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The RecA Binding Locus of RecBCD Is a General Domain for Recruitment of DNA Strand Exchange Proteins

Short Article

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Summary

RecBCD enzyme facilitates loading of RecA protein onto ssDNA produced by its helicase/nuclease activity. This process is essential for RecBCD-mediated homologous recombination. Here, we establish that the C-terminal nuclease domain of the RecB subunit ($RecB^{nuc}$) forms stable complexes with RecA. Interestingly, $RecB^{nuc}$ also interacts with and loads noncognate DNA strand exchange proteins. Interaction is with a conserved element of the RecA-fold, but because the binding to noncognate proteins decreases in a phylogenetically consistent way, species-specific interactions are also present. $RecB^{nuc}$ does not impede activities of RecA that are important to DNA strand exchange, consistent with its role in targeting of RecA. Modeling predicts the interaction interface for the RecA-RecBCD complex. Because a similar interface is involved in the binding of human Rad51 to the conserved BRC repeat of BRCA2 protein, the RecB-domain may be one of several structural domains that interact with and recruit DNA strand exchange proteins to DNA.

Introduction

At the initial stage of homologous genetic recombination and recombinational DNA repair in *Escherichia coli*, double-stranded DNA breaks (DSBs) are processed by the combined helicase/nuclease action of RecBCD enzyme (see Arnold and Kowalczykowski [1999] and Spies and Kowalczykowski [2005] for review). In wild-type *E. coli*, ~99% of the recombination events occurring at DSBs require RecBCD function. The enzyme is a heterotrimeric protein complex (330 kDa) consisting of three nonidentical subunits: RecB, RecC, and RecD (Amundsen et al., 1986). RecBCD displays ATPase, helicase, and nuclease activities, the latter of which is attenuated by a specific DNA locus (5′-GCTGGTGG-3′), called χ (Chi, *c*rossover *h*otspot *i*nstigator) (Lam et al., 1974). Alteration of enzymatic activity is manifest only when RecBCD is translocating through DNA and only when it approaches a χ sequence from its 3′ side relative to DSB entry site (Taylor et al., 1985). Upon interaction with a properly oriented χ site, degradation of the 3′-terminated strand is downregulated, whereas a lesser degradation of the 5′-terminated strand is upregulated (Anderson and Kowalczykowski, 1997a; Dixon and Kowalczykowski, 1991, 1993). This change in both the polarity and frequency of degradation results in production of a 3′-terminated ssDNA tailed duplex with the χ sequence at its end, an ideal substrate for the RecA-mediated DNA strand invasion (Dixon and Kowalczykowski, 1991).

To assemble on ssDNA, RecA protein not only needs to bind ssDNA, but it must also displace ssDNA binding (SSB) protein (see Kowalczykowski et al. [1994] and Spies et al. [2003] for review), which is a competitor for ssDNA binding (Kowalczykowski and Krupp, 1987). RecBCD facilitates this displacement by actively loading RecA onto the ssDNA in response to χ recognition (Anderson and Kowalczykowski, 1997b). This loading results in formation of the RecA nucleoprotein filament on the χ containing ssDNA, which subsequently invades homologous dsDNA (Dixon and Kowalczykowski, 1991).

Mutations within the RecB subunit impair RecA-loading activity. For example, the $RecB^{1–928}C(D)$ enzyme in which RecB subunit is truncated by removal of the C-terminal nuclease domain, leaving only the helicase domain, fails to mediate RecA loading in vitro (Churchill and Kowalczykowski, 2000). This finding suggested that the 30 kDa C-terminal domain of the RecB protein ($RecB^{928–1180}$) (Yu et al., 1998) harbors a site of interaction with RecA (Churchill and Kowalczykowski, 2000). Unfortunately, attempts to detect a direct protein-protein interaction between RecA and RecBCD were unsuccessful (Anderson and Kowalczykowski, 1997b), possibly because the interaction with χ is required for a conformational change in RecBCD that exposes the RecA binding site. However, the RecBC enzyme, which acts as a constitutively χ modified RecBCD in that it does not require the χ induced modification to load RecA onto ssDNA, also failed to interact with RecA in gel filtration chromatography and coimmunoprecipitation assays (J.J. Churchill and S.C.K., unpublished data). Thus, it seems most likely that the interaction between RecA and RecBCD (or RecBC) enzyme is transient, requiring RecBCD to either bind to, or actively translocate on, a DNA substrate.

