Published in final edited form as:

J Mol Biol. 2009 August 14; 391(2): 269–274. doi:10.1016/j.jmb.2009.06.042.

The RecB Nuclease Domain Binds to RecA-DNA Filaments: Implications for Filament Loading

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Abstract

The *E. coli* RecBCD enzyme facilitates the loading of RecA onto single-stranded DNA produced by the combined helicase/nuclease activity of RecBCD. The nuclease domain of RecB protein, RecB^{nuc}, has been previously shown to bind RecA. Surprisingly, RecB^{nuc} also binds to phage and eukaryotic homologs of RecA, leading to the suggestion that RecB^{nuc} interacts with the polymerization motif that is present in all three proteins. This mode of interaction could only be with monomeric RecA, as this motif would be buried in filaments. We show that RecB^{nuc} binds extensively to the outside of RecA-DNA filaments. Three-dimensional reconstructions suggest that RecB^{nuc} binds to the ATP-binding core of RecA, with a displacement of the C-terminal domain of RecA. Solution experiments confirm that the interaction of RecB^{nuc} is only with the RecA core. Since the RecA C-terminal domain has been shown to be regulatory, the interaction observed may be part of the loading mechanism where RecB displaces the RecA C-terminal domain and activates a RecA monomer for polymerization.

Keywords

recombination; electron microscopy; helical polymers

The RecBCD complex plays a key role in homologous genetic recombination in *E. coli*. It serves to act as both a helicase and nuclease that together generate single-stranded DNA (ssDNA) tails that become covered with the RecA protein and which then act in strand invasion reactions¹. It has been shown that RecBCD also plays a specific role in the loading of RecA onto these ssDNA tails^{2–4}, and the RecA-binding portion of RecBCD has been shown to reside in the RecB nuclease (RecB^{nuc}) domain^{3,5}. Remarkably, it has been shown that RecB^{nuc} also interacts with the phage and eukaryotic RecA homologs, UvsX and Rad51, respectively⁵. RecA and UvsX have a C-terminal domain⁶ that is absent in Rad51, while Rad51 has an N-terminal domain⁷ that is absent in RecA and UvsX. All three of these proteins, however, share a

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structurally conserved ATP- and DNA-binding core that is also present in such disparate proteins as the F1-ATPase⁸ and many helicases⁹.

Since RecA, UvsX and Rad51 appear to have a common “polymerization” motif that forms a part of the subunit-subunit interface in the filaments that are formed by these proteins^{7,10,11}, it was suggested that RecB^{nuc} binds to this motif⁵. Such a mode of interaction might be similar to that put forward for the Rad51-binding BRC motifs within BRCA2, since it was suggested¹⁰ that the BRC motifs mimic the polymerization motif in Rad51, bind to Rad51 monomers, and block polymerization of Rad51¹². These BRC-Rad51 interactions appear to be more complicated, because it has now been shown that some BRC motifs can also bind to Rad51-DNA filaments¹³, an interaction that must involve a region of the Rad51 subunit that is not part of a buried polymerization motif within the filament.

