Domain Structure of the Red β Single-Strand Annealing Protein: the C-terminal Domain is Required for Fine-Tuning DNA-binding Properties, Interaction with the Exonuclease Partner, and Recombination *in vivo*.

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Abstract

Red β is a component of the Red recombination system of bacteriophage λ that promotes a single strand annealing (SSA) reaction to generate end-to-end concatemers of the phage genome for packaging. Red β interacts with λ exonuclease (λ exo), the other component of the Red system, to form a “synaptosome” complex that somehow integrates the end resection and annealing steps of the reaction. Previous work using limited proteolysis and chemical modification revealed that Red β consists of an N-terminal DNA binding domain, residues 1-177, and a flexible C-terminal “tail”, residues 178-261. Here, we quantitatively compare the binding of the full-length protein (Red β^{FL}) and the N-terminal domain (Red β^{177}) to different lengths of ssDNA substrate and annealed duplex product. We find that in general, Red β^{FL} binds more tightly to annealed duplex product than to ssDNA substrate, while Red β^{177} binds more tightly to ssDNA. In addition, the C-terminal region of Red β corresponding to residues 182-261 was purified and found to fold into an α -helical domain that is required for the interaction with λ exo to form the synaptosome complex. Deletion analysis of Red β revealed that removal of just eleven residues from the C-terminus disrupts the interaction with λ exo as well as ssDNA and dsDNA recombination *in vivo*. By contrast, the determinants for self-oligomerization of Red β appear to reside solely within the N-terminal domain. The subtle but significant differences in the relative binding of Red β^{FL} and Red β^{177} to ssDNA substrate and annealed duplex product may be important for Red β to function as a SSA protein *in vivo*.

Introduction

Together with homologous recombination (HR) and non-homologous end joining (NHEJ), single-strand annealing (SSA) is one of three main pathways for repair of dsDNA breaks^{1,2}. SSA is performed by a complex and highly regulated network of proteins in eukaryotes, including several to recognize and process dsDNA ends^{3,4}, and a central protein, Rad52, to perform the strand annealing step⁵. Many bacteriophage and oncogenic dsDNA viruses encode a simple two-component “SynExo” recombination system that performs a streamlined version of SSA to generate end-to-end concatemers of their genome, to facilitate viral replication and packaging^{6,7}. The most well-studied SynExo system is *Red* (recombination defective) from bacteriophage λ , in which the exonuclease component is λ exonuclease (λ exo), a 26 kDa protein encoded by the *red α* gene, and the synaptase component is Red β , a 30 kDa protein encoded by the *red β* (or *bet*) gene⁸. λ exo forms a ring-shaped homotrimer that binds to dsDNA ends and processively digests the 5' strand to form a long 3'-overhang⁹⁻¹¹. Red β is a SSA protein that assembles onto the resulting 3'-overhang to anneal it to a complementary strand from a homologous duplex^{12,13}. λ exo and Red β interact with one another to form a “synaptosome” complex that integrates the end resection and annealing steps of the reaction, by an unknown mechanism¹⁴⁻¹⁶. Related SynExo pairs, most notably RecET from the *E. coli* *rac* prophage^{17,18}, are found in a wide variety of bacteriophage and in bacterial genomes as integrated prophage^{19,20}. Due to their relative simplicity, high efficiency, and ability to work on short (~50 bp) regions of homology, Red, RecET, and other SynExo recombination systems have been exploited

in powerful methods for genetic engineering known as recombineering and MAGE (Multiplex Automated Genome Engineering)²¹⁻²⁵.

Previous work by Radding and colleagues revealed an unusual DNA binding behavior for Red β ²⁶. In gel-shift assays with 33-mer oligonucleotides, Red β bound weakly to ssDNA, not at all to pre-formed dsDNA, but tightly to the duplex product (or intermediate) of annealing formed when two complementary oligonucleotides were added to Red β sequentially. Visualization of Red β by electron microscopy (EM) closely paralleled these results²⁷. The protein formed oligomeric rings of 10-12 subunits in the absence of DNA, slightly larger rings in the presence of ssDNA, and a left-handed helical filament upon the addition of heat-denatured dsDNA. The filaments were often seen to extend from rings, suggesting that the annealing process began on the ring and was extended by the filament. Based on these observations, a model for the mechanism was proposed in which the oligomeric ring form of Red β binds and presents the ssDNA substrate with its bases exposed for homology recognition, while the filament assembles on the duplex product of annealing as it is formed, to drive the reaction forward. Consistent with this model, crystal structures of the N-terminal DNA-binding domain of the eukaryotic Rad52 SSA protein revealed an 11-mer ring with an outer positively-charged groove with appropriate dimensions for binding ssDNA^{28,29}.

Although the RecT/Red β family of SSA proteins was originally thought to be distinct from Rad52 based on the absence of detectable sequence similarity, more recent comparisons indicate that the two families of proteins are in fact related³⁰⁻³³. Together with Erf from phage P22³⁴, Sak from *Lactobacillus lactis* phage ϕ 136³⁵, and the mitochondrial recombinase Mgm101³⁶, RecT/Red β and Rad52 appear to form a large

family of SSA proteins with similar structures and possibly similar mechanisms of action. However, given the absence of a crystal structure for a RecT/Red β family member, and the low level of sequence similarity with the Rad52 domain that has been crystallized, detailed structural comparisons are tenuous at best, and it has proven difficult to predict the residues of Red β that are involved in oligomerization and/or DNA binding³³.

Previous UV crosslinking experiments identified an N-terminal 20 kDa peptide of Red β that is implicated in binding to ssDNA³⁷. Subsequently, our group performed a thorough domain mapping study using limited proteolysis, chemical modification, and mass spectrometry to define a protease-resistant N-terminal DNA-binding domain comprised of residues 1-177³⁸. The C-terminal residues 178-261 of Red β were predicted to form a more flexible C-terminal “tail,” due to their susceptibility to protease and the absence of residues implicated in DNA binding. The N-terminal domain of Red β , which we will refer to as Red β^{177} , was purified and found to bind ssDNA and form the complex with annealed duplex in a similar way as the full-length protein, which we will refer to as Red β^{FL} . Further, nitrocellulose filter binding experiments revealed that Red β^{177} binds more tightly and stably than Red β^{FL} to ssDNA, suggesting a role for the C-terminal tail of Red β in modulating the DNA binding properties of the N-terminal domain.

Previous comparisons of Red β^{FL} and Red β^{177} were only qualitative. In the present study we use gel-shift and fluorescence polarization (FP) to quantitatively compare the binding of Red β^{FL} and Red β^{177} to different lengths of ssDNA substrate and annealed duplex product. In general, we find that Red β^{FL} binds with approximately two-fold higher affinity to annealed duplex product than to ssDNA substrate, while Red β^{177} binds with

two-fold higher affinity to ssDNA. A deletion analysis demonstrates that Red β^{177} is completely defective for both dsDNA and ssDNA recombination *in vivo*, suggesting that the small but significant differences in DNA binding seen *in vitro* are likely to be important for the function of Red β as a SSA protein *in vivo*. We further demonstrate that the C-terminal residues 182-261 of Red β fold into a small α -helical domain that is both required and sufficient for binding to λ exo to form the synaptosome complex. Finally, we find that the residues of Red β involved in self-oligomerization reside predominantly, if not entirely, within the N-terminal domain.

Results

Gel-shift assays reveal different DNA binding behavior of Red β^{FL} and Red β^{177}

For all DNA binding assays, Red β^{FL} was expressed and purified with an N-terminal 6xHis-tag, which was subsequently removed with thrombin protease, leaving three non-native N-terminal residues (Gly-Ser-His). Red β^{177} was insoluble when expressed with a N-terminal 6x-His tag, so it was expressed and purified as an untagged protein with its native N-terminal sequence.

The DNA sequences used for all DNA binding experiments were derived from M13 mp18 DNA as described previously²⁶ and shown in Table S1. Figure 1 compares the binding of Red β^{FL} and Red β^{177} to three different lengths of 5' ³²P-labeled ssDNA (33+, 48+, and 83+). Data from the plot of fraction DNA bound vs. protein concentration were fit to the Hill equation to determine *apparent* K_d values, which are indicated in the figures and summarized in Table 1. The data reveal that Red β^{177} binds with about two-

to three-fold higher affinity than Red β^{FL} to each ssDNA. The K_d values measured for Red β^{FL} , which range from 790 – 1600 nM, are consistent with a previous study reporting a K_d of 1800 nM for binding of Red β^{FL} to a 36-mer oligo³⁷. The shifted bands for the complexes formed by Red β^{FL} and Red β^{177} also appeared qualitatively different. For all three lengths of ssDNA, a relatively diffuse band of higher mobility was seen for Red β^{FL} , while a more distinct band of lower mobility was seen for Red β^{177} , suggesting that it forms a larger, more stable protein-DNA complex. We did not observe a large dependence of binding affinity on DNA length for either Red β^{FL} or Red β^{177} . This contrasts previous filter binding experiments in which Red β bound with higher affinity to shorter oligos, suggesting a nucleating reaction with DNA ends²⁶.