The C-terminal domain of RecB protein also comprises a nuclease domain and, hence, is referred to as $RecB^{nuc}$ (Zhang and Julin, 1999). This domain has a structural fold similar to that of lambda exonuclease, and it is connected to the helicase domain of the RecB subunit by an unstructured 70 amino acid linker (Singleton et al., 2004). Because $RecB^{nuc}$ is a stable structural domain with a defined nucleolytic function, it was surprising that this domain was also implicated, by deletion analysis, in RecA loading. To determine whether this region is indeed involved in RecBCD-facilitated loading of RecA, we purified 6×His-tagged $RecB^{nuc}$ and investigated its ability to interact with RecA directly. We found that purified $RecB^{nuc}$ binds to and forms stable complexes with purified RecA. Interestingly, $RecB^{nuc}$ also bound to noncognate RecA-like DNA strand exchange proteins, suggesting that interaction is via a structural feature common to all RecA-like proteins. Modeling the interaction between the $RecB^{nuc}$ and RecA core structures predicts that the interaction interface includes

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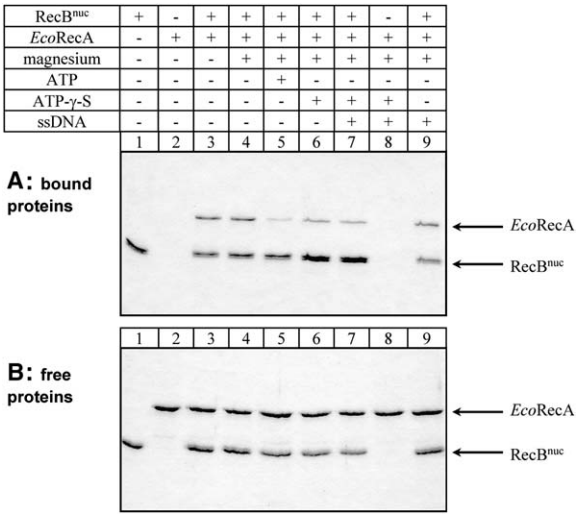

Figure 1. RecA Forms Stable Complexes with RecB^{nuc}

Pull-down assays using Ni-NTA beads were carried out as described in the Experimental Procedures and analyzed by SDS-PAGE.

(A) The proteins adsorbed on the bead either by direct chelation (the 6×His-tagged RecB^{nuc}) or through interaction with the immobilized 6×His-tagged RecB^{nuc} protein were eluted with 300 mM imidazole.

(B) Excess proteins that were free in solution.

elements of the surface that are involved in the RecA oligomerization. Interestingly, in human Rad51 protein, a similar surface is involved in the interaction with the BRC repeat of BRCA2 protein (Pellegrini et al., 2002). A model for RecBCD-facilitated targeting of the RecA onto the χ containing ssDNA, which may apply to the recruitment and loading of DNA strand exchange proteins in general, is discussed.

Results

Interaction between RecA and the C-Terminal Domain of RecB (RecB^{nuc}) Can Be Detected by Coelution from Ni-NTA Beads

To determine whether the RecA binding site of RecBCD is contained within the C-terminal domain of the RecB subunit (RecB^{nuc}), this putative interaction was examined by pull-down assays using Ni-NTA beads (Figure 1). Based on trial experiments, an excess of both 6×His-tagged RecB^{nuc} and untagged RecA, relative to the bead capacity, was used. After extensive washing to remove unbound proteins, the proteins that were retained on the beads were eluted with imidazole and analyzed by SDS-PAGE (Figure 1A); the free proteins that did not bind to the beads are shown in Figure 1B. Where RecB^{nuc} was present, the pull-down experiments revealed the presence of bound RecA indicative of stable complex formation between RecB^{nuc} and RecA (Figure 1A, lanes 3–7 and 9). Interaction with RecA slightly reduced the binding of RecB^{nuc} to the beads (compare lanes 1 and 3 in Figure 1). When the RecB^{nuc} was omitted, no RecA was retained by nonspecific adsorption to the beads (Figure 1, lanes 2 and 8). Complex formation between RecB^{nuc} and RecA was independent of magnesium ion and ssDNA but was reduced in the presence of nucleotide cofactors, ATP, and ATP-γ-S.

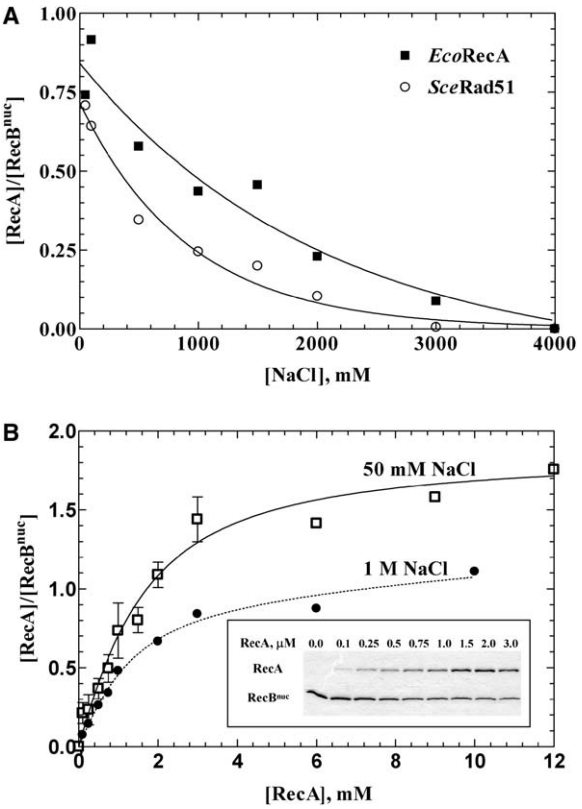

Figure 2. RecA-RecB^{nuc} Complexes Are Resistant to Disruption by Increasing Salt Concentration and Saturate at a ~1:1 Stoichiometry

Assays were carried out as described in the Experimental Procedures at the indicated concentrations of NaCl.

