DNA Binding by the Substrate Specificity (Wedge) Domain of RecG Helicase Suggests a Role in Processivity*

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RecG differs from most helicases acting on branched DNA in that it is thought to catalyze unwinding via translocation of a monomer on dsDNA, with a wedge domain facilitating strand separation. Conserved phenylalanes in the wedge are shown to be critical for DNA binding. When detached from the helicase domains, the wedge bound a Holliday junction with high affinity but failed to bind a replication fork structure. Further stabilizing contacts are identified in full-length RecG, which may explain fork binding. Detached from the wedge, the helicase region unwind junctions but had extremely low substrate affinity, arguing against the "classical inchworm" mode of translocation. We propose that the processivity of RecG on branched DNA substrates is dependent on the ability of the wedge to establish strong binding at the branch point. This keeps the helicase motor in contact with the substrate, enabling it to drive dsDNA translocation with high efficiency.

Helicases are ubiquitous enzymes essential in all stages of DNA and RNA metabolism, including replication, transcription, recombination, and repair (1, 2). They are motor proteins that generally translocate along one or both strands of dsDNA,1 dsRNA, or a DNA-RNA hybrid in an ATP-dependent manner and separate some or all parts of the molecule into its component strands. Some enzymes are highly processive, driving strand separation for long distances without dissociating from the template, a property that is especially important for enzymes involved in chromosome replication (3).

A variety of mechanisms has evolved to ensure processivity. The prokaryotic helicase DnaB, for example, forms a hexameric ring that completely encircles the DNA, which allows one-dimensional motion while preventing dissociation (4, 5). Such a strategy is common to many of the hexameric helicases (6). Other helicases are thought to achieve the same effect by interacting with the "sliding clamp" that encircles the DNA strands during replication. Examples include Wrr and Rrm3, which have been shown to have enhanced processivity in the presence of PCNA (7, 8). TheRec component of the RecBCD helicase has no helicase activity in itself, but its role may be similar to a sliding clamp, preventing dissociation of the RecBCD complex from the DNA (9–11).

A second strategy is for the helicase to have two binding sites, such that one site can release and move along the strand while the second stays bound. This can be achieved by having a dimeric structure, with one binding site per monomer and has led to the proposal of the "hand over hand" or "rolling" method. Rep helicase is thought to translocate using this mechanism (12). The Rep dimer binds to DNA, with one monomer behind the other. ATP hydrolysis alters the conformation of the helicase, radically changing the position of one monomer relative to the other, as well as causing a change in its nucleic acid binding properties (12–14). This movement means the second monomer can now bind the DNA in front of the first monomer, and thus translocation is achieved.

An alternative method, which does not rely on a dimeric structure, is the "inchworm" method of translocation. This also requires at least two binding sites, but they can be within a single monomer. The best characterized example of this is in PcrA helicase (15, 16). The PcrA helicase region contains two RecA-like helicase domains, 1A and 2A, that move relative to each other on ATP binding and hydrolysis. The two domains bind ssDNA bases in pockets that open and close with this conformational change. Initially the ssDNA is bound tightly by domain 1A. ATP binding causes the pockets in domain 2A to open, binding the ssDNA, while the pockets in domain 1A close, releasing ssDNA. At the same time, the two domains move closer together. Upon ATP hydrolysis, the pockets in 1A open, binding ssDNA, and the pockets in 2A close, releasing ssDNA. The domains also move apart, causing the ssDNA to be pulled along the DNA binding channel relative to domain 2A. Thus the DNA is effectively moved along the channel and translocation occurs. However, this "classical inchworm" method of translocation is reliant on one of the two RecA-like domains of the helicase region alternating between strong and weak DNA binding in the ATP-bound and ATP-free conformations, with the other domain conversely alternating between weak and strong DNA binding.

In this study, we have investigated the RecG helicase of Escherichia coli. RecG was first identified as a protein involved in DNA recombination and repair (17). This role was supported by the discovery that it has specificity for branched DNA molecules, in particular, Holliday junctions and replication forks (18–21). It does not unwind linear duplex DNA and has extremely low activity on classical linear, partial duplexes. Biochemical studies revealed that RecG is active as a monomer (22) and catalyzes the interconversion of forks and junctions (20, 23, 24), a process that facilitates interplay between DNA replication, recombination, and repair (25, 26). The conversion of a replication fork into a Holliday junction requires the simultaneous unwinding of the leading and lagging strands followed by the reannealing of the two parental strands and the annealing of the two nascent strands. An understanding of how this could be achieved has come from the crystal structure of

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1 The abbreviations used are: ds, double-stranded; ss, single-stranded; RF, replication fork.
Thermatoga maritima RecG in complex with a replication fork substrate (27). RecG has standard helicase domains linked to a novel “wedge” domain that provides the specificity for branched DNA. The structure has suggested models for how RecG could translocate on double-stranded DNA (27–29).