Importantly, binding of both proteins to all three lengths of ssDNA was cooperative, with n values from fits to the Hill equation ranging from 2.5-5.6. The extent of cooperativity did not depend on DNA length, but was slightly higher for Red β^{177} than for Red β^{FL} . The fact that the binding is cooperative suggests that both proteins bind to ssDNA as an oligomeric complex, as seen in previous DNA binding³⁷ and EM experiments²⁷. By contrast, non-cooperative binding was suggested by recent atomic force microscopy (AFM) experiments in which Red β was seen to bind to ssDNA as dispersed monomers³¹. We do not at present understand the reason for these differing results, although the proteins in each study were purified with different affinity tags.

Next we performed the gel-shift assay to compare the binding of Red β^{FL} and Red β^{177} to the annealed duplex product formed by adding two complementary oligonucleotides (33+ and 33-) to the protein sequentially. Previous experiments showed that Red β^{FL} forms a remarkably stable complex when the two strands are

added in this way, and that deproteinization of the complex with SDS releases the DNA as duplex product²⁶. Interestingly, as compared to the result described above with ssDNA, the opposite trend was seen, as shown for the 33-mer oligos in Figure 2: Red β^{FL} bound with about two-fold higher affinity to the annealed duplex product (K_d 520 $\pm$ 20 nM) than Red β^{177} (K_d 1200 $\pm$ 200 nM). For both proteins, the shifted complex appears as a discrete band of lower mobility, similar to the band seen for the complex of Red β^{177} with ssDNA. Similar results were seen for the annealed duplex formed with longer oligos (48+/- and 83+/-, data not shown), and binding of both proteins to all three lengths of DNA was cooperative, with n values ranging from 2.7-4.5. No systematic dependence of n on the length of DNA was observed, but this time Red β^{FL} bound more cooperatively than Red β^{177} . Importantly, the order of addition of the two complementary strands to the protein did not have a significant effect on the result, for either protein. It should also be noted that although we are treating the binding to annealed duplex as an equilibrium experiment to extract apparent dissociation constants to compare the two proteins, neither Red β^{FL} nor Red β^{177} binds at all to pre-formed dsDNA (see below), and thus the system is presumably not at equilibrium.

In Figures 1 and 2, we have plotted the data for Red β^{FL} and Red β^{177} together, so as to compare the binding of the two proteins to each type of DNA. However, the data can also be plotted to compare the binding of each protein to the two different types of DNA (ssDNA substrate vs. annealed duplex product), as shown for the 33-mers in Figure S1. These plots reveal an interesting difference: while Red β^{FL} binds with about three-fold higher affinity to annealed duplex product (K_d 520 $\pm$ 20 nM) than to ssDNA (K_d 1600 $\pm$ 200 nM), Red β^{177} has the opposite preference, binding with two-fold higher

affinity to ssDNA (K_d 580 ± 50 nM) than to annealed duplex (K_d 1200 ± 200 nM). Thus, although Red β^{177} forms complexes with ssDNA substrate and annealed duplex product in a similar manner as Red β^{FL} , the relative binding is reversed. The C-terminal region of Red β does not bind to DNA on its own (see below), but somehow modulates the inherent DNA binding properties of the N-terminal domain³⁸.

Nitrocellulose filter binding assay

Next we determined if the differences in binding of Red β^{FL} and Red β^{177} to ssDNA substrate and annealed duplex product seen by gel-shift also held true when a double-filter method was used³⁹. In this experiment, ³²P-labeled DNA bound to Red β is trapped by the nitrocellulose filter, while unbound DNA passes through the nitrocellulose and is trapped by the underlying DEAE filter. The binding of Red β^{FL} and Red β^{177} to 33-mer ssDNA and 33-mer annealed duplex were compared (Fig. S2). As expected, Red β^{FL} bound 1.6-fold more tightly to annealed duplex (K_d 150 ± 10 nM) than to ssDNA (K_d 250 ± 10 nM), while Red β^{177} had the opposite preference, binding more tightly to ssDNA (K_d 160 ± 10 nM) than to annealed duplex (K_d 190 ± 20 nM). The latter difference was small, but statistically significant. Binding was again cooperative, with slightly higher cooperativity for Red β^{FL} than for Red β^{177} . Thus, compared to Red β^{FL} , the increased affinity of Red β^{177} for ssDNA seen by gel-shift was recapitulated in the filter-binding assay, although the differences were smaller. Similar results were obtained with the longer 33-mer and 83-mer oligonucleotides, but the experiments were not performed in triplicate, and are not reported in Table 1 (data not shown). Note that the dissociation constants measured by the double-filter method were about 5-fold lower

(on average) than those measured by gel-shift, for all complexes tested (please see Table 1 for a summary of all binding data). The weaker binding seen by gel-shift could conceivably be due to partial dissociation of the complexes during migration through the gel in the electric field.

Fluorescence polarization DNA binding assay

To further compare the binding of Red β^{FL} and Red β^{177} to different lengths of ssDNA substrate and annealed duplex product, we used fluorescence polarization (FP) with 5'-fluorescein (5'FluorT)-labeled oligonucleotides having the same sequences used above. We first compared the binding of Red β^{FL} and Red β^{177} to 33+, 48+, and 83+ ssDNA. As seen by gel-shift, Red β^{177} bound to all three lengths of ssDNA with about two-fold higher affinity than Red β^{FL} (Fig. 3). Binding to all three lengths of ssDNA was again cooperative for both proteins, with n values ranging from 3.1-4.2, and there was no strong dependence of either binding affinity or cooperativity (n) on the length of the ssDNA, for either protein. Finally, similar to nitrocellulose filter binding, the dissociation constants measured by FP were about 5-fold lower (on average) than those measured by gel-shift, for all complexes tested (Table 1).

Next we used FP to compare the binding of Red β^{FL} and Red β^{177} to different lengths of annealed duplex product (Fig. 4). As was seen by gel-shift, Red β^{FL} bound to 33-mer and 83-mer annealed duplex with approximately two-fold higher affinity than Red β^{177} . However, for the 48-mer annealed duplex, the binding of Red β^{FL} and Red β^{177} was not statistically different. We do not understand the basis for this discrepancy, but given that all of the differences we have observed are small (consistently on the order of

two- to three-fold), it is possible that other factors could affect the comparison. Fits to the Hill equation resulted in n values ranging from 3.6-5.3, again indicating cooperative binding of both proteins to all three lengths of annealed duplex.

As was shown for the gel-shift, the FP data can also be plotted to compare the binding of each individual protein (Red β^{FL} or Red β^{177}) to the two different types of DNA, ssDNA substrate and annealed duplex product (Fig. S3). For the 33-mer and 83-mers, Red β^{FL} bound to annealed duplex about 2-fold more tightly than to ssDNA, while for Red β^{177} , the opposite is true. The same trend is seen for the 48-mers, although the difference is considerably smaller, but still statistically significant. Curiously, the baseline anisotropy seen at lower protein concentrations for the 48-mers is higher when both oligos are present than when only when oligo is present, suggesting that the two oligos may be annealing to one another on their own, when the concentration of protein is too low to bind and have an effect. This was not seen for the 33-mers and 83-mers, although the GC-content for the three different oligonucleotides is similar (37-45%). We do not at present understand the basis for the effect, but it could at least in part explain the anomalous behavior seen above for the 48-mers as compared to the 33-mers and 83-mers.

The FP assay was also used to demonstrate that neither protein binds at all to pre-formed dsDNA, prepared by annealing the two complementary oligos to one another before adding the protein. This is shown by the green data points in Figure S3 for 33-mer dsDNA, but also confirmed for the 48-mer and 83-mer dsDNA (data not shown). This lack of binding to pre-formed dsDNA was seen previously by gel-shift²⁶,

but the more sensitive FP assay confirms that neither Red β^{FL} nor Red β^{177} exhibit any binding at all to dsDNA, at concentrations as high as 2.5 μ M.

In summary, we have used three different methods to compare the binding of Red β^{FL} and Red β^{177} to three different lengths of ssDNA substrate and annealed duplex product (Table 1). For all of the experiments, a small but significant difference in the binding preference for the two different proteins is seen. In general, whereas Red β^{FL} binds more tightly to annealed duplex product than to ssDNA substrate, Red β^{177} has the opposite preference. This difference can be attributed to both an increase in the affinity of Red β^{177} for ssDNA, as well as a decrease in its stability on the annealed duplex product.