(A) The molar ratio of bound *E. coli* RecA (open squares) or *S. cerevisiae* Rad51 protein (closed circles) relative to the amount of RecB^{nuc} bound to the bead at various concentrations of NaCl.

(B) The molar ratio of RecB^{nuc} and RecA coeluted from Ni-NTA beads was calculated from analysis of SDS-PAGE. Where present, the error bars indicate the standard deviation for several independent experiments. The assays were carried out in standard interaction buffer containing either 50 mM (open squares) or 1 M (closed circles) NaCl. A typical gel depicting a RecA titration in the presence of 50 mM NaCl is shown in the inset.

The Complexes between RecB^{nuc} and RecA Are Stable to Disruption by Salt and Form with a Defined Stoichiometry

One indicator of a specific protein-protein interaction is the stability of the complex to disruption by increasing concentrations of salt. Therefore, we carried out the pull-down assays in the presence of various concentrations of NaCl (Figure 2A, solid squares). The RecA-RecB^{nuc} complexes were surprisingly stable, with interaction detectable at even 3 M NaCl, thereby supporting the conclusion that the interaction between nuclease domain of RecB and RecA is specific.

Another indicator of interaction specificity is the formation of a complex with a defined, saturable stoichiometry. Because RecA exists as a heterogeneous population of multimers, whose distribution depends on protein concentration, nucleotide cofactors, pH, and ionic environment (Brenner et al., 1988; Ogawa et al., 1978), we carried out titrations with RecA at two different salt concentrations (Figure 2B). First, the assays were

carried out at 50 mM NaCl, where RecA is active in DNA strand exchange but exists in oligomeric forms that range, depending on concentration, from monomers to filaments (Brenner et al., 1988). The ratio of RecA to $RecB^{nuc}$ in the coeluted complexes increased with increasing concentration of RecA (Figure 2B, open squares). The apparent K_d of the $RecB^{nuc}$-RecA interaction under these conditions was ~1 μM. The stoichiometry reached a plateau at ~1.8 ± 0.1 RecA monomers per $RecB^{nuc}$, indicating that various oligomeric forms of RecA can bind to the C-terminal domain of $RecB^{nuc}$.

At 1 M NaCl, RecA exists predominantly as a monomer (Ogawa et al., 1978) (see also Figure S1 available in the Supplemental Data with this article online). When the pull-down assays were carried out in buffer containing 1 M NaCl, the ratio of coeluted RecA and $RecB^{nuc}$ proteins reached a plateau at 1.2 ± 0.1 RecA per $RecB^{nuc}$, whereas the apparent K_d was 0.9 ± 0.2 μM (Figure 2B, solid circles). Thus, the complex between $RecB^{nuc}$ and RecA has a defined, saturable molecular composition, and an interaction dissociation constant that is comparable to the ~1–10 μM concentration of RecA in vivo (Karu and Belk, 1982).

$RecB^{nuc}$ Interacts with Noncognate RecA-Like Proteins

The RecA of *E. coli* is a member of family of structurally, functionally, and genetically similar proteins that are essential to homologous recombination (Bianco et al., 1998; Brendel et al., 1997). To further examine the interaction specificity, we tested several proteins closely related to *E. coli* RecA, the *Bacillus subtilis* RecA, *Saccharomyces cerevisiae* Rad51, and bacteriophage T4 UvsX protein, for their ability to interact with $RecB^{nuc}$. We found that *B. subtilis* RecA can form a complex with $RecB^{nuc}$ (Figures 3A and 3B, compare lanes 4 and 5) as can UvsX and Rad51 (Figure 3C, gel; data not shown); however, the amounts bound are reduced relative to the *E. coli* protein. Nevertheless, the interaction with Rad51, at least, appears to be specific in that the Rad51-$RecB^{nuc}$ complexes are also relatively salt stable (Figure 2A, open circles). However, as inferred from the lower level of complex formation, the Rad51-$RecB^{nuc}$ complexes are less stable in increasing concentrations of NaCl than the RecA-$RecB^{nuc}$ complexes.

To determine whether the interaction with $RecB^{nuc}$ was specific to DNA strand exchange proteins rather than a general attribute of DNA binding proteins, three DNA binding proteins were examined. Neither *E. coli* SSB protein nor T4 phage gene 32 protein (gp32), both ssDNA binding proteins, interact with $RecB^{nuc}$; the same is true for LexA repressor, a dsDNA binding protein (Figure 3C). These observations showed that the interaction with the DNA strand exchange proteins is not likely via a DNA binding site.