The monomeric nature of the active RecG helicase precludes the use of a physical barrier to prevent dissociation, as employed by the hexameric helicas. In addition, it also excludes the use of a rolling method of translocation to maintain contact with the DNA. We have found no evidence of an interaction with a sliding clamp complex. The poor binding observed with non-branched DNA molecules provides evidence against a classical inchworm method, which necessitates having a strong binding site within the conserved helicase region of the protein. We have dissected the RecG domain structure and identified the main structural features facilitating DNA binding. From the results presented, we suggest the wedge domain not only provides the specificity for branched DNA structures and the means to separate DNA strands but also prevents dissociation of RecG from branched DNA during the translocation process. In this sense, the wedge can be regarded as both a strand separation module and a processivity factor.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The E. coli strains used are all derivatives of MG1655 (30), except AM1125, a ΔrecG263 derivative of BL21(DE3) plysS (31). N4256 (ΔrecG263::kan), N4583 (ΔruvABC::cat), and N4971 (ΔrecG263 ΔruvABC) have been described (32). The pT7-7-derived plasmid, pM210, carries a cassette version of wild-type RecG, allowing convenient restriction fragment exchange (28). Mutant RecG genes encoding RecG proteins with defined amino acid substitutions were made by site directed mutagenesis using QuikChange mutagenesis (Stratagene) as described (31). Mutations were transferred into pM210 by fragment exchange, confirmed by DNA sequencing, and subsequently engineered into the E. coli chromosome essentially as described (33). The mutated chromosomal recG allele was then transferred into AM1418, a ΔrecG263::kan ΔpyrE::hfr derivative of MG1655 by P1vir-mediated transduction, selecting for Pyr+ colonies. Inheritance of the donor recG allele was indicated by loss of the kanamycin resistance of the recipient ΔrecG263::kan and confirmed by sequencing PCR products amplifying using recG-specific primers. The ΔruvABC::cat allele was subsequently introduced by P1vir-mediated transfer from strain N4971, selecting resistance to chloramphenicol. To make a construct encoding a Δwedge derivative of RecG, the DNA encoding the N-terminal region of RecG from Met-1 to Thr-48 was amplified from pM210 using a 5′ primer covering the Ndel site and a 3′ primer inserting a SpeI site, which modified the Thr-48 codon ACC for ACT (silent mutation) and added a codon for Ser-49. This was cloned into pBluescript SK+ as an Ndel-SpeI fragment to form pGB005. A further region of RecG, from Glu146 to Arg223, was amplified from pM210 using primers that added a 5′ SpeI site and encompassed the 3′ Sall site. This was inserted into pGB005 as a SphI-Sall fragment to form pGB008. The entire Ndel-Sall fragment was then transferred into pM210 by fragment exchange to form the plasmid pGB010. A construct encoding the wedge domain of RecG was amplified from pM210, using primers that modified Leu24 to Met by the addition of an Ndel site and a 3′ stop codon, TAA, by the addition of a 3′ HindIII site. This was cloned into pET28 as Ndel-HindIII fragment to form the plasmid pGB023.

Media and General Methods—LB broth and agar media and methods for P1 transduction and for determining sensitivity to UV light were as described (34). Data for UV survival are based on the means of at least two, but in most cases three or more, independent experiments. Values are very reproducible, with standard errors being less than 10% of the mean.

Protein Expression and Purification—Full-length wild-type RecG mutants, carrying defined amino acid substitutions, and the Δwedge derivative were purified as described (31). The RecG wedge domain linked to a His6 tag at the N terminus was expressed as described (31). Induced cells were resuspended in Buffer A (50 mM Tris-HCl, pH 7.5, 0.5 mM NaCl) containing 10 mM imidazole and lysed by sonication, and the supernatant was collected after centrifugation. This was loaded onto a N32-charged Hitrap chelating column and eluted with a gradient A containing 10 mM imidazole. After buffer exchange into Buffer B (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM diithiothreitol) using a PD-10 column, the protein was loaded onto a heparin-Sepharose column and eluted with a 0–1 mM NaCl gradient. Peak fractions of the wedge protein was dialyzed against Buffer B containing 100 mM NaCl and 50% glycerol and stored at −80 °C.