Red β^{177} is severely compromised for recombination *in vivo*

To determine if the small but significant differences in DNA binding seen for Red β^{177} *in vitro* affect its ability to promote SSA *in vivo*, dsDNA and ssDNA recombineering assays were performed. A schematic of these assays is shown in Figure 5. For these experiments, Red β^{FL} and Red β^{177} were expressed with their native N-terminal sequences, beginning with Met-1. In the dsDNA assay (linear plus circular recombineering), a linear 2 kB PCR-generated neomycin cassette carrying homologies at each end to a target site within a p15A-*Cm^R* plasmid was electroporated into GB2005 *E. coli* cells (*recA*-) expressing the Red recombination functions⁴⁰ (Fig. 5a). Once inside the cells, the neomycin cassette is inserted by Red recombination into the p15A-*Cm^R* plasmid to generate chloramphenicol and kanamycin-resistant colonies. In the ssDNA assay (single-strand oligonucleotide repair, or ssOR), a synthetic 100-mer

oligonucleotide was electroporated into cells to delete a 4 nt insertion within a neomycin gene on a bacterial artificial chromosome (BAC) (Fig. 5b). The ssDNA assay can utilize oligos targeting either the leading (Ld) or lagging (Lg) strand of the replication fork at the target site, with the latter being about 10-fold more efficient⁴¹⁻⁴⁴. With this experimental setup, WT Red β (Red β^{FL}) produced about 10^4 recombinants per 10^8 viable cells in the dsDNA assay, and 10^3 - 10^4 recombinants in the ssDNA assay (Table 2). For the ssDNA assay, the expected lagging-strand bias was observed, with approximately 10-fold higher efficiency for oligos targeting the lagging strand. Surprisingly, despite its ability to bind to ssDNA substrate and form the complex with annealed duplex *in vitro*, Red β^{177} gave only very low numbers of recombinants ($\sim 10^1$ - 10^2), for both the dsDNA and ssDNA assays (Table 2). In fact, the number of recombinants produced per 10^8 viable cells was within the range of background levels, as determined in the absence of arabinose induction, or with a Red β deletion mutant, two negative controls that gave the same low number of colonies. No colonies were formed when no DNA was added (both for the dsDNA and the ssDNA experiments), indicating that all of the recombination events, including those for Red β^{177} and the negative controls, involved the electroporated DNA.

PCR and restriction analysis were performed to determine the percentage of colonies formed with Red β^{FL} and Red β^{177} that contained the expected recombinant, as compared to the two negative controls (Figures S4 and S5). For dsDNA recombineering (Figure S4), while Red β^{FL} produced correct recombinants for 10/10 selected colonies, none of the colonies produced by Red β^{177} or the Red β deletion contained the correct recombinant (ten colonies were tested for each). Two of the ten colonies tested for the

no-arabinose control contained the correct recombinant, suggesting the possibility of leaky expression, although the number of colonies was not significantly higher than for the Red β deletion. For ssDNA recombineering (Figure S5), at least 9/10 colonies contained the correct recombinant, indicating successful oligo repair, for all of the protein variants tested, including the negative controls. This result is consistent with previous findings in which ssDNA recombination was seen to occur at a low but significant level, even in the absence of RecA or Red β ^{45,46}. It must be emphasized that even though the correct ssDNA recombinants are formed with Red β ¹⁷⁷, there is no evidence that this protein is actively promoting the oligo repair, since the number of colonies was not above the background measured for the negative controls.

To determine the region of the C-terminus that is required for *in vivo* activity more precisely, a series of Red β C-terminal deletions was generated, to express proteins ending at amino acid residue 250, 240, 220, 200, and 188 (Red β ^{FL} extends to the C-terminal residue 261). Surprisingly, all of these C-terminally truncated proteins were as defective as Red β ¹⁷⁷ for both dsDNA and ssDNA recombineering (Table 2). Soluble expression of each truncated protein was confirmed by Western blot (Fig. S6a), indicating that the lack of activity observed *in vivo* was not due to the absence of soluble protein. However, based on the intensities of the bands in the Western blot, the level of each of the truncated proteins was reduced by approximately 2.5- to 3-fold relative to Red β ^{FL} (assuming similar reactivity with the antibody). Interestingly, probing with an antibody raised against both λ exo and Red β revealed a significant reduction in the level of λ exo protein, for all of the Red β C-terminal truncations (Figure S6b). The decrease in the level of λ exo caused by alterations of Red β could conceivably arise from a

disruption of the complex formed by the two proteins, if the complex is required for their stability in the cell.

These data indicate that, remarkably, removing as few as 11 amino acids from the C-terminus of Red β results in complete loss of activity for both dsDNA and ssDNA recombineering. The lack of activity in the dsDNA assay could conceivably be attributed to a disruption of the interaction with λ exo (see below), the protein that resects the ends of the electroporated dsDNA prior to annealing by Red β ¹⁵. However, a lack of interaction with λ exo could not explain the reduced activity observed in the ssDNA assay, which does not require λ exo^{24,41,42,44}. Thus, it appears that the subtle but significant differences in DNA binding we have observed for Red β^{FL} and Red β^{177} *in vitro* may be important for the function of Red β as a SSA protein *in vivo*.

Red β^{177} does not interact with λ exo to form the synaptasome complex

Red β forms a protein-protein interaction with λ exo that appears to be required for full dsDNA recombineering activity *in vivo*¹⁵. However, the regions of each protein involved in the interaction are currently unknown. To determine if the C-terminal region of Red β is involved in the interaction, a Ni-spin pull-down assay was performed using N-terminally 6xHis-tagged λ exo to pull down a subset of the un-tagged C-terminally truncated Red β proteins used above in the recombineering assays. Proteins were overexpressed from pET vectors in separate batches of BL21-AI *E. coli*, which were then mixed and lysed by sonication, loaded onto Ni-NTA spin columns, washed with 30 mM imidazole, and eluted with 500 mM imidazole. Figure 6 shows that untagged Red β^{FL} forms a strong interaction with 6xHis- λ exo, as it is abundantly present in the

elution fractions. The remaining C-terminally truncated proteins fail to show a significant interaction with λ exo. Removal of just 11 amino acids from the C-terminus (to form Red β^{250}) significantly eliminates the interaction with λ exo, and further truncations from the C-terminus (to form Red β^{200} , Red β^{188} , and Red β^{177}) completely eliminate the interaction. Similar results were obtained using purified proteins instead of proteins from mixed cell lysates (Fig. S7a), or when the N-terminal 6xHis-tag was placed on Red β instead of on λ exo (Fig. S7b-c). These data show that the C-terminal region of Red β is required to form the complex with λ exo.

Red β^{177} forms similar oligomers as Red β^{FL}

A common feature of SSA proteins is their ability to form oligomeric rings and filaments²⁷⁻³⁶. As seen previously by EM, Red β alone forms rings of 10-12 subunits, rings of 15-18 subunits with ssDNA, and left-handed helical filaments with heat-denatured dsDNA²⁷. To determine if Red β^{177} is able to form similar oligomers as Red β^{FL} , analytical gel filtration chromatography was performed on purified Red β^{FL} and Red β^{177} loaded at concentrations of 100 μ M. Figure 7a shows that both proteins elute from the column at volumes corresponding to large oligomers, with estimated molecular masses of 676 and 368 kDa, respectively. While these masses are larger than expected from the number of subunits seen by microscopy^{27,31}, likely due to the non-spherical shape of the rings, the data indicate that the two proteins form oligomers comprised of a similar number of subunits, since the smaller Red β^{177} protein runs at a smaller size than Red β^{FL} . Running Red β^{FL} or Red β^{177} proteins as complexes with 33-mer ssDNA substrate and annealed duplex product results in the elution from the column of

progressively larger oligomers, indicating that the DNA has the same effect on both proteins (Fig. S8). These data show that Red β^{177} is able to form similar oligomeric species as Red β^{FL} , both in the presence and absence of DNA. Thus, the N-terminal domain of Red β appears to have most, if not all of the determinants necessary for self-oligomerization and DNA binding.

We further assessed the ability Red β^{FL} and Red β^{177} to self-oligomerize using the Ni-spin pull-down assay with mixed cell lysates expressing 6xHis-Red β^{FL} or 6xHis-Red β^{177} and the corresponding un-tagged protein. For comparison, we also expressed both tagged and un-tagged versions of the C-terminal residues 182-261 of Red β , which we will refer to as Red β^{CT} . Figure 7b-d shows that 6xHis-Red β^{FL} pulls down un-tagged Red β^{FL} , and 6xHis-Red β^{177} pulls down un-tagged Red β^{177} , indicating that both proteins are able to self-oligomerize, as expected from the gel filtration data. By contrast, 6xHis-Red β^{CT} is unable to pull down un-tagged Red β^{CT} indicating that the C-terminal domain does not oligomerize on its own. The pull-down assay was also performed on pairwise mixtures of the three different constructs. Figure S9 shows that 6xHis-Red β^{177} is able to pull down un-tagged Red β^{FL} (and *vice versa*, data not shown), indicating a stable interaction involving the N-terminal domain. By contrast, 6xHis-Red β^{CT} is not able to pull down un-tagged Red β^{FL} or Red β^{177} , indicating that there is no stable interaction between the N- and C-terminal domains of Red β . Taken together, these data show that the determinants for self-oligomerization of Red β reside predominantly, if not entirely, within the N-terminal domain. Moreover, there is not likely to be a substantial interaction between the N- and C-terminal domains of Red β , either within or between subunits of an oligomer.

Further characterization of the Red β C-terminal domain

The C-terminal residues 178-261 of Red β are required for the interaction with λ exo and for full recombination activity *in vivo*, but whether or not they form a stable, folded structure is unknown. To determine if this is the case, 6xHis-Red β^{CT} (consisting of residues 182-261) was expressed in *E. coli* and purified by Ni-affinity chromatography, thrombin digestion to remove the 6xHis tag, and finally gel filtration chromatography. Red β^{CT} elutes from a Superdex 200 gel filtration column as a single, well-defined peak corresponding to a folded monomer (estimated mass of 9.5 kDa), in contrast to the large oligomers formed by Red β^{FL} and Red β^{177} (Fig. S10). This is consistent with the pull-down assay, which showed that Red β^{CT} is unable to self-associate (Fig. 7d). To determine if purified Red β^{CT} is folded, the protein was subjected to circular dichroism (CD) spectroscopy at room temperature. Figure 8a shows the far-UV spectrum, with minima at 208 and 222 nm indicating a folded, predominantly α -helical structure⁴⁷. This result is consistent with the secondary structure prediction from the amino acid sequence of this region of Red β , which shows strong hits for three α -helices (Fig. S11).