The binding of DNA strand exchange proteins, but not DNA binding proteins, with *E. coli* $RecB^{nuc}$ raised the possibility that the interaction might be via the nucleotide binding site present in each DNA strand exchange protein. Therefore, the binding of $RecB^{nuc}$ to *E. coli* RecQ and *B. stearothermophilus* PcrA helicases, each of which possesses a nucleotide binding site (the nucleotide binding site of RecQ protein has an adenylate kinase fold, whereas PcrA helicase has a RecA fold),

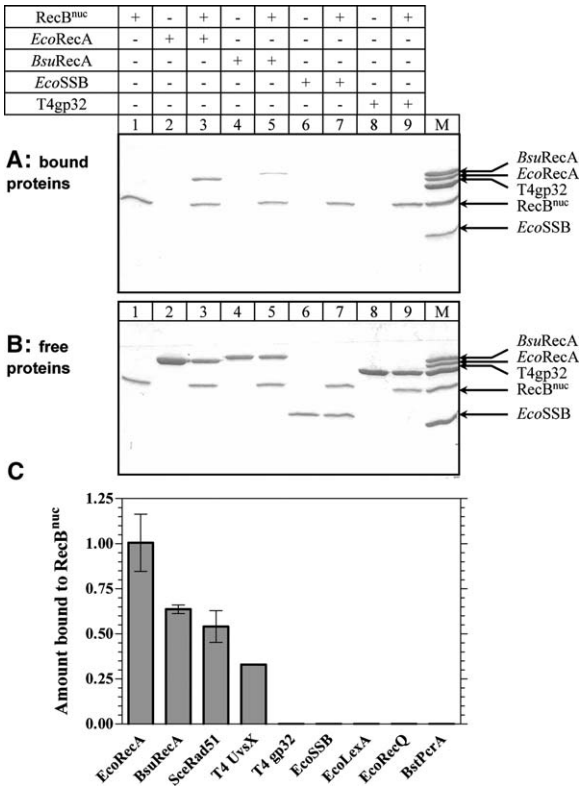

Figure 3. $RecB^{nuc}$ Binds to and Forms Complexes with Noncognate DNA Strand Exchange Proteins

Pull-down assays were carried out as described in the Experimental Procedures at 100 mM NaCl.

(A) The proteins adsorbed to the bead, were eluted with 300 mM imidazole, and were analyzed by SDS-PAGE.

(B) Excess proteins that remained free in solution.

(C). The bar graph shows the molar ratio for the various complexes eluted, determined by quantifying gels such as (A); where present, the error bars indicate a standard deviation for three independent experiments.

was also tested. No interaction between these helicases and $RecB^{nuc}$ was detected (Figure 3C), establishing that a nucleotide binding site was insufficient for interaction. These results argue that recognition and binding of RecA by the C-terminal domain of RecB protein requires a structural element that is highly conserved in all RecA-like DNA strand exchange proteins, perhaps a unique structure within the highly conserved core region of DNA strand exchange proteins (Bianco et al., 1998; Brendel et al., 1997). Interestingly, such an interaction was reported for the complex of a BRC-repeat peptide from the BRCA2 protein with the Rad51 core domain (Pellegrini et al., 2002). Finally, consistent with our interpretations, we found that RecBCD could also load noncognate DNA strand exchange proteins onto ssDNA in response to χ activation (Figure S2).

Discussion

We demonstrated here that the C-terminal domain of the RecB subunit of the RecBCD helicase/nuclease is involved in a direct protein-protein interaction with RecA. It is through this interaction that RecBCD recruits RecA to the χ containing ssDNA, which is produced by the

helicase/nuclease activity of RecBCD. The interaction with $RecB^{nuc}$ does not impair the activities of RecA that are important for homologous recombination. In the presence of $RecB^{nuc}$, RecA efficiently forms a presynaptic complex and mediates DNA strand exchange between circular ssDNA and homologous linear dsDNA (Figure S3). The failure to inhibit any activity of RecA is consistent with the interaction between $RecB^{nuc}$ and RecA being specific, because the loading interaction should not preclude interaction of RecA with ssDNA, ATP, or an adjacent RecA monomer.

Besides *E. coli* RecA, $RecB^{nuc}$ forms complexes with several noncognate DNA strand exchange proteins. This finding was unexpected, but the presence and specificity of these interactions were confirmed by direct and indirect methods (Figures S2 and S3). However, and more importantly, these experiments showed that the cognate interaction is the strongest. Our in vitro observations correlate well with genetic data, which established that interspecies combinations of RecA and RecBCD from several enteric bacteria partially restored recombination in *E. coli* deleted for these enzymes and that complementation was best when both enzymes were from the same species (de Vries and Wackernagel, 1992).

One can envision several mechanisms by which interaction with the nuclease domain of the RecB subunit facilitates RecA loading. The most likely explanation is that physical interaction with RecBCD brings RecA close to the newly produced ssDNA. This delivery helps it to compete with SSB protein by increasing the local concentration of RecA in the vicinity of ssDNA via formation of the transient ternary complex: RecBCD-DNA-RecA. An alternative hypothesis, namely, that the interaction with $RecB^{nuc}$ induces a conformational change within RecA, which endows the protein with a greater affinity for ssDNA, has no supporting experimental evidence: we did not detect any alteration in either the ability to bind ssDNA or to displace SSB protein in the presence of $RecB^{nuc}$ (Figure S4).