DNA Substrates—DNA substrates J12 and RF were made by annealing synthetic oligonucleotides as described (18, 21, 35). In each case, one strand was labeled at the 5′-end with [γ-32P]ATP prior to annealing. The Holliday junction, J12, has a homologous core of 12 bp flanked by 19–20-bp heterologous arms. The partial replication fork (RF) has a 25-bp duplex lagging strand arm (31). All DNA concentrations are given in moles of junction or fork structure.

DNA Binding and Unwinding—Assays were conducted essentially as described (21, 31). For binding assays, RecG and 32P-labeled substrate DNA were mixed in binding buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM diithiothreitol, 100 μg/ml bovine serum albumin, and 6% (v/v) glycerol) and incubated on ice for 15 min before loading onto a prechilled 4% native polyacrylamide gel in a low ionic strength buffer (6.7 mM Tris-HCl pH 8.0, 3.3 mM sodium acetate, and 2 mM EDTA). Electrophoresis was at 160 V for 90 min at 4 °C. For DNA unwinding assays, RecG at the concentration indicated was mixed in helicase buffer (20 mM Tris-HCl, pH 7.5, 2 mM diithiothreitol, 100 μg/ml bovine serum albumin, 5 mM ATP, and 5 mM MgCl2) and kept on ice for 5 min prior to the addition of labeled substrate DNA to 0.2 nM. An aliquot was removed immediately and deproteinized by the addition of 0.2 volumes of stop buffer (2.5% (w/v) SDS, 200 mM EDTA, and 10 mg/ml proteinase K) and incubating for a further 10 min at 37 °C. This was taken as the zero time point. The remaining reaction was then placed at 37 °C, and samples were removed at intervals, and deproteinized. All samples were then analyzed by electrophoresis using a 10% polyacrylamide gel and a Triton borate buffer system. For both DNA binding and unwinding assays, gels were dried and quantified using x-ray film and a phosphor screen (Molecular Dynamics).

RESULTS

RecG-DNA Interactions at the DNA Branch Point—The crystal structure of RecG from T. maritima identified two residues as candidates in the binding of branched DNA molecules. Phe204 was thought to stabilize the orphan base of the leading strand template by base stacking, effectively capping the parental duplex. Tyr208 was thought to play a similar role in the stabilization of the lagging strand duplex (27). A multiple sequence alignment of this region of RecG (Fig. 1A) shows that Phe204 in T. maritima is highly conserved and is likely to be equivalent to Phe97 in E. coli. However, Tyr208 is less conserved, and there is no equivalent aromatic residue in E. coli RecG at position 101. However, those sequences that do not have an aromatic residue at position 101 (E. coli numbering) do have an aromatic residue at position 99. Based on the published T. maritima RecG-DNA structure, we modeled the E. coli RecG in a complex with a DNA structure mimicking a replication fork but without a leading strand at the branch point (Fig. 1, B and C). Analysis of this model suggests that the E. coli residue Phe99 may also have a stabilizing effect equivalent to that of Phe204 in T. maritima, whereas Phe99 could act as an equivalent of Tyr208. In addition, Phe75 may be in position to stabilize the separation of the leading strand duplex, and Arg178 may be in position to interact with the phosphate backbone of the leading strand template. To test these hypotheses, we mutated phenylalanine residues 75, 96, 97, and 99 to alanine and Arg98 to glutamine and studied the resulting proteins in vivo and in vitro. A ΔrecG allele confers extreme sensitivity to UV radiation when combined with ΔruvABC allele but only very modest sensitivity in a ruv− background (Fig. 2A), consistent with the overlapping roles of the RecG and RuvABC proteins in the processing of DNA repair intermediates (26, 36). We used this difference to investigate the activities of the mutant RecG

2 A. A. Mahdi, unpublished observations.
proteins in vivo, replacing the chromosomal wild-type recG with a mutated copy. We used chromosomal constructs because previous studies have shown that the increased expression of RecG from a high copy number recG plasmid construct reduces the copy number of that plasmid quite severely. This effect is reversed to varying degrees by mutations that reduce or eliminate RecG activity (31, 37). Such effects can complicate the in vivo analysis of RecG mutant proteins expressed from a plasmid, especially as RecG appears to be expressed normally but Phe97 has only a 4-fold reduction. The double mutant has a 20-fold reduction in the ability to bind the partial (missing the leading strand) RF substrate, and the double mutant has an ~50-fold reduction (Fig. 3A). Reduced binding is also seen with a Holliday junction substrate, J12 (Fig. 3, B and C). However, in this case there is a significant difference between the mutants; Phe97 has a 50-fold reduction in binding, but Phe97 only has a 4-fold reduction. The double mutant has a 500-fold reduction in affinity.