Binding to RecA-DNA filaments

We have examined the interaction of RecB^{nuc} with RecA-DNA filaments using electron microscopy (EM). Naked RecA-DNA filaments (Fig. 1a) display the characteristic striations arising from the $\sim 90\text{--}95$ Å pitch RecA helix. After incubation with RecB^{nuc} (with a 1:1 stoichiometry of RecA:RecB^{nuc}) the filaments become less smooth (Fig. 1b), suggesting a binding of RecB^{nuc} along the entire length of these filaments. The extensive binding was confirmed using three-dimensional reconstruction. We used the Iterative Helical Real Space Reconstruction (IHRSR) method^{14,15} to reconstruct the RecA-DNA-RecB^{nuc} filaments from these EM images of negatively stained samples. We started the procedure with 48,633 overlapping segments (each 70 pixels or 332 Å in length). The IHRSR approach failed to converge to the same solution from different starting points, which is an indication of heterogeneity in structure^{16,17}. The heterogeneity might come from partial binding of RecB^{nuc} to RecA-DNA filaments, with incomplete occupancy resulting in structural heterogeneity. The heterogeneity might also arise from different binding modes, similar to what is seen for certain actin-binding proteins that can interact with F-actin polymorphically¹⁸⁻²¹. We therefore used three reference volumes to sort these segments based both on occupancy and binding modes: a naked RecA-DNA filament, a RecA-DNA filament with additional mass on the outside of the filament, and a RecA-DNA filament with additional mass in the groove of the filament. A multi-reference sorting against projections of these reference volumes was then used to classify image segments into three different groups. Slightly more than a third of the segments were classified as naked RecA-DNA filaments, $\sim 40\%$ of the segments were characterized as having additional mass on the outside of the RecA-DNA filaments, and the remaining quarter of the segments were classified as having additional mass in the groove. Such classification does not necessarily mean that the sorting is valid. One independent test of this sorting was using the same reference volumes to sort images of naked RecA-DNA filaments (Fig. 1a). Surprisingly, a significant amount ($\sim 25\%$) of the naked RecA-DNA segments were classified as having additional mass in the groove, comparable to the fraction of RecA-DNA-RecB^{nuc} segments sorted into this category. This suggested to us that this category may simply be an artifact of incomplete stain penetration into the groove. However, no significant fraction of naked RecA-DNA segments was classified as having additional mass on the outside of the filament, the largest category in the RecA-DNA-RecB^{nuc} filaments.

The classification method just described is model-dependent, so we also used a model-independent approach to sort the RecA-DNA-RecB^{nuc} filaments based upon the projected density at different radii. By extracting segments showing the highest density on the outside of the RecA-DNA filament, a more homogeneous sample was obtained that showed extensive binding by RecB^{nuc} (Fig. 2b) when compared to a control reconstruction of naked RecA-DNA filaments (Fig. 2a). The helical parameters of the RecA-DNA filaments bound by RecB^{nuc} (an

axial rise of 14.8 Å and a twist of 59.0° per subunit) were very similar to those found for naked RecA-DNA filaments, showing that the binding of RecB^{nuc} introduces no major change in the helical geometry. A high degree of confidence exists in the interpretation of the domain structure within a low-resolution RecA-DNA reconstruction (Fig. 1a), since our model for this filament²² has been confirmed by a recent high-resolution crystal structure of an active RecA-DNA filament²³. We have found no way to fit both RecA (PDB 3CMW) and the RecB^{nuc} structure²⁴ (PDB 1W36) into the reconstructed volume (Fig. 2b). The simplest explanation is that RecB^{nuc} binds to the core of RecA, with the C-terminal domain of RecA shifted extensively by RecB^{nuc} into different positions so that it is not seen in the helically averaged reconstruction. Such motions of the RecA C-terminal domain would be consistent with what has previously been observed by both EM²² and x-ray crystallography²⁵. A molecular model showing RecB^{nuc} bound to the core of RecA (Fig. 2b) suggests that the full molecular volume of RecB^{nuc} cannot be accommodated. We suggest that the reconstruction is an average of naked RecA subunits (where the RecA C-terminal domain is normally positioned) and RecA subunits where RecB^{nuc} is bound and the C-terminal domain is displaced.

This model for RecB^{nuc} binding to a RecA-DNA filament suggests that RecB^{nuc} should bind to the core of RecA in solution but not to the C-terminal domain. We have tested this by examining the interaction between the RecB^{nuc} domain and the N-or C-terminal fragments of RecA. A construct was produced that contained the N-terminal region of RecA, comprising the first 268 residues which encompasses the N-terminal and the ATPase domains (Δ RecA), fused with SUMO to both facilitate solubility and provide a hexahistidine tag. Pull-down assays were performed and an excess of untagged RecB^{nuc} was mixed with Δ RecA and Ni-agarose beads. After extensive washing, proteins retained on the beads were eluted and a complex between RecB^{nuc} and Δ RecA was detected (Fig. 3a). We also undertook pull-down assays with an alternative construct, in which the C-terminal domain of RecA (δ NRecA, residues 269–352) was fused to SUMO. In this case, no interactions were detected (Fig. 3b), supporting the proposal that it is the core of RecA that interacts with the nuclease domain of RecB.