The presence of specific interactions between the nuclease domain of RecB and the noncognate DNA strand exchange proteins most likely reflects recognition by $RecB^{nuc}$ of some common structure present in all RecA-like proteins. In spite of a modest overall homology between bacterial RecA, bacteriophage UvsX, and eukaryotic Rad51 protein (Karlin et al., 1995; Story et al., 1993), all of the DNA strand exchange proteins contain a highly conserved core domain (Brendel et al., 1997). This common core most likely contains the site of interaction with $RecB^{nuc}$. The core domain includes a nucleotide binding fold that is referred to as the "RecA fold." Recent crystallographic studies confirmed that the nucleotide binding folds of Rad51, RadA, and RecA are topologically identical (Conway et al., 2004; Datta et al., 2000; Pellegrini et al., 2002; Shin et al., 2003).

The presence of a RecA fold, however, is not sufficient to mediate an interaction with RecA, because we showed that neither PcrA (an SF1 helicase) nor RecQ helicase (an SF2 helicase) interacted with $RecB^{nuc}$, indicating that the structural element recognized by $RecB^{nuc}$ is specific to the core region of DNA strand exchange proteins. Thus, a RecA fold is necessary but insufficient for interaction.

While this work was in progress, the crystal structure of RecBCD bound to DNA was solved (Singleton et al., 2004). To see if we could define the location of the RecA-$RecB^{nuc}$ interaction interface based on the structural information, we used a docking algorithm to determine whether we could obtain a model for the predicted RecA-$RecB^{nuc}$ complex. Docking was performed by using the soft protein docking algorithm BiGGER (Palma et al., 2000). This algorithm fixes the spatial coordinates of the "probe" and docks it with the "bait," repeating the process for programmed angular rotations. The probability that each docking solution represents an accurate complex between the two proteins is estimated based on geometric complementarity, on both electrostatic potential and hydrophobicity of the surfaces, and on the pair-wise interactions between amino acid side chains. The central core domain (amino acids 33–240) of RecA, which is conserved across all major kingdoms of life (Brendel et al., 1997), was used as the docking partner for $RecB^{nuc}$. The top scoring solution had a global score of 67%, which is the estimated probability, in percentage, that this particular docking solution represents an accurate model (rmsd < 4 Å) of the complex. (This probability is based on extensive comparisons of the structure of known interactions to those calculated by the docking program [Palma et al., 2000]). The predicted interface comprises 925 $Å^2$ (Figure 4A) and includes 23 amino acids of RecA that reside on one side of the RecA subunit-subunit interface (see Figure S5 and the legend to Figure 4A for additional details) and does not involve regions of RecA responsible for DNA, ATP, or repressor binding (reviewed in McGrew and Knight [2003]); therefore, binding in the predicted complex should not interfere with biochemical activities of RecA. It is also notable that the predicted interface corresponds to the surface in the RecA nucleoprotein filament that would be at the 3′ -end of the ssDNA. Because RecA polymerizes on the ssDNA in the 5′ → 3′ direction, the contact between $RecB^{nuc}$ domain and RecA would need to be disrupted after RecA-loading to permit growth of the RecA-ssDNA nucleoprotein filament in the expected direction (Churchill et al., 1999). The affinity (K_d) between RecA and the $RecB^{nuc}$ domain is in the micromolar range (Figure 2B), suggesting that, once loaded onto ssDNA, the interactions between RecA and $RecB^{nuc}$ are replaced by the stronger cooperative RecA-RecA interactions that result in growth of the RecA nucleoprotein filament. Intriguingly, the same structural elements of human Rad51 protein are involved in its binding to the BRC peptide of BRCA2 protein (Pellegrini et al., 2002), a protein whose role is to facilitate Rad51 nucleoprotein assembly in mammalian cells.

The predicted interaction surface on $RecB^{nuc}$ is, interestingly, the surface that interacts with the RecC subunit in the structure (Figure 4A). Consequently, when the predicted RecA-$RecB^{nuc}$ complex is superimposed on the structure of RecBCD-DNA complex (Singleton et al., 2004), RecA is found in the same location as the 2B subdomain of the RecC subunit. Because of the obvious physical clash, a conformational change would be required that involves swiveling, or release, of the nuclease domain of RecB to uncover the RecA binding site. This prediction would offer a rational explanation for why the RecA-loading domain is attached by a 70 amino