To determine if the isolated C-terminal domain of Red β is able to form a complex with λ exo on its own, the Ni-spin pull-down assay was performed with purified proteins. As seen in Figure 8d, 6xHis-Red β^{CT} is indeed able to pull down un-tagged λ exo in a similar manner as Red β^{FL} , indicating a stable λ exo-Red β^{CT} complex is formed. Experiments using proteins from mixed cell lysates instead of purified proteins gave similar results (data not shown). However, the interaction was not observed when

6xHis- λ exo was used to pull down untagged Red β^{CT} . It is possible that the interaction involves the N-terminus of λ exo, and that the N-terminal 6xHis tag on λ exo disrupts the complex with Red β^{CT} but not with Red β^{FL} . The complex with Red β^{FL} could be more stable, either by avidity through oligomer formation, or if there is a stabilizing interaction involving the Red β N-terminal domain that is not detectable with Red β^{177} alone. Regardless, the Ni-spin pull-down demonstrates a stable interaction between 6xHis-Red β^{CT} and λ exo, but not between 6xHis-Red β^{177} and λ exo (or Red β^{177} and 6xHis- λ exo), indicating that most if not all of the elements for forming the synaptosome complex are located within the Red β C-terminal domain.

Finally, the ability of Red β^{CT} to bind to DNA was assessed using the FP assay with 5'-fluorescein-labeled 33-mer oligos (Fig. 8b-c). Addition of Red β^{CT} , even at concentrations as high as 2 μ M, did not lead to any observable increase in anisotropy, for either ssDNA substrate or annealed duplex product. This indicates that Red β^{CT} does not bind to DNA on its own, although an interaction with DNA in the context of full-length Red β cannot be excluded.

Effect of the extra N-terminal Gly-Ser-His residues on Red β^{FL}

We have observed that a seemingly small difference, removal of the last 11 residues of Red β , has a profound effect on the activity of the protein *in vivo*. The Red β^{FL} protein we have used for the DNA-binding experiments was purified with an N-terminal 6xHis-tag, and the final purified protein contains an extra N-terminal Gly-Ser-His sequence after removal of the 6xHis tag with thrombin. To determine if the Gly-Ser-His sequence has an effect on the DNA-binding activity *in vitro*, an un-tagged version of

Red β^{FL} was purified using similar procedures as for Red β^{177} , which was also purified without a tag. The fluorescence polarization experiment of Figure S12 using 33-mer oligos shows that the un-tagged Red β^{FL} protein exhibits essentially identical behavior as GSH-Red β^{FL} , indicating that the extra GSH sequence has little to no effect on DNA binding. To determine if the Gly-Ser-His sequence affects the activity of Red β *in vivo*, the nucleotides encoding the extra N-terminal Gly-Ser-His sequence were inserted into the pSC101 vector, and dsDNA and ssDNA recombineering assays were performed (Table 2). The results show that the GSH-Red β exhibits activity that is equal to native Red β^{FL} (WT) in the dsDNA assay, and only slightly reduced in the ssDNA assay (lagging strand). PCR analysis confirmed that all of the colonies produced by the GSH-Red β contained the correct recombinant product for both dsDNA and ssDNA recombineering experiments (Fig. S4c and S5c). These results indicate that the GSH sequence has little to no effect on the activity of Red β^{FL} *in vitro* or *in vivo*.

Biochemical properties of the Red β^{250} variant missing the last eleven residues.

Our DNA binding experiments revealed subtle but significant differences between Red β^{FL} and Red β^{177} that may be important for *in vivo* activity. Our deletion analysis revealed that removal of just the last 11 residues from the C-terminus of Red β renders it completely inactive for both dsDNA and ssDNA recombination. However, whether or not the inactivity of Red β^{250} (and the other deletion constructs) *in vivo* could be due to the same alteration of DNA binding properties as Red β^{177} is unknown. We therefore purified the Red β^{250} protein as an untagged protein (with a native N-terminus), and determined whether its DNA-binding properties are more like Red β^{FL} or Red β^{177} . The FP DNA-

binding experiment with 33-mer oligos shows that Red β ²⁵⁰ binds with 1.5-fold higher affinity to ssDNA than to annealed duplex, making it more like Red β ¹⁷⁷ (Fig. S13). However, the affinity of Red β ²⁵⁰ for both types of DNA was reduced by about 5-fold compared to Red β ^{FL} and Red β ¹⁷⁷, for unknown reasons. Nonetheless, these data support the conclusion that the inactivity of Red β ¹⁷⁷ and the other C-terminal truncations may be due to a change in the relative binding to ssDNA substrate and annealed duplex product.

Discussion

Despite limited sequence homology, Red β of bacteriophage λ is now believed to be a member of a growing superfamily of SSA proteins that likely share similar structural and mechanistic features³¹⁻³³. In a previous study, we used limited proteolysis and chemical modification to identify a protease-resistant DNA-binding domain of Red β formed by residues 1-177³⁸. This domain corresponds precisely to the region of the protein that is predicted to be conserved in Rad52 and related SSA proteins³¹⁻³³. The N-terminal domain of Red β (Red β ¹⁷⁷) was purified and found to bind to ssDNA and form the complex with annealed duplex in a similar way as Red β ^{FL}. A nitrocellulose filter binding experiment showed that Red β ¹⁷⁷ actually binds to ssDNA (a 33-mer oligo) with significantly higher affinity and stability than Red β ^{FL}, indicating that the C-terminal residues of Red β somehow modulate the inherent DNA binding of the N-terminal domain. However, these experiments were only qualitative, and moreover, the extent to which Red β ¹⁷⁷ is functional *in vivo* was not determined.

In the present study, we sought to compare the functional properties of Red β^{FL} and Red β^{177} more fully and more quantitatively. Although we again find that Red β^{177} exhibits similar DNA binding properties as Red β^{FL} *in vitro*, it is completely inactive for both ssDNA and dsDNA recombineering *in vivo*. The lack of activity seen for Red β^{177} in dsDNA recombineering could be explained by its lack of interaction with λexo , which is thought to physically load Red β onto the 3'-overhang as it is generated. However, the lack of activity of Red β^{177} in the ssDNA assay cannot be explained by the loss of interaction with λexo , since λexo is dispensable for ssDNA recombineering^{24,41,42,44}. This points to a change in the properties of the Red β^{177} protein itself for the loss of *in vivo* recombination. Interestingly, removal of just 11 residues from the C-terminus of Red β (to form Red β^{250}) results in the same complete loss of activity that is seen when the C-terminal region is deleted entirely, as in Red β^{177} . Thus, whatever the alteration is in Red β^{177} that results in it's being inactive, seems to be recapitulated in Red β^{250} .

One possibility is that Red β^{177} and the other C-terminally truncated proteins are less stable or less soluble than Red β^{FL} when expressed in the GB2005 cells used in the recombineering assay. Western blots show soluble expression of Red β^{177} and other C-terminally truncated mutants (Fig. S6), but at a level that appears to be reduced by 2.5-3 fold compared to Red β^{FL} . Although this is not a large difference compared to the difference in activity, it is conceivable that some threshold level of Red β is required for full activity, and that the levels of Red β^{177} and the other C-terminally truncated proteins are below this threshold. Expression levels of λexo were also reduced in all truncated Red β mutants, suggesting that its stability may rely on the formation of the complex with Red β . Qualitatively, we have noticed that when overexpressed in *E. coli* for protein

purification, Red β^{177} (and Red β^{250}) require low-temperature expression, and use of higher concentrations of NaCl and glycerol in the lysis buffer, to obtain optimal amounts of soluble protein in the supernatant. As this is not the case for Red β^{FL} , the loss of *in vivo* activity for truncated Red β could indeed be due to alteration of the solubility or other physico-chemical properties of the protein. Along these lines, we also noticed a propensity of Red β^{177} to aggregate during gel filtration chromatography (as seen by a peak in the void volume), which was not seen with Red β^{FL} (Fig. S10).

A second possibility is that the subtle yet significant differences in DNA binding we have observed for Red β^{177} *in vitro* somehow limit its ability to function as a SSA protein *in vivo*. As noted previously, Red β^{FL} binds only weakly to ssDNA substrate, and much more tightly to the complex with annealed duplex formed when two complementary oligos are added to the protein sequentially²⁶. Tight binding of Red β to the product (or intermediate) of annealing was suggested to be important for its function as a SSA protein. Using three different assays and three different lengths of oligos, we observed that in general Red β^{177} exhibits the opposite DNA binding preference as Red β^{FL} , binding more tightly to ssDNA substrate than to annealed duplex product. Although the differences are small (2-3 fold at most), they are statistically significant when the experiments are performed in triplicate. We observed this same trend with the Red β^{250} protein, although it has significantly reduced affinity for both types of DNA, for unknown reasons. As enzymes in general have evolved to bind to substrate, transition state, and product with relative affinities that are finely tuned to catalyze their reactions efficiently, it is possible that the tighter binding of Red β^{177} (and Red β^{250}) to ssDNA that we have observed *in vitro* could be responsible for its lack of activity *in vivo*.