The rates of unwinding of the RF substrate reflect the differences in the binding affinities. All three proteins have detectable activity when used at 10 nM, but when used at 0.5 nM it is clear that both F96A and F97A have 3–5% of the wild-type RecG activity, whereas unwinding by the double mutant is too low to be accurately measured (Fig. 4A). The F97A protein has a much greater ability to unwind the Holliday junction substrate at 0.5 nM, having ~20% of wild-type activity as opposed to 5% for F96A. It appears that a mutation at Phe97 has less effect on both binding and unwinding activity with the Holliday junction substrate than with the RF substrate, whereas substrate type appears to have little effect on F96A activity. This implies their activities are not functionally identical. These data suggest that the mutant proteins would be less effective than the wild-type protein in targeting branched DNA structures in vivo, thus accounting for the observed deficiency in DNA repair.

**Deletion of the RecG Wedge Domain**—Our studies on the phenylalanine mutations F96A and F97A imply that the binding of branched DNA is almost entirely due to interactions with the wedge domain. Analysis of the crystal structure of RecG from

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3 P. McGlynn and R. G. Lloyd, unpublished work.
T. maritima and our model of RecG from E. coli indicated it might be possible to delete the entire wedge domain while retaining a correctly folded protein with an unaffected DNA duplex translocation channel. We constructed such a deletion version of RecG (Δwedge) by replacing residues 49–145 with a single serine residue (Fig. 5). A plasmid construct designed to express the mutant protein failed to confer resistance to UV light and mitomycin C in a Δwedge recG strain and also failed to eliminate the extreme UV sensitivity conferred by Δwedge recG in a Δruv background (data not shown), indicating that deletion of the wedge disables the ability of RecG to promote DNA repair. After over-expression in an E. coli strain deleted for native RecG, the Δwedge protein was found to purify under conditions identical to the full-length protein and was stable under normal assay conditions. As might be predicted from the structure of a RecG-DNA complex and the failure of the mutant protein to function in vivo, band shift assays showed that the Δwedge protein has a drastically reduced affinity for both the J12 and RF substrates (Fig. 6C and data not shown). The binding of J12 appears to be ~10,000-fold weaker than with wild-type RecG (Fig. 6B). Unwinding activity is barely detectable at protein concentrations normally used for wild-type RecG (1–10 nM), even though significant unwinding activity is seen with the phenylalanine mutants under these conditions (Fig. 4). Unwinding activity is detectable at high protein concentrations (100–500 nM) and is broadly identical for both the J12 and the RF substrates (Fig. 6, C and D). Furthermore, the products of unwinding are the same as those generated by full-length RecG (Fig. 6C and data not shown). These results demonstrate that the Δwedge protein retains some translocation activity and so is likely to be folded correctly. Surprisingly, the poor unwinding activity can be attributed entirely to the reduced DNA binding affinity, as there is an ~5000-fold reduction in observed unwinding activity (500 nM Δwedge has 20% of 0.5 nm wild-type RecG activity), which correlates well with the 10,000-fold reduction in binding. Thus, while bound to the junction substrate, Δwedge can successfully translocate. Interestingly, this also suggests that the loss of the ssDNA leading and lagging parental strand channels is relatively unimportant to the unwinding activity.

The Isolated Wedge Domain Binds a Holliday Junction—The results presented in the previous section indicate that the wedge domain is almost entirely responsible for the binding of branched DNA molecules by RecG. To test whether this is indeed the case, we expressed and purified the wedge domain separately. This construct covered residues 43–151 of wild-type RecG, with Leu42 converted to methionine (Fig. 7A). An N-terminal His6 tag was added to aid purification. It was hoped that this construct would
allow the β-sheets from residues 43–48 and 147–151 to form correctly, forming the ssDNA binding channel for the lagging strand template (Fig. 7A). Band shift assays showed that the wedge domain binds very strongly to J12, although the affinity is clearly reduced relative to full-length RecG (Fig. 7, B and C). This not only confirmed that the purified domain had folded correctly but also confirmed our assumption that the wedge domain was responsible for much of the junction binding by RecG. Interestingly, no binding of the RF substrate was observed (data not shown), implying that there are other contacts necessary for DNA binding between RecG and the fork that are not located in the wedge domain. The loss of these contacts is probably responsible for the difference in affinities of full-length RecG and the isolated wedge domain for the Holliday junction. The binding of the Holliday junction but not the replication fork also demonstrates that the binding is not an artifact of any nonspecific DNA interactions arising from the addition of a histidine tag to the wedge protein.