Binding to Rad51-DNA filaments

It was shown⁵ that RecB^{nuc} also interacts with human Rad51 (hRad51), so we have used similar procedures to look at the binding of RecB^{nuc} to hRad51-DNA filaments. When naked hRad51-DNA filaments (Fig. 1c) are compared with filaments after incubation with RecB^{nuc} (Fig. 1d), filaments are seen to be decorated by RecB^{nuc} in a similar manner to the decoration of RecA-DNA filaments (Fig. 1b). We used the IHRSR method to reconstruct the hRad51-DNA-RecB^{nuc} filaments from EM images of negative stained samples. As with the RecA-DNA-RecB^{nuc} filaments, the hRad51-DNA-RecB^{nuc} filaments were judged to be heterogeneous by the failure of the IHRSR approach to converge to the same solution from different starting points. Similar sorting methods were used as for the RecA-DNA-RecB^{nuc} filaments, and we were able to generate a three-dimensional reconstruction (Fig. 2d) from a relatively homogeneous subgroup. As with the case for RecA, our interpretation of the low-resolution domain structure (Fig. 2c) of the naked Rad51-DNA filaments²⁶ has been confirmed by high-resolution x-ray crystal structures of these filaments^{7,11}. What is also similar to the situation with the RecA-DNA-RecB^{nuc} filaments is that the reconstruction of the decorated volume (Fig. 2d) is unable to accommodate both a molecule of hRad51 and a molecule of RecB^{nuc}. However, the reconstructed density can be explained by RecB^{nuc} bound to the core of Rad51 (Fig. 2d), with the Rad51 N-terminal domain completely missing. In this model, the interaction of RecB^{nuc} with the Rad51 core would be very similar to the interaction between RecB^{nuc} and the RecA core (Fig. 2b).

Is it reasonable to imagine that the Rad51 N-terminal domain would be invisible in Fig. 2d, presumably due to disorder? The RecB^{nuc} bound to a Rad51 subunit one turn above would be

in steric conflict with the N-terminal domain of another Rad51 subunit, and this would generate large displacements of the N-terminal domains. There are many observations showing that this domain is highly mobile. In a crystal structure of a related protein, archaeal RadA, only one N-terminal domain is seen in seven subunits due to large disorder²⁷. In a crystal structure of the related Dmc1 protein, no N-terminal domains are seen in an octamer of subunits²⁸. Electron microscopic reconstructions of yeast Rad51 filaments have shown that in the presence of a point mutation (G103E) within the N-terminal domain, the N-terminal domain is never seen after helical averaging²⁹. Thus, the large displacements of the N-terminal domain that would be generated by the binding of RecB^{nuc} to hRad51-DNA filaments appear to be consistent with many previous observations.

Implications of the results

Although the complex of RecB^{nuc} bound to hRad51-DNA filaments is not of biological significance, such binding must arise from RecB^{nuc} recognizing a conserved structural surface that is also present in RecA, as suggested previously⁵. In contrast to the original prediction, however, this surface is not part of the subunit-subunit interface in the filament. On the other hand, the complex of RecB^{nuc} with a RecA-DNA filament has great biological relevance, and provides insights into how RecBCD loads RecA onto ssDNA⁴. Although the stoichiometric binding of a RecB domain to a RecA polymer is not in itself expected to recapitulate what happens in a cell, stoichiometric binding of actin-binding proteins to F-actin and microtubule-binding proteins to microtubules have been extremely useful in understanding the structural basis of the interactions that will occur biologically at much lower stoichiometries. Many observations suggest that the RecA C-terminal domain plays a regulatory role in RecA filament formation and homologous recombination³⁰⁻³³. This regulatory role appears mainly to be negative, because truncations of the C-terminal domain can lead to enhanced binding to DNA and other activities *in vitro*, while C-terminal truncated RecA proteins can lead to constitutive induction of the SOS response *in vivo*³⁴. Presumably, the C-terminal truncation of RecA leads to a protein that will more readily form activated filaments on double-stranded DNA within the cell, thereby cleaving the LexA repressor in the absence of the normal signal for DNA damage, the presence of naked ssDNA. We therefore think that it may be significant that the binding of RecB^{nuc} to the core of RecA displaces the C-terminal domain. This may be the first step in the activation of a RecA subunit for polymerization, a process that is normally negatively regulated by the presence of the C-terminal domain.