In our previous study, we concluded that the C-terminal residues 178-261 of Red β likely form a flexible “tail” due to their high sensitivity to protease and absence of residues implicated in DNA binding³⁸. However, it is also possible that these residues form a small folded domain that is connected to the rest of the protein by a flexible linker. New data presented here support this conclusion: residues 182-261, which we refer to as Red β^{CT} , behave as a folded protein when expressed and purified, and elute from a gel filtration column at a volume corresponding to a folded monomer. In addition, CD spectroscopy of Red β^{CT} indicates a predominantly α -helical secondary structure, in agreement with secondary structure predictions that give strong hits for three α -helices. Interestingly, secondary structure prediction also flags residues 180-197 as a low complexity region, as expected for a flexible linker (Fig. S11).

C-terminal truncations of Red β reveal that removal of just the last 11 amino acids (to form Red β^{250}) results in a protein that is functionally similar to Red β^{177} , with stronger binding to ssDNA than to annealed duplex, no activity for dsDNA and ssDNA recombineering *in vivo*, and little to no interaction with λ exo. The third predicted α -helix of Red β^{CT} extends to amino acid residue 256 (5 amino acids from the C-terminus), such that deletion of 11 residues may disrupt this helix and prevent the entire C-terminal domain from folding. If the proper folding of Red β^{CT} is required for the interaction with λ exo and its effects on DNA binding, this could explain why truncating the last 11 residues has the same effect as deleting the domain entirely.

If the C-terminal domain of Red β does have an important role in modulating DNA binding, how does it exert its effect? The purified Red β^{CT} protein does not bind at all to DNA on its own, and an interaction of the C-terminal domain with DNA in the context of

the full-length protein seems unlikely. As the C-terminal domain has a net charge of -5, it could conceivably weaken binding to ssDNA through non-specific electrostatic repulsions. Interestingly, several other ssDNA binding proteins, most notably *E. coli* RecA and SSB, have flexible C-terminal “tails” that modulate DNA binding and contain sites for binding to other proteins^{48,49}. Removal of the negatively charged C-terminal tail of RecA results in increased binding to dsDNA⁵⁰, and in a similar manner, we found that removal of the C-terminal domain of Red β results in increased binding to ssDNA. While for RecA and SSB it appears that the C-terminal residues are unstructured, for Red β they appear to form a small folded domain.

We cannot attribute the reduced activity of Red β^{177} *in vivo* to the inability of the protein to form oligomers. Gel filtration shows that Red β^{177} forms similar oligomers as Red β^{FL} , both alone and in the presence of ssDNA and annealed duplex (Fig. S8). Ni-spin pull-down revealed that all of the determinants for self-oligomerization appear to reside within the N-terminal domain (Fig. 7). However, a notable difference was observed in the gel-shift assay with ssDNA substrate. Whereas Red β^{FL} binds weakly to ssDNA to form a complex that migrates as a diffuse band of higher mobility, Red β^{177} binds more tightly to ssDNA to form a complex that migrates as a distinct band of lower mobility. The same difference was observed for all three lengths of DNA tested. This suggests that Red β^{FL} and Red β^{177} form different types of complexes on the ssDNA.

The band for the Red β^{177} -ssDNA complex appeared similar to the bands that both proteins form with annealed duplex product, when two complementary oligos are added to the protein sequentially. EM data show that Red β forms two distinct types of oligomers: an oligomeric ring for binding to ssDNA substrate, and a helical filament for

binding to annealed duplex product²⁷. It is conceivable that, based on the bands in the gel shift, Red β ¹⁷⁷ may bind as a helical filament to both forms of DNA. If the oligomeric ring is required for SSA activity *in vivo*, this then could explain why Red β ¹⁷⁷ is inactive *in vivo*.

On the other hand, previous AFM data suggested that Red β binds to ssDNA not as an oligomeric ring, but rather as dispersed monomers³¹. The fact that the complexes of Red β ^{FL} with ssDNA seen in the gel shift were of higher mobility than those with Red β ¹⁷⁷ is consistent with this possibility, since the higher mobility suggests a smaller size. Moreover, as the concentration of Red β was increased, the mobility of the diffuse band decreased, suggesting the gradual binding of additional monomers to the DNA. However, binding of both Red β ^{FL} and Red β ¹⁷⁷ to all types of DNA tested was cooperative, with values for the Hill coefficient (n) ranging from 2.0-5.6. This suggests that all of the complexes are formed by protein oligomers, as opposed to monomers.

In fitting the DNA binding data to the Hill equation, we have not made any assumptions as to the number of monomers of Red β present in each type of complex. In previous experiments it was determined that Red β binds to ssDNA with a stoichiometry of 4-5 nt per monomer³⁷. Similarly, based on the crystal structure and on footprinting experiments, Rad52 appears to bind to ssDNA with a stoichiometry of four nucleotides per monomer^{28,29,51}. For Red β ^{FL} and Red β ¹⁷⁷, we did not observe a consistent or large dependence of the cooperativity parameter (n) on either the type of DNA used (ssDNA substrate vs. annealed duplex product) or the length of the oligonucleotide. While the use of the Hill equation allowed us to compare the relative binding of the two proteins to three different lengths of each type of DNA, the data do

not provide information on the type of complex formed. In future studies, measurement of the stoichiometry, and fitting of the data to a lattice model instead of the Hill equation, could provide additional information.

It is also important to note that although the binding of Red β to ssDNA can be treated as an equilibrium process to extract binding affinities, the complex with annealed duplex cannot. Formation of this complex requires sequential addition of the two complementary oligos to the protein, and the protein does not bind at all to pre-formed dsDNA. Although we have treated the data for this complex as an equilibrium for purposes of comparing the two proteins, the “tighter” binding we have observed for Red β^{FL} in this complex likely reflects higher stability, as opposed to higher affinity. Kinetic measurements using other methods will be required to dissect the differences in binding more precisely.

In summary, the functional data presented here indicate that the domain structure of Red β is highly modular. Whereas the N-terminal domain defined by residues 1-177 appears to exhibit all of the determinants for DNA binding and oligomerization, the C-terminal domain defined by residues 182-261 appears to exhibit all (or most) of the determinants for the interaction with λ exo. Based on the lack of *in vivo* activity observed for Red β^{177} and the other C-terminally truncated proteins, and on the altered DNA binding properties of Red β^{177} observed *in vitro*, the C-terminal domain may also have a less well-defined role in modulating DNA binding, to fine-tune the relative affinities for ssDNA substrate and annealed duplex product. Interestingly, several other SSA proteins that are believed to be distantly related to Red β , including Erf and Sak, have a strikingly similar domain structure, in which an N-terminal domain is

responsible for DNA binding and oligomerization, while a flexibly-tethered and more variable C-terminal domain is implicated in interactions with other proteins^{52,53}.

Materials & Methods

Materials. All chemicals were purchased from Fisher Scientific unless otherwise noted. All enzymes were purchased from New England Biolabs.

Protein Expression and Purification. Red β ^{FL} and Red β ¹⁷⁷ were expressed and purified as described previously³⁸. Briefly, Red β ^{FL} was expressed from a pET-14b vector as an N-terminally 6xHis-tagged protein in BL21-AI *E. coli* cells for 4 h at 37 °C. Cells were harvested, re-suspended in Sonication Buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0), lysed by sonication, and purified on a Ni²⁺-charged HisTrap column (GE Healthcare). Collected fractions were dialyzed into Phosphate Buffered Saline (PBS; 150 mM NaCl, 20 mM NaH₂PO₄, pH 7.4) and treated with thrombin to remove the N-terminal 6xHis-tag. A second (reverse) HisTrap column was run to remove any remaining His-tagged protein, and pooled fractions were dialyzed into 20 mM Tris-HCl, pH 8.0, and purified further by anion-exchange on a Q sepharose FF column (GE Healthcare). Pooled fractions were dialyzed into 20 mM Tris-HCl, pH 8.0, 1 mM DTT, concentrated to > 10 mg/ml in and stored in small aliquots at -80 °C. The final purified protein contains an extra Gly-Ser-His sequence at the N-terminus (GSH-Red β ^{FL}).

Red β ¹⁷⁷ was expressed as an un-tagged protein from a pET-9a vector in BL21-AI cells at 15 °C for 18 h. Cleared cell lysates were purified on DEAE sepharose, Q sepharose, and Heparin ion-exchange columns (GE Healthcare), dialyzed into 20 mM

Tris-HCl, pH 8.0, 1 mM DTT, concentrated to > 10 mg/ml, and stored in small aliquots at -80 °C. Un-tagged versions of Red β ^{FL} and Red β ²⁵⁰ were expressed and purified by the same procedures used for Red β ¹⁷⁷.