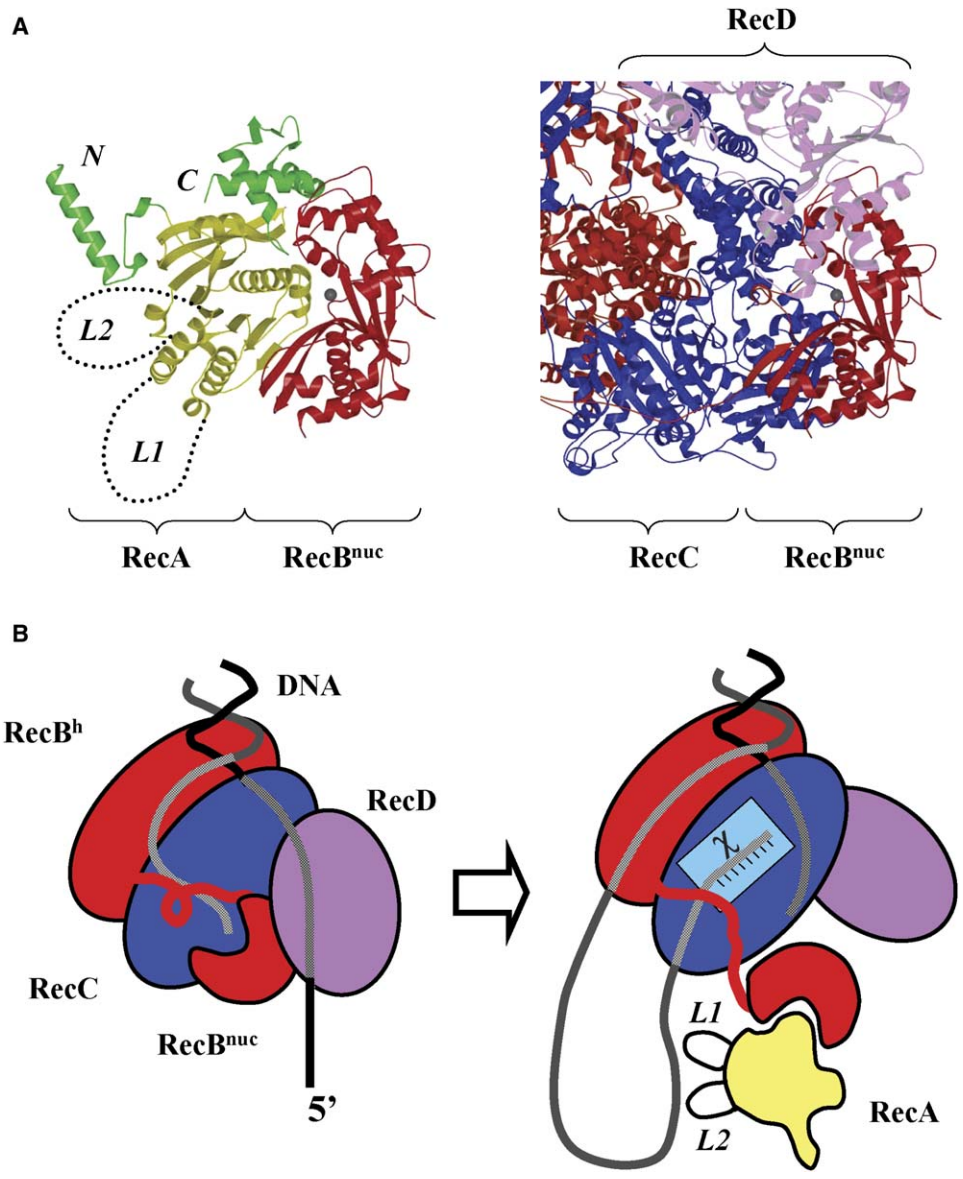

Figure 4. Model for χ Induced Loading of RecA Protein onto ssDNA by RecBCD Enzyme

(A) Model of the RecA-RecB^{nuc} complex predicted by the protein docking algorithm BiGGER (left). The "probe" used was the core domain of RecA (yellow), and it was docked with the "bait" RecB^{nuc} (red). The N- and C-terminal domains of RecA (green), and loops L1 and L2 (dotted lines, and which are not defined in the crystal structure) were added to the top-scoring complex to show that these structural elements do not interfere with proposed binding to RecB^{nuc} domain. The predicted interface includes 23 amino acids of the RecA (Phe 92, Asp94, His97 → Ile102, Arg105 → Gly108, Asp 110 → Asn113, Leu115, Glu118, Glu124, Ile128, Leu132, Ser135, and Ala137) that reside on one side of the RecA subunit-subunit interface; the main structural elements involved in the interaction are α helix D, β sheet 3, and α helix E. On the RecB^{nuc} side, 29 amino acids are found within 4 Å of residues in RecA: Ala928, Ala929, Ala949, Gly952, Thr953, His956, Ser957, Glu960, Leu979, Val1019, Glu1020, Glu1022, Tyr1024, Ile1027, Phe1055, Met1056, Val1058, Phe1065, Lys1082, His 1105 → Tyr1107, Leu1113, and Tyr1114. For comparison, the right panel shows the position of RecB^{nuc} within RecBCD initiation complex. RecB subunit is shown in red, RecC subunit is dark blue, and RecD subunit is magenta.