Acknowledgements

This work was supported by NIH grant GM035269 (to E.H.E.) and CRUK (D.B.W.). The clone for overexpression of the 6xHis-tagged RecB^{nuc} was a generous gift from Dr. S. Kowalczykowski.

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Figure 1.

Electron micrographs of negatively-stained RecA-dsDNA filaments (a), hRad51-dsDNA filaments (c), RecA-DNA-RecB^{nuc} complexes (b), and hRad51-dsDNA-RecB^{nuc} complexes (d). RecA-dsDNA-ATP- γ -S complexes were incubated in 25 mM triethanolamine-HCl (Fisher) buffer (pH 7.2) at 37° C for 10 min, with a RecA concentration of 3.5 μ M, RecA to calf thymus dsDNA (Sigma) ratio of 40:1 (w/w), 1.25 mM ATP- γ -S (Boehringer), 2 mM magnesium acetate (Sigma). Then 3.5 μ M RecB^{nuc} was added incubated at 37° C for an additional 10 min. The complexes with hRad51 were prepared the same way, except that the hRad51 concentration was 6 μ M. Samples were applied to carbon-coated grids and stained with 2% uranyl acetate (w/v). Images were recorded on film with a Tecnai 12 electron microscope operating at 80 keV with a nominal magnification of 30,000x. Negatives were scanned with a Nikon Coolsan 8000 densitometer at a raster of 4.2 Å/pixel. The 23 Å pitch helix of the Tobacco Mosaic Virus (a, lower left and lower right) was used as an internal magnification standard. The scale bar in (a) is 1,000 Å.

Figure 2.

Three-dimensional reconstructions of naked RecA-DNA filaments (a) and naked hRad51-DNA filaments (c) are compared with reconstructions of RecA-DNA-RecB^{nuc} (b) and hRad51-DNA-RecB^{nuc} complexes (d). The reconstructions are displayed as grey transparent surfaces, with ribbon models for the components shown within each. In (a) the RecA core is shown in blue, with the C-terminal domain shown in green. The same coloring is used in (b), with one RecB^{nuc} domain²⁴ shown in red. The binding of RecB^{nuc} to the bottom of the RecA core (in the orientation shown) would require a large displacement of the RecA C-terminal domain. In (c) the Rad51 core is shown in cyan, while the N-terminal domain is shown in magenta. The yeast Rad51 crystal structure⁷ has been used due to the fact that no crystal structure exists for

full length hRad51, but all evidence suggests that at this resolution the two are very similar²⁹. In (d) the RecB^{nuc} domain is shown as a red ribbon. The binding of RecB^{nuc} to the core of one hRad51 would be in steric conflict with the N-terminal domain of a subunit one turn below, leading to a displacement of the N-terminal domains so that they are not seen in this reconstruction.

Figure 3. Evidence for RecB^{nuc} binding to the RecA core

Pull-down assays using Ni-agarose beads were performed and analyzed by SDS-PAGE. (a) Lane 1: δ CRecA; lane 2: RecB^{nuc}; lane 3: sample loaded into the column; lane 4: flow through; lane 5: eluate; lane 6: RecB^{nuc} load; lane 7: RecB^{nuc} flow through; lane 8: RecB^{nuc} eluate. Lanes 6–8 are controls to show that RecB^{nuc} does not interact with the beads. (b) Lane 1: δ NRecA; lane 2: δ NRecA treated with Ulp1; lane 3: RecB^{nuc}; lane 4: RecB^{nuc} treated with Ulp1; lane 5: flow through; lane 6: flow through treated with Ulp1; lane 7: eluate; lane 8: eluate treated with Ulp1. Only the SUMO-tagged δ NRecA construct is cleaved by Ulp1 protease. The RecB^{nuc} domain was expressed and purified as described previously⁴. A 4 °C overnight thrombin digestion was performed in order to cleave the 6 $\times$ His-tag. The sample was then loaded onto a NiNTA column (GE Healthcare) equilibrated in 50 mM Tris (pH 7.5), 300 mM NaCl and 50 mM imidazole. The flow through was concentrated using a Vivaspinn concentrator (5 kDa cutoff) and loaded onto a gel filtration column (Superdex 75 16/60) (GE Healthcare) equilibrated with 150 mM NaCl, 50 mM Tris (pH 7.5). This purified, untagged protein was used for protein interaction experiments. The clone for the overexpression of the SUMO-tagged