DNA encoding the C-terminal residues 182-261 of Red β , referred to as Red β ^{CT}, was cloned between the *Nde*I and *Bam*HI sites of pET-28b, and expressed as an N-terminally 6xHis-tagged protein with an intervening thrombin cleavage site. Expression and purification were performed as described above for Red β ^{FL}. The final Red β ^{CT} protein contains an extra Gly-Ser-His sequence at the N-terminus. Protein concentrations were measured by absorbance at 280 nm using extinction coefficients calculated from the amino acid sequences. These were: 34,950 M⁻¹cm⁻¹ for Red β ^{FL} and Red β ²⁵⁰, 27,960 M⁻¹cm⁻¹ for Red β ¹⁷⁷ and 6,990 M⁻¹cm⁻¹ for Red β ^{CT}. Molecular masses were: 29,969 g/mol for GSH-Red β ^{FL}, 29,688 g/mol for Red β ^{FL}, 28,535 g/mol for Red β ²⁵⁰, 20,527 g/mol for Red β ¹⁷⁷, and 9,742 g/mol for Red β ^{CT}.

Oligonucleotides. Oligonucleotides for DNA binding assays were purchased HPLC-purified from Integrated DNA Technologies. Oligonucleotides for gel-shift assays were 5'-end labeled with [γ -³²P] ATP using bacteriophage T4 polynucleotide kinase and purified from reaction components on G25 Microspin columns (GE Healthcare). Oligonucleotides for fluorescence polarization assays were ordered with a 5'-fluorescein label (5FluorT). The sequences of the oligonucleotide, which were derived from M13 mp18 DNA, are shown in Table S1. Complementary oligos for each DNA length were used for assays involving annealed duplex product (sequentially added oligos) or pre-formed dsDNA. Pre-formed dsDNA was prepared by mixing equimolar amounts of

complementary oligos, heating for 5 min at 95 °C, and slowly cooling in a water bath to 22 °C. Oligo concentrations are given in nucleotides unless otherwise specified.

Gel-shift DNA binding assays. For ssDNA-binding, varying concentrations of purified Redβ^{FL} or Redβ¹⁷⁷ were incubated in Binding Buffer (20 mM Tris-HCl pH 7.5, 1 mM MgCl₂, 1 mM DTT, 0.1 mg/ml BSA) with 0.5 μM of ³²P 5'-end-labeled DNA at 37 °C for 15 min. 10 μl reactions were quenched with 5X Loading Buffer (20% glycerol, 0.12% bromophenol blue, 0.12% xylene cyanol) and electrophoresed in 1X TBE (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.3) at 4 °C on 15% native polyacrylamide gels. For binding to annealed duplex product, proteins were incubated at 37 °C for 15 min with 0.5 μM of one ³²P 5'-end-labeled oligo, followed by subsequent addition of 0.5 μM of the un-labeled complementary oligo, and incubated for an additional 15 min at 37 °C prior to electrophoresis. Gels were dried, autoradiographed on a Storm 860 PhosphorImager (Amersham Biosciences) and quantified using ImageQuant software (Molecular Dynamics). Data from plots of fraction of DNA bound vs. protein concentration were fit to the Hill equation in Kaledagraph to determine the apparent dissociation constants (K_d) and Hill coefficients (n)⁵⁴:

$$B = B_{\min} + (B_{\max} - B_{\min}) \times [1/(1+(K_d/B_{\min})^n)]$$

where B is the fraction of DNA bound, $B_{\min}$ is the minimum fraction of DNA bound and $B_{\max}$ is the maximum fraction of DNA bound in the titration. Reported mean and standard deviations for K_d and n are based on three independent titrations.

Double-filter binding assay. DNA binding of Redβ was also performed using a double-filter method described previously^{38,39}. Briefly, 50 μl reactions in Binding Buffer contained 0.5 μM ³²P 5'-end-labeled oligonucleotide and varying concentrations (0-5

μM) of Redβ^{FL} or Redβ¹⁷⁷. Each binding reaction was performed in triplicate, incubated at 37 °C for 15 min, and loaded onto a Minifold I 48-well slot-blot apparatus (Schleicher & Schuell) containing nitrocellulose (Bio-Rad) and diethylaminoethyl (DEAE, Whatman) filters. Wells were pre-rinsed with 100 μl of Binding Buffer before loading individual reactions. Samples were pulled through under vacuum, and wells were then washed with 500 μl of Binding Buffer. Filters were dried and autoradiographed on a Storm 860 PhosphorImager (Amersham Biosciences). Radioactivity was quantified using ImageQuant software. The percent of protein-bound DNA was determined from the measured radioactivity on nitrocellulose (bound) and DEAE (unbound) filters as:

$$\% \text{ Bound} = B_{\text{nitro}} / (B_{\text{nitro}} + B_{\text{DEAE}})$$

Plots of % DNA bound vs. protein concentration were fit to the Hill equation as described above for gel-shift assays. Reported mean and standard deviations for K_d are based on three independent titrations.

Fluorescence polarization. For ssDNA binding, 10 nM (molecules) of 5FluorT-labeled oligonucleotide was incubated with varying concentrations (2-2000 nM) of serially diluted Redβ^{FL} or Redβ¹⁷⁷ for 15 min at 25 °C in 50 mM Tris-HCl pH 7.5, 1 mM DTT, 0.1 mg/ml BSA. For binding to annealed duplex, 10 nM (molecules) of the complementary un-labeled DNA was added subsequently, and incubated for an additional 15 min at 25 °C. Fluorescence anisotropy at 490 nm excitation and 515 nm emission were measured for each 20 μl sample in triplicate in Corning 384 well round bottom black polystyrene plates at 25 °C with a Spectra Max M5 Microplate Reader (Molecular Devices). Mean fluorescence anisotropy was plotted against protein

concentration in KaleidaGraph and fit to the following (Hill) equation to determine apparent dissociation constants (K_d) and Hill coefficients (n):

$$A = A_{\min} + (A_{\max} - A_{\min}) \times [1/(1+(K_d/A_{\min})^n)]$$

Where A is the measured anisotropy, $A_{\min}$ is the minimum anisotropy, and $A_{\max}$ is the maximum measured anisotropy for the titration.

In vivo recombineering assays. Activities of Red β^{FL} , Red β^{177} , and other C-terminal truncations of Red β were compared using dsDNA and ssDNA recombineering assays established previously⁴⁰, except that all recombination experiments were carried out in the recombination-deficient (*recA*-) *E. coli* DH10B derivative GB2005 (F-mcrA Δ (mrr-hsdRMS-mcrBC) ϕ 80lacZM15 Δ lacX74 *recA1 endA1 araD139* Δ (ara, leu) 7697 galU galK l rpsL nupG *fhuA::IS2 recET red α* , phage T1-resistant)⁵⁵. Red recombineering functions (*gam-bet-exo*) were expressed from a tetracycline-resistant, arabinose-inducible pSC101-BAD- $\gamma\beta\alpha$ -tet plasmid. The plasmid replicates at 30 °C and can be removed after induction by shift in growth temperature to 37 °C. The C-terminal truncations of Red β were generated in pSC101 by inserting stop codons at the desired locations using the QuikChange protocol (Stratagene). The Red β deletion mutant was generated by inserting a stop codon in place of Glu-14. Correct sequences were confirmed by Sanger dideoxy DNA sequencing (Plant-Microbe Genomics Facility at The Ohio State University). For recombination experiments, which were performed in triplicate, single colonies of GB2005 transformed with pSC101 vector and target DNA (p15A-*Cm^R* plasmid for the dsDNA assay, or MII-BAC-*Cm^R-neo** for the ssDNA assay) were grown in 1.2 ml Luria Broth (LB) cultures supplemented with 10 μ g/ml chloramphenicol and 4 μ g/ml tetracycline (*Cm¹⁰Tet⁴*) for 16 h at 30 °C. Approximately

30 μ l of each overnight culture was diluted into 1.2 ml LB (with Cm¹⁰Tet⁴) to give a starting OD₆₀₀ of 0.09, and grown at 30 °C for 2 h. Red functions were then induced with 0.2% L-arabinose and grown at 37 °C for 40 min. Cultures were then rapidly chilled on ice, centrifuged, and washed twice with 1 ml ice-cold ddH₂O. Pellets were then re-suspended in 50 μ l ice-cold ddH₂O and 100 ng of targeting DNA was added (dsDNA PCR product for the dsDNA assay, or single-stranded oligonucleotide for the ssDNA assay). Cells were electroporated at 1.45 kV in 0.1 cm cuvettes (MidSci EC1) using a BioRad GenePulser, recovered in 1 ml LB at 37 °C for 70 min, and plated on LB agar with 10 μ g/ml chloramphenicol and 15 μ g/ml kanamycin (Cm¹⁰Kan¹⁵). Experiments were performed several times for each protein in triplicate, the number of colonies were counted, and reported as the # of colonies formed per 10⁷ viable cells, as determined by plating on LB-agar with no antibiotics.

For the dsDNA recombineering assay (linear plus circular), the p15A-Cm^R plasmid used as the target DNA was purified by QIAprep® Spin Miniprep Kit (Qiagen) and eluted in ddH₂O. The EM7-*neo* PCR product was amplified from pR6K-UCB-EM7-*neo* plasmid using the forward and reverse primers 5'-GGA TGA ATG GCA GAA ATT CGA AAG CAA ATT CGA CCC GGT CGT CGG TTCA GCG GAA CAG GCG AGG AAA AGT AG-3' and 5'-AAT TCT CAT GTT TGA CAG CTT ATC ATC GAT AAG CTT TAA TGC GGT AGT TTG GCG CCA GCA ACC GCA CCT GTG-3', respectively. The 5' portion of the primer is homologous to the target site in p15A-Cm^R plasmid, while the underlined 3' end is homologous to the neomycin resistance gene (*neo*). PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen) and eluted in ddH₂O.

