecA protein onto ssDNA during unwinding. The loading of RecA protein can be demonstrated (Anderson & Kowalczykowski, 1997b) by protection of the ssDNA in the RecA protein filament from degradation by the $3' \rightarrow 5'$ exonuclease activity of *E. coli* exonuclease I (ExoI). Figure 3 shows a typical *Exo*I protection assay for RecA protein-loading, comparing the susceptibility of full-length ssDNA produced by RecBC enzyme with that produced by $RecB_{1\text{-}929}C$ enzyme. Although a small amount of dsDNA resists unwinding by $RecB_{1\text{-}929}C$ enzyme, the bulk of the dsDNA has been unwound by both enzymes prior to the addition of *Exo*I. The difference in the types of protein-ssDNA complexes produced by the two enzymes becomes evident at five minutes after addition of *Exo*I, at which point it becomes apparent that the ssDNA produced by RecBC enzyme consists of two populations, one which is completely resistant to *Exo*I degradation, and one which is susceptible. In triplicate reactions, 42(±3)% of the ssDNA produced by RecBC enzyme was resistant to *Exo*I degradation. The *Exo*I-resistant population represents ssDNA from the strand terminating with a 3′-end at RecBC enzyme's entry site and is the strand onto which RecA protein was loaded by the enzyme (Churchill *et al*., 1999). In contrast, only 3(±2)% of the ssDNA produced by $RecB_{1\text{-}929}C$ enzyme persists after 20 minutes; thus, RecA protein is not loaded onto ssDNA produced during unwinding by the truncated enzyme.

Our results demonstrate that although $RecB_{1\text{-}929}C$ enzyme is able to unwind DNA, it is unable to facilitate the loading of RecA protein onto its ssDNA product. This enzyme is missing the 251 amino acid residues which correspond to the 30 kDa C-terminal domain of the RecB subunit. This domain is known to be critical for the nuclease activity of RecBCD enzyme (Yu *et al*., 1998a,b). We conclude that this domain is also critical for the RecA protein-loading function of both RecBC enzyme and χ-modified RecBCD enzyme. Furthermore, the constitutive RecA protein-loading capacity of RecBC enzyme cannot be attributed solely to its absence of dsDNA exonuclease activity, since $RecB_{1\text{-}929}C$ enzyme is also completely devoid of nuclease activity, but does not load RecA protein.

The C-terminal domain of RecBCD enzyme comprises the region essential for RecA-loading function

The loading of RecA protein onto ssDNA by RecBCD enzyme is not only an important *in vitro* aspect of homologous pairing activity, but is likely to be an important cellular function as well. Genetic data indicate that cells expressing the truncated $RecB_{1\text{-}929}$ subunit are deficient in RecBCD-dependent recombination (A. Taylor, S. Amundsen, and G. Smith, personal communication), and that a *Rec*$^-$ mutant (the *recB2109 allele*), with a substitution near the hinge region (at position 807) of the RecB subunit, results in a RecBCD holoenzyme that can process dsDNA to produce 3′-overhanging ssDNA tails, but that fails to load RecA protein onto this ssDNA (Arnold & Kowalczykowski, 2000). We currently favor the idea that loading of RecA protein by RecBCD enzyme involves a direct interaction between these proteins that occurs only during DNA unwinding, and that the 30 kDa C-terminal domain of the RecB subunit is the site for this interaction. All evidence argues that this interaction is essential for efficient homologous recombination.

We can now imagine a possible mechanism for the phenomenon of RecA protein-loading. We postulate that the 30 kDa C-terminal domain of the RecB subunit harbors a binding site which interacts specifically with RecA protein and actively facilitates its binding to ssDNA. Consistent with this view is the observation that interspecies complementation *in vivo* yields optimal recombination when both RecBCD enzyme and RecA protein are from the same species (de Vries & Wackernagel, 1992; Rinken *et al*., 1991). However, to date we have not been able to show a direct interaction

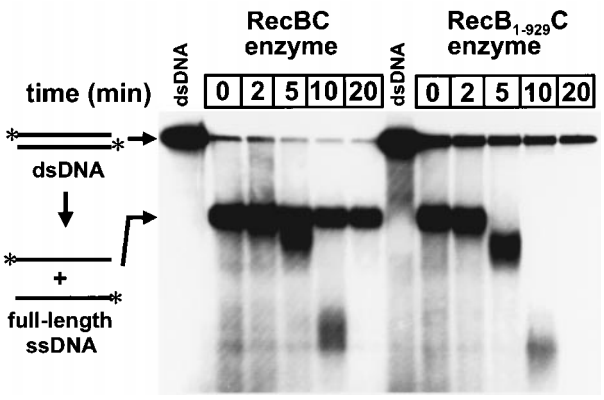

Figure 3. The $RecB_{1\text{-}929}C$ enzyme does not load RecA protein onto ssDNA. Unwinding was initiated using standard reaction conditions by the addition of either 5 nM RecBC enzyme or 50 nM reconstituted $RecB_{1\text{-}929}C$ enzyme. After three minutes, a mixture of ATPγS and M13mp7 ssDNA was added, to stabilize RecA protein bound to ssDNA, and to sequester any remaining free RecA protein (Anderson & Kowalczykowski, 1997b). After two minutes of further incubation, ExoI was added. The zero time point was taken just prior to addition of ExoI. Samples were taken at the indicated times following the addition of ExoI. Standard reaction conditions were used. After deproteinization, samples were loaded on 1.2% agarose gels and electrophoresed for ten hours at 2.2 V/cm in TAE.

between RecA protein and RecBCD enzyme (Anderson & Kowalczykowski, 1997b) or RecBC enzyme (J. J. C., unpublished results). It is possible that the interaction between RecB and RecA proteins is a transient one that may also require active translocation by the helicase.