C-terminal deletion RecA (residues 1–268, δ CRecA) was obtained by PCR amplification using the following primers: 5' - CGCGGATCCATGGCTATCGACGAAAACAAACAGAAAGCGTTG – 3' and 5' - CGCGGATCCATGGGTATCAACTTCTACGGCGAACTGGTTGAC – 3'. The amplified construct and the pSMT3 vector were digested at 37 °C with Bam HI and HindIII and then ligated. δ CRecA was expressed in *E.coli* B834+ cells. Cells were grown at 37°C until OD = 0.6, induced with 1 mM IPTG then grown overnight at 18°C. After harvesting, the cell pellet was resuspended in 50mM phosphate (pH 6.8), 500mM NaCl, 20mM imidazole, 0.1% Triton X-100, protease inhibitors and lysozyme. After sonication, the lysate was cleared by centrifugation and the supernatant was loaded onto a NiNTA column. After extensive washing with 50 mM phosphate (pH 6.8), 500 mM NaCl, 20 mM imidazole, the protein was eluted in the same buffer but with 300 mM imidazole. The sample was concentrated and loaded onto a Superdex 200 (10/300) in 20 mM phosphate (pH 6.8), 500 mM NaCl. The clone for the overexpression of the SUMO-tagged, C-terminal domain of RecA (Δ NRecA, residues 269–352) was obtained by PCR amplification using the following primers: 5' - CGTATCGTCGAAATCTACGGACCGGAATCT – 3' and 5' - CCGCTCGAGTTAAAAATCTTCGTTAGTTTCTGCTACGCCTTC – 3'. The amplified construct and the pSMT3 vector were digested at 37°C with NdeI and XhoI and ligated. The RecA C-terminal SUMO-tagged construct (RecA_C-term) was expressed in *E.coli* BL21 cells. Cells were grown at 37°C until OD = 0.6, induced with 1 mM IPTG, then left to grow for a further three hours. After harvesting, the cell pellet was resuspended in 50 mM Tris (pH 8.0), 500mM NaCl, 20mM imidazole, 0.1% Triton X-100, protease inhibitors and lysozyme. After sonication, the lysate was cleared by centrifugation and loaded onto a NiNTA column. After extensive washing with 50 mM Tris (pH 7.5), 300 mM NaCl, 20 mM imidazole, the protein was eluted in the same buffer but with 300 mM imidazole. The fractions containing the protein were pooled, dialysed against 20 mM Tris (pH 7.5), 200 mM NaCl, then concentrated and loaded onto a Superdex 75 (16/60) equilibrated with 20 mM Tris (pH 7.5), 200 mM NaCl. An excess of RecB^{nuc} was mixed with δ CRecA in 50 mM Tris (pH7.5), 150 mM NaCl, 20 mM imidazole, and incubated at 37°C for one hour. Ni-agarose beads were then added and the mixture was incubated for another hour at room temperature. After extensive washing to remove unbound proteins, the protein was eluted with 50 mM Tris (pH7.5), 150 mM NaCl, 300 mM imidazole. Samples were collected and analyzed by SDS-PAGE. This procedure was repeated by mixing RecB^{nuc} to δ NRecA but with an additional step because the SUMO-tagged δ NRecA and RecB^{nuc} ran at the same place on SDS gels so could not be distinguished from one another. Incubation of the samples with Ulp1 protease released the δ NRecA from the SUMO tag but was unable to digest the RecB^{nuc} domain alone, allowing the identity of the protein bands to be established.