To confirm successful recombination, colonies from Cm¹⁰Kan¹⁵ plates were used as templates to PCR-amplify the EM7-*neo* insert using the forward and reverse primers 5'-GCA AAT TCG ACC CGG TCG TCG GTT CAG-3' and 5'-CAT CGA TAA GCT TTA ATG CGG TAG TTT GG-3' respectively, which anneal to the p15A-Cm^R plasmid outside of the EM7-*neo* insert. PCR reactions were analyzed on 0.8 % agarose gels, and successful recombinants were identified by the presence of a 2 kB band, representing the amplified EM7-*neo* insert.

For the ssDNA assay (oligonucleotide repair), the MII-BAC-Cm^R-*neo** bacterial artificial chromosome used as target DNA contains a four nucleotide insertion in the neomycin resistance gene (*neo**) from transposon Tn5. The targeting DNA was a 100-mer oligonucleotide targeting either the lagging strand, *neo*-Lg (5'-CAG AAA AGC GGC CAT TTT CCA CCA TGA TAT TCG GCA AGC AGG CAT CGC **CAT GGG** TCA CGA CGA GAT CCT CGC CGT CGG GCA TGC GCG CCT TGA GCC TGG C-3') or the leading strand of the target site, *neo*-Ld (5'-GCC AGG CTC AAG GCG CGC ATG CCC GAC GGC GAG GAT CTC GTC GTG ACC **CAT GGC** GAT GCC TGC TTG CCG AAT ATC ATG GTG GAA AAT GGC CGC TTT TCT G-3'), where nucleotides in bold are absent from the MII-BAC-Cm^R-*neo** target.

Successful ssDNA recombination was confirmed by PCR-amplifying the *neo* gene from the MII-BAC-Cm^R-*neo** construct using selected colonies as the templates and the forward and reverse primers 5'-GCA TGG GAT CGG CCA TTG AAC AAG ATG G-3' and 5'-GCT CAG AAG AAC TCG TCA AGA AGG CGA TAG-3', respectively. PCR products were then digested with *Nco*I at 37 °C for 1 h and run on 0.8% agarose gels. Successful oligo repair introduces an *Nco*I restriction site within the *neo* gene, and

yields two DNA fragments of 600 and 300 bp, while failed repair leaves the *neo* gene intact (900 bp).

Western blot analysis. GB2005 cultures expressing Red β^{FL} and the C-terminally truncated Red β proteins from the pSC101 plasmid were prepared as described above for the recombineering experiments. Cells were harvested (at the stage immediately prior to when target DNA would have been added), suspended in Sonication Buffer, lysed, and centrifuged to generate soluble lysates. A portion of the soluble fraction was run on a 13.5% SDS-PAGE gel, and transferred to a nitrocellulose membrane (Bio-Rad) in transfer buffer (25 mM Tris pH 8.3, 192 mM Glycine, 0.05% SDS, 20% methanol) for 80 min at 20 V (constant voltage) and 4 °C. Nitrocellulose membranes were treated with Blocking Buffer (1X PBS, 0.05% Tween® 20, 5% non-fat milk) for 1 h at room temperature, incubated with anti-Red β primary antibody (generously provided by Dr. Francis Stewart) or anti-Red $\alpha\beta$ antibody (generously provided by Dr. Lynn Thomason, Dr. Donald Court, , and Dr. Kenan Murphy) in Blocking Buffer for 1 h 30 min at room temperature. Membranes were then washed three times for 10 min in 1X PBS, 0.05% Tween® 20, incubated with horseradish peroxidase-conjugated Goat anti-Rabbit secondary antibody (Jackson ImmunoResearch) in Blocking Buffer for 1 h at room temperature, and washed again as described above. Chemiluminescence was detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and visualized on x-ray film.

Ni-spin pull-down assays. Ni-spin pull-down assays were performed with either purified proteins or proteins from mixed cell lysates. For experiments with purified proteins, 100 μ l of 20 μ M N-terminal 6xHis-tagged λ exo was mixed with an equimolar

amount of un-tagged Red β in Sonication Buffer and incubated for 1 h at 37 °C. Samples were loaded onto Ni-NTA spin columns (Qiagen), washed twice with 600 μ l of Sonication Buffer with 30 mM imidazole, and eluted twice with 200 μ l of Sonication Buffer with 500 mM imidazole. Flow-through, wash, and elution fractions were analyzed on 13.5% SDS-PAGE stained with Coomassie brilliant blue. For Ni-spin pull-down experiments with proteins from cell lysates, 6xHis- λ exo and un-tagged Red β^{FL} or C-terminal deletion mutants were overexpressed separately from pET-28b or pET-9a vectors in BL21-AI cells in 50 ml cultures grown in LB. Cells were harvested and re-suspended separately in Sonication Buffer. Cell suspensions were mixed, supplemented with 1 mg/ml lysozyme, incubated on ice 30 min, lysed by sonication, centrifuged, and cleared lysates loaded onto Ni-NTA columns. Wash, elution, and SDS-PAGE analysis were performed as described above for experiments with purified proteins.

Size exclusion chromatography. For the experiments of Figure 8a and S3, 500 μ l of purified Red β^{FL} or Red β^{177} were injected at 100 μ M onto a Superose 6 10/300 GL gel filtration column (GE Healthcare) equilibrated at 0.4 ml/min in GF buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl). For Red β samples with ssDNA, Red β was incubated with 133 μ M of (33+) single-stranded oligo at room temperature for 15 min before applying to the column. For Red β samples with annealed duplex product, Red β was incubated with 133 μ M of the 33+ oligo at room temperature for 15 min followed by the addition of 133 μ M of the 33- oligo, and the mixture was incubated for an additional 15 min before applying to the column. Molecular masses were determined using calculated K_{av} values ($[K_{av}=(V_e-V_0)/(V_t-V_e)]$, where V_e is the elution volume of the species, V_0 is the void

volume of the column, and V_t is the total column volume) and a calibration curve of $\log(\text{MW})$ vs. K_{av} of known standards: ferritin (440 kDa), catalase (232 kDa), albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen (25 kDa). The experiments of Figure S3 were performed under the same conditions, except that higher concentrations (5-10 mg/ml) of Red β^{FL} , Red β^{177} , and Red β^{CT} were injected onto a Superdex 200 Hi-Load prep grade column.

Circular dichroism spectroscopy. Far-UV CD spectra of purified Red β^{FL} , Red β^{177} , and Red β^{CT} proteins were recorded on a Jasco J-815 Spectrometer from 260-190 nm at a scan speed of 100 nm/min at room temperature in a 0.1 cm path-length quartz cuvette at 0.5 mg/ml protein in 1X PBS, pH 7.4. A scan of the buffer alone was collected and subtracted from spectra of protein samples. Plots of mean residue ellipticity $[\theta]$ were reported in $\text{deg cm}^2 \text{ dmol}^{-1}$.

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Keywords: DNA repair; DNA recombination; DNA binding; Recombineering; Single strand annealing.

Abbreviations used: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; λ exo, λ exonuclease; HR, homologous recombination; NHEJ, non-homologous end joining; SSA, single strand annealing; FP, fluorescence polarization; BAC, bacterial artificial chromosome; EM, electron microscopy; AFM, atomic force microscopy; CD, circular dichroism.

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Figure Legends

Fig. 1. Red β^{177} binds more tightly than Red β^{FL} to ssDNA substrate. Gel-shift assay using 0.5 μ M (6-15 nM molecules) of 5' ^{32} P-labeled 33-mer **(a)**, 48-mer **(b)**, or 83-mer **(c)** ssDNA oligos and varying concentrations (0, 0.4, 0.6, 0.9, 1.4, 2, 3, 4.5 μ M) of Red β^{FL} (top gel, blue curve) or Red β^{177} (bottom gel, red curve). The lower band on each gel is unbound DNA, up-shifted bands are protein-ssDNA complexes. Data were plotted as fraction DNA bound vs. protein concentration, and fit to the Hill equation to determine the apparent dissociation constant (K_d) and Hill coefficient (n). Mean and standard deviations are based on fits from three independent titrations for each of the six experiments, a representative of which is shown in the figure. Notice that Red β^{177} binds more tightly than Red β^{FL} to all three lengths of ssDNA, and that the two complexes migrate differently on the gel.

Fig 2. Red β^{FL} binds more tightly than Red β^{177} to annealed duplex product. Gel-shift assay with varying concentrations (0, 0.4, 0.6, 0.9, 1.4, 2, 3, 4.5 μ M) of Red β^{FL} **(a)** or Red β^{177} **(b)** and sequentially added complementary 33-mer oligonucleotides. The protein was first incubated with 0.5 μ M (15 nM molecules) of ^{32} P 5'-end-labeled 33+ for 15 min, and then 0.5 μ M of the un-labeled complementary oligo (33-) was added and incubated for an additional 15 min prior to electrophoresis. The lower band on each gel is unbound DNA, while up-shifted bands are protein-DNA complexes. Data were plotted as fraction DNA bound vs. protein concentration and fit to the Hill equation to determine the apparent dissociation constant (K_d) and Hill coefficient (n). Mean and standard

deviations are based on fits from three independent titrations for each of the two experiments, a representative of which is shown in the figure. Notice that Red β^{FL} binds with about two-fold higher affinity than Red β^{177} to the 33-mer annealed duplex. Also notice that the two complexes migrate similarly on the gel.