(B) The illustration shows the proposed conformational changes involved in RecA loading by RecBCD. Prior to χ recognition, the C-terminal domain of the RecB subunit is positioned so that the 3′ terminated strand is translocated to the nuclease site, whereas the RecA binding site is sequestered by virtue of its interaction with the RecC subunit. Recognition of a properly oriented χ sequence results in release of the RecB^{nuc} domain, revealing the RecA-loading surface. This RecA binding site of RecB^{nuc} remains close to the 3′ terminated strand due to the 70 amino acid tether connecting it to the RecB helicase domain; in addition, reorientation of the RecB^{nuc} domain permits access of the 5′ terminated strand to nucleolytic site.

acid linker to the helicase domain: such a physical connection would ensure that the RecB^{nuc} domain remains tethered to the holoenzyme despite being completely detached from RecC. Furthermore, this view also offers a possible explanation for why a direct interaction between RecBC or RecBCD and RecA is not detected in solution.

Before the crystal structure of RecBCD was determined, we attempted to narrow the RecA binding interface by deletion analysis of RecB^{nuc}. We aligned the

amino acid sequences of the nuclease domains from several RecB and AddA proteins and found five conserved motifs distributed along the length of $RecB^{nuc}$ (Figure S6). In a preliminary attempt to determine whether any of these motifs comprised the RecA binding interface, we generated a series of truncated $RecB^{nuc}$ peptides and analyzed their ability to interact with RecA. Six of the resultant peptides were soluble, and each was capable of binding RecA in pull-down experiments. The region contained within each of these truncations is amino acids 1034–1100, thereby defining a minimal region required for this interaction. This minimal region is adjacent to the RecC subunit and is a part of the interface in the computed model of the RecA-$RecB^{nuc}$ complex. Other truncations were insoluble (data not shown). Now that the three-dimensional structure of $RecB^{nuc}$ is available, a more refined analysis of our predictions is possible and is in progress.

The mechanism by which interaction with χ activates RecBCD for RecA loading is emerging as the result of bulk-phase biochemistry, recent single-molecule work, and resolution of the crystal structure (Handa et al., 2005; Singleton et al., 2004; Spies et al., 2003). In Figure 4B, we present a model reflecting our current understanding of the RecBCD-facilitated RecA loading. It appears now that the χ sequence itself binds to the holoenzyme, serving as the allosteric effector that gives rise to the structural changes that underlie the multiple changes in RecBCD activity (Kulkarni and Julin, 2004; Singleton et al., 2004; Spies et al., 2003). Based on the crystal structure and on our modeling results, we envision that, prior to χ recognition, the RecA binding site is sequestered within the holoenzyme via interaction with RecC subunit and also perhaps blocked by the RecD subunit (Figure 4B). The DNA is translocated through the RecBCD so that the two DNA strands are separated by RecC subunit and channeled to each of the motor subunits. Upon separation at the RecC subunit, the 3′ terminated DNA strand passes through the channel that leads to the helicase domain of RecB subunit and then exits at the nuclease site of $RecB^{nuc}$ domain, resulting in preferential degradation of 3′ terminated DNA strand (Figure 4B). We propose that binding of the χ sequence by the RecC subunit triggers a structural change that includes a repositioning of the C-terminal domain of the RecB subunit. Even though there are no physical interactions between RecB and RecD subunits in the structure of the preinitiation complex with DNA (Singleton et al., 2004), we note that there might be added structural changes induced upon ATP binding and translocation. We offer this added speculation to explain why RecBCD requires interaction with χ to activate loading of RecA, whereas the RecBC enzyme does not, being a constitutive loader of RecA. This proposal suggests that the RecD subunit restricts the mobility of the $RecB^{nuc}$ domain in the translocating complex. Many existing models of χ induced modification of RecBCD propose that the RecD subunit of the RecBCD is modified, ejected, or inactivated at χ, which enables RecBCD to facilitate RecA-loading (Anderson et al., 1997; Churchill and Kowalczykowski, 2000; Myers et al., 1995; Taylor and Smith, 1999), but it is now known that the RecD subunit is not lost upon recognition of χ (Handa et al., 2005). However, the χ induced conformational change may result in an alteration in the position of RecD relative to the other subunits so that the $RecB^{nuc}$ domain is freed to swing along the flexible linker connecting the helicase and nuclease domains of the RecB subunit (Figure 4B). As a result, the RecA binding site would be revealed and it would be in close proximity to the 3′ terminated strand, thereby affording an efficient mechanism for deposition of RecA onto that strand (Figure 4B).

The mechanism by which a DNA strand exchange protein is delivered to the site of a recessed DSB is of universal importance. In multicellular eukaryotes, the BRCA2 protein is proposed to recruit Rad51 protein to sites of DNA damage. BRCA2 protein contains BRC repeats that physically interact with Rad51 protein and are proposed to be responsible for this targeted recruitment (Chen et al., 1998, 1999; Wong et al., 1997). Recent structural and biochemical studies established a direct physical link between the recruitment of Rad51 protein to ssDNA by BRCA2 protein (Kowalczykowski, 2005; Pellegrini et al., 2002; Yang et al., 2005). The structure of a fusion between the BRC4 peptide and the core region of human Rad51 protein (Pellegrini et al., 2002) revealed that the BRC peptide mimics the conserved structural element of the RecA/Rad51 core, which is involved in monomer-monomer contacts between adjacent subunits within the RecA and Rad51 nucleoprotein filament. This interaction was proposed to be responsible for the delivery of Rad51 protein to damaged DNA by BRCA2 and, upon release, mediating assembly of the Rad51 nucleoprotein filament on the ssDNA that had been produced by processing of the DSB by a helicase/nuclease (see Kowalczykowski [2002] for review). Recent studies with a BRCA2 homolog, Brh2 protein from *Ustilago maydis*, showed that Brh2 protein loads Rad51 protein onto ssDNA at ssDNA-dsDNA junctions (Yang et al., 2005). Interestingly, both the structural and biochemical characteristics of BRCA2 function have parallels to the RecBCD-mediated loading of RecA onto ssDNA (see Figure S7). In both cases, a direct protein-protein interaction with a DNA strand exchange protein is involved. More specifically, however, this interaction involves the core domain of the DNA pairing protein, which contains a highly conserved structure, and an interaction site mimic. Thus, such an interaction may represent a universal structural basis for the recruitment of DNA pairing proteins to the sites of DNA damage.