We speculate that, in the RecBCD holoenzyme prior to interaction with χ, the putative RecA protein binding site in the RecB subunit is occluded by the RecD subunit (Figure 4(a)). The χ-induced modifications to the holoenzyme clear this block, thereby exposing this pocket, which results in the recruitment of RecA protein to the incipient ssDNA during subsequent unwinding (Figure 4(b)). Since the C-terminal domain of the RecB subunit is also critical for the nuclease activity of the holoenzyme (Yu *et al*., 1998b), it seems probable that this conformational shift is also the basis for the switch in exonuclease polarity and overall attenuation of nuclease activity that follows interaction with χ.

In parallel with this work on the truncated RecBC enzyme, we also characterized the mutant RecBCD enzyme in which a single aspartate residue (Asp1080) is mutated to alanine (Yu *et al*., 1998b). We demonstrated that this $RecB_{D1080A}CD$ enzyme is also unable to load RecA protein (Anderson *et al*., 1999). We were also able to establish that, even though this mutant enzyme does not produce χ-specific fragments, it does retain an ability to recognize the χ sequence. These results allowed us to conclude either: (1) that Asp1080 in RecB is required to transmit the χ-recognition signal to whatever element in the RecBCD holoenzyme is responsible for RecA protein loading; or, alternatively, (2) that Asp1080 is an essential component of the RecA protein-loading machinery. Unfortunately, because RecA-loading by the RecBCD holoenzyme requires productive interaction with χ and the consequent conformational change, we were unable to distinguish between these two possible interpretations. However, because the RecBC enzyme loads RecA protein constitutively and independently of χ, analysis of the $RecB_{1\text{-}929}C$ enzyme allowed us to test directly the hypothesis that the C-terminal domain of the RecB subunit is involved in RecA protein-loading (and not merely in transmission of the χ-recognition signal). Thus, we now can conclude that the elimination of this domain interferes directly with the RecA protein-loading function and that it, therefore, likely constitutes the region that physically interacts with RecA protein.

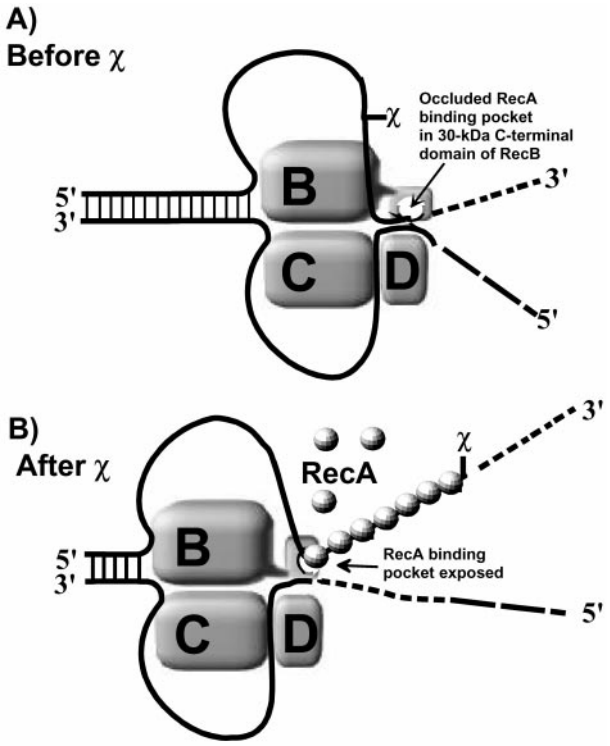

Figure 4. Model for χ-induced RecA protein loading by RecBCD enzyme. Before interaction with χ, the RecA protein binding pocket in the 30 kDa C-terminal domain of RecB protein is blocked by the RecD subunit. Following interaction with χ, a conformational change in the enzyme exposes the binding pocket, allowing RecBCD enzyme to bind RecA protein and to recruit it to the χ-containing strand. The model also shows how switching of the enzyme's asymmetric exonuclease activity leads to preservation of the χ-containing strand downstream of the χ sequence (Anderson & Kowalczykowski, 1997a).

RecA protein can be viewed as the "cargo" of the RecBCD helicase

RecBCD enzyme is a linear motor protein, a class of proteins characterized by their ability to couple energy from hydrolysis of nucleoside triphosphates to translocation along a polymer lattice. The microtubule motor proteins, such as kinesin, translocate along microtubule polymers to transport cargo (such as membranous organelles) from one location to another, whereas DNA helicases translocate along a polymer lattice of DNA (Lohman *et al*., 1998). Despite the obvious differences in function between helicases and microtubule motor proteins, certain mechanistic parallels exist for members of these two different classes of motor proteins (Lohman *et al*., 1998). Our findings demonstrate that the RecBCD enzyme is the first DNA-based motor protein that is capable of targeting a particular material, RecA protein, to a specific site on ssDNA, suggesting the possibility that other proteins of this class may yet be identified, along with the targets that they recognize.

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