Fig. 3. Binding of Red β^{FL} and Red β^{177} to different lengths of ssDNA measured by fluorescence polarization (FP). 10 nM (molecules) of 5FluorT-labeled 33+ (a), 48+ (b), or 83+ (c) ssDNA oligos were incubated with serially diluted (2-2000 nM) Red β^{FL} (blue) or Red β^{177} (red), incubated for 15 min, and anisotropy was measured at 25 °C. For each of the six experiments, data from three independent titrations (one of which is shown here) were fit to the Hill equation to determine apparent dissociation constants (K_d) and Hill coefficients (n). Notice that Red β^{177} binds to all three lengths of ssDNA with higher affinity than Red β^{FL} .

Fig. 4. Binding of Red β^{FL} and Red β^{177} to three different lengths of annealed duplex product measured by fluorescence polarization (FP). 10 nM (molecules) of 5FluorT-labeled 33+ (a), 48+ (b), or 83+ (c) oligonucleotide was incubated with serially diluted (2-2000 nM) Red β^{FL} (blue) or Red β^{177} (red) for 15 min at 25 °C, after which, 10 nM (molecules) of the corresponding un-labeled complementary oligo was added and incubated for an additional 15 min prior to measuring anisotropy. For each of the six experiments, data from three independent titrations (one of which is shown here) were fit to the Hill equation to determine apparent dissociation constants (K_d) and Hill coefficients (n). Notice that Red β^{FL} binds more tightly than Red β^{177} to the 33-mer and

83-mer annealed duplex, whereas the two proteins bind equally to the 48-mer annealed duplex.

Fig. 5. Schematic of recombineering experiments. (a) dsDNA recombination (linear plus circular) between p15A-*Cm^R* plasmid and electroporated EM7-*neo* dsDNA. Regions of homology between the substrate and plasmid are colored in red and green. The dsDNA substrate is processed by λ exo and Red β and inserted into the p15A-*Cm^R* plasmid at the regions of homology. Cells harboring the recombinant plasmid are chloramphenicol and kanamycin resistant. (b) ssDNA recombineering between a BAC and a homologous 100-mer ssDNA oligo. Electroporated oligos targeting either the leading (*neo*-Ld) or lagging strand (*neo*-Lg) repair a 4 nt insertion in the BAC *neo** gene and restore kanamycin resistance. In both experiments, the Red functions (λ exo, Red β , and gam) are expressed from a temperature-sensitive pSC101 plasmid under the control of an arabinose-inducible promoter.

Fig. 6. Ni-spin pull-down assay to detect binding of C-terminally truncated forms of Red β to λ exo. Cell lysates expressing 6xHis-tagged λ exo were mixed with untagged Red β^{FL} (a), Red β^{250} (b), Red β^{200} (c), Red β^{188} (d), or Red β^{177} (e), loaded onto Ni-NTA spin columns, washed with 30 mM imidazole buffer, and eluted with buffer containing 500 mM imidazole. Left panels show negative controls without 6xHis- λ exo present. Gel lanes: “Load” is input to the column; “FT” is flow-through of the column; “wash” are low imidazole fractions; “elute” are high imidazole fractions. Notice that while

un-tagged Red β^{FL} is abundantly present in the elution fractions, all of the C-terminally truncated forms are not.

Fig. 7. Self-oligomerization of Red β^{FL} , Red β^{177} , and Red β^{CT} . (a) Elution profile from a Superose 6 gel filtration column of purified Red β^{FL} (blue) or Red β^{177} (red) loaded at 100 μ M shows that the proteins elute as large oligomers in solution. Column void volume (V_0) and approximate MW markers are indicated from running standards of known molecular mass. (b-d) Ni-spin pull-down of mixed cell lysates containing un-tagged and 6xHis-tagged versions of Red β^{FL} (b), Red β^{177} (c), and Red β^{CT} (d) shows that the N-terminal domain is required for oligomerization: notice that while un-tagged Red β^{FL} and Red β^{177} are present in the elution fractions (indicating formation of oligomers), un-tagged Red β^{CT} is not.

Fig. 8. Characterization of Red β^{CT} . (a) Far-UV CD spectrum of purified Red β^{FL} (blue), Red β^{177} (red), and Red β^{CT} (green) recorded at 0.5 mg/ml (50 μ M) protein at 25 °C in 1X PBS. Local minima at 208 and 222 nm indicate a folded, α -helical structure for Red β^{CT} ⁴⁷. (b) Titration of 10 nM (molecules) of 5FluorT-labeled 33+ with serially diluted (2-2000 nM) Red β^{FL} (blue) or Red β^{CT} (green) shows that Red β^{CT} does not bind at all to ssDNA substrate. (c) Similar FP experiment performed with sequentially added 33-mers shows that Red β^{CT} does not bind to annealed duplex product. (d) Ni-spin pull-down between purified 6xHis-Red β^{CT} and un-tagged λ exo indicates formation of a stable complex. The gel on the left shows that λ exo does not bind to the Ni-spin column when

6xHis-Red β^{CT} is absent. Gel lanes: “Load” is input to the column; “FT” is flow-through of the column; “wash” are low imidazole fractions.

Table 1. Summary of DNA binding parameters.

Gel Shift		K_d (nM)		n	
DNA ^a	bp	Red β^{FL}	Red β^{177}	Red β^{FL}	Red β^{177}
ss	33	1600 $\pm$ 200	580 $\pm$ 50	3.2 $\pm$ 0.3	4.8 $\pm$ 0.4
	48	960 $\pm$ 30	480 $\pm$ 40	4.6 $\pm$ 0.5	4.8 $\pm$ 1.2
	83	790 $\pm$ 110	440 $\pm$ 70	2.5 $\pm$ 0.5	5.6 $\pm$ 0.8
seq ^b	33	520 $\pm$ 20	1200 $\pm$ 200	4.5 $\pm$ 0.9	2.7 $\pm$ 0.5
Filter binding					
ss	33	250 $\pm$ 10	160 $\pm$ 10	4.9 $\pm$ 0.3	2.0 $\pm$ 0.3
seq ^b		150 $\pm$ 10	190 $\pm$ 20	4.1 $\pm$ 0.8	2.8 $\pm$ 0.3
Fluorescence Polarization					
ss	33	202 $\pm$ 12	95 $\pm$ 10	3.5 $\pm$ 0.1	3.1 $\pm$ 0.2
	48	210 $\pm$ 10	140 $\pm$ 12	3.5 $\pm$ 0.5	4.2 $\pm$ 0.6
	83	240 $\pm$ 30	120 $\pm$ 7	4.0 $\pm$ 0.1	3.2 $\pm$ 0.4
seq ^b	33	103 $\pm$ 20	190 $\pm$ 5	3.6 $\pm$ 0.4	5.3 $\pm$ 0.9
	48	170 $\pm$ 20	190 $\pm$ 20	3.6 $\pm$ 0.5	4.7 $\pm$ 1.9
	83	150 $\pm$ 20	250 $\pm$ 8	3.7 $\pm$ 0.1	4.0 $\pm$ 1.0

^a DNA sequences are given in detail in Figure S1. “ss” refers to ssDNA substrate, and “seq” refers to annealed duplex product. Apparent K_d and n values were obtained from fits to the Hill equation. Values for mean and standard deviation were determined from at least three independent titrations for each experiment. ^b Apparent K_d values for Red β with sequentially added oligos are not true equilibrium measurements since the complementary strands are added sequentially and neither protein binds at all to pre-formed dsDNA.

Table 2. *In vivo* recombination activity of Red β proteins

	Recombinants per 10 ⁸ viable cells ^a		
	dsDNA	ssDNA <i>Ld</i>	ssDNA <i>Lg</i>
-DNA/-arabinose	0	0	0
-DNA	0	0	0
-arabinose	104 $\pm$ 40	110 $\pm$ 70	120 $\pm$ 40
Red β ^{FL} (WT)	22420 $\pm$ 1580	4670 $\pm$ 1260	49630 $\pm$ 13010
Red β ¹⁷⁷	110 $\pm$ 50	60 $\pm$ 40	130 $\pm$ 50
Red β ¹⁸⁸	90 $\pm$ .60	100 $\pm$ 70	250 $\pm$ 110
Red β ²⁰⁰	80 $\pm$ .30	60 $\pm$ 30	130 $\pm$ 60
Red β ²²⁰	120 $\pm$ 40	60 $\pm$ 20	120 $\pm$ 20
Red β ²⁴⁰	60 $\pm$ 40	110 $\pm$ 50	160 $\pm$ 60
Red β ²⁵⁰	80 $\pm$ 40	90 $\pm$ 50	120 $\pm$ 60
Red β Deletion	70 $\pm$ 30	100 $\pm$ 40	110 $\pm$ 60
GSH-Red β	22160 $\pm$ 5380	6990 $\pm$ 1950	31270 $\pm$ 4020

^aNumber of recombinants on chloramphenicol plus kanamycin plates were compared to the number of colonies obtained after plating cultures on antibiotic-free LB agar. Reported averages and standard deviations are from four independent experiments.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8