Experimental Procedures

Materials and Reagents

Chemicals were of a reagent grade. ATP and ATPγS (Sigma) were dissolved as concentrated stock solutions at pH 7.5, whose concentrations were determined spectrophotometrically by $\varepsilon_{260} = 1.54 \times 10^4\ M^{-1}\ cm^{-1}$.

Proteins

To study protein-protein interactions using proteins immobilized on magnetic beads, a 6×His-tagged, C-terminal 30 kDa domain of the RecB ($RecB^{nuc}$) was used. The clone for overexpression of the 6×His-tagged $RecB^{nuc}$ was a generous gift from Dr. D. Julin (University of Maryland). *B. stearothermophilus* PcrA, *B. subtilis* RecA, and T4 UvsX were generous gifts from Dr. D.B. Wigley (Clare Hall Laboratories, CRUK, UK), Dr. F.R. Bryant (Johns Hopkins University), and Dr. S.W. Morrical (University of Vermont), respectively. *E. coli* RecA,

LexA, RecQ and SSB, *S. cerevisiae* Rad51, and T4 gp32 proteins were purified as described in Harmon and Kowalczykowski (1998), Kowalczykowski et al. (1981), LeBowitz (1985), New et al. (1998), and Rehrauer et al. (1996), respectively.

Protein-Protein Interaction Assay Using Ni-NTA Magnetic Beads

Protein-protein interactions between 6×His-RecB^{nuc} and untagged proteins were studied by pull-down assays with Ni-NTA (Ni^{2+}-charged nitriloacetic acid) magnetic beads (QIAGEN). Unless otherwise indicated, reaction mixtures containing 50 mM Tris-acetate (pH 7.5), 50 mM NaCl, 50 mM imidazole, 0.2% Triton X-100, and 1 μM of the assayed proteins were preincubated for 15 min at 37ºC. When present, ATP or ATP-γ-S were present at 2 mM, and poly(dT) was present at 5 μM nucleotides. Ni-NTA magnetic beads were added to final concentration of 1%. Immobilization of the histidine-tagged proteins was carried out according to the manufacturer's specifications.

Beads were separated from the solution phase by a QIAGEN "12-Tube Magnet." Aliquots (20 μl) from each assay mixture containing unbound proteins were analyzed by 10% SDS-PAGE. The beads were washed with 3 × 300 μl of interaction buffer (50 mM Tris-acetate [pH 7.5], 50 mM NaCl, 50 mM imidazole, and 0.2% Triton X-100) to remove any trace of free proteins. Proteins bound to the beads were eluted with 20 μl of elution buffer (pH 7.5), containing 150 mM Tris acetate, 100 mM NaCl, and 300 mM imidazole and were analyzed by using 10% SDS-PAGE.

The gels were stained with Coomassie brilliant blue, and the relative amounts of each protein were measured by comparison to known quantities of each protein. The amounts of protein eluted from beads were evaluated by "Gel Pro Analyzer" (Media Cybernetics), and binding data were fit to a hyperbola by using Prism v. 4 for Windows (GraphPad Software).

Modeling of the RecA-RecB^{nuc} Interaction Interface

The coordinates for the core domain of RecA were obtained from the structure of RecA (PDB accession number 2REB) (Story et al., 1992). Coordinates for the C-terminal nuclease domain of RecB protein were obtained from the structure of RecBCD (Singleton et al., 2004). Molecular interaction simulations were performed with the "Chemera" molecular graphics and modeling environment (http://www.cqfb.fct.unl.pt/bioin/chemera/Chemera/Intro.html), employing the soft protein docking algorithm BiGGER (Bimolecular Complex Generation with Global Evaluation and Ranking) (Palma et al., 2000). Calculations were performed with the following settings: the central core domain of RecA (amino acids 33–240) and nuclease domain of RecB subunit (amino acids 928–1180) were selected as the target and probe, respectively, the surface matching was carried out with an angular step of 10º, the minimum contact area was set to 300 Å^2, and the maximum number of solutions was set to 5000. Control docking experiments are detailed in the Supplemental Data (Figure S7).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and seven figures and can be found with this article online at http://www.molecule.org/cgi/content/full/21/4/573/DC1/.

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