A Conserved GVG Motif Stabilizes the Lagging Strand Duplex—Multiple sequence alignments identified a conserved motif, Gly16-Val17-Gly18 (GVG), which may be involved in binding the lagging strand duplex (Fig. 8A). This region appears to form a binding pocket in the crystal structure, with the hydrophobic valine buried in the protein core (Fig. 8B). We mutated Val17 to a glutamate, which we predicted would flip out into the solvent, destroying the pocket in a manner similar to that seen recently with a DNA polymerase (38). We also mutated Gly18 to an aspartate, which would also destroy the pocket. The negative charges introduced by these mutations were expected to also interfere with any association between the GVG pocket and the negatively charged DNA phosphate backbone. Both proteins show a similar, moderate decrease in binding affinity for J12 (Fig. 8C), consistent with most of the stabilizing interactions occurring at the wedge. However, both have a vastly reduced binding affinity for the RF substrate, suggesting that the mutations perturb a binding interaction that is much more important in this case. Surprisingly, there is little difference in the unwinding of the J12 and the RF substrates (Fig. 8D); both proteins have ~50% of wild-type activity on both. However, it should be noted that once the RF substrate begins to unwind, the unwound lagging strand might make additional contacts with the wedge domain such that the binding affinity now approaches that seen with J12.

**DISCUSSION**

RecG differs from most other helicases in that it is thought to unwind branched DNA molecules via translocation of a monomer on one of the duplex arms, with the wedge domain fixed at the branch point to facilitate strand separation (27). One consequence of its monomeric nature is that it cannot achieve the processivity required for unwinding by exploiting the methods employed by dimeric and hexameric enzymes. We dissected RecG and established that the wedge is a major DNA binding feature. A second binding site that interacts with the lagging strand of a fork structure was identified outside the wedge. The data presented demonstrate not only how RecG identifies branched DNA structures but also how the protein-DNA interactions established are necessary to the unwinding activity. We...
propose that the processivity of RecG on branched DNA substrates depends on the ability of the wedge to establish strong binding at the branch point.

Only one of the junction binding residues proposed from the T. maritima RecG structure is conserved in E. coli RecG, the phenylalanine residue at position 97 (E. coli numbering). We confirmed that this residue is important for RecG activity, as mutation of this position affects junction and fork binding in vitro and DNA repair in vivo. We have also shown that the adjacent phenylalanine residue, Phe96, appears to be important and that substituting both residues almost completely inactivates the protein. The surprising aspect of these mutations is the difference seen with the fork and junction substrates. Substitutions at either position 96 or 97 affect the activity with the replication fork substrate equally. However, the mutation at Phe97 is far less deleterious than at Phe96 when the Holliday junction substrate is used. These data suggest that Phe96 is not functionally equivalent to Phe97, as we originally hypothesized. Phe97 appears to be relatively unimportant in the binding of a Holliday junction, probably because of the number of other interactions available between the wedge domain and the four strands of the junction that encircle it. However, these additional interactions are not present in the RecG:fork complex, and so the loss of Phe97 has a more serious effect on complex stability. The equivalent residue to Phe96 in the T. maritima structure, Trp203, is partially buried within the hydrophobic core of the wedge domain, and so a mutation at this position is likely to effect the conformation of the wedge domain as a whole. Such a conformational change is likely to be more detrimental to the binding of J12, as it would affect a number of interactions rather than just that between Phe97 and the DNA. This would explain why the affinity of the double mutant for the J12 substrate is much weaker than for the RF substrate. The correlation between binding affinities and unwinding activities suggests that the translocation mechanism is not affected by mutations at the wedge domain; the reduction in activity is due to the decreased concentration of the RecG-DNA complex.

The extremely low affinity of the ∆wedge protein for branched DNA confirms that the helicase domains do not contain a strong DNA binding site, as would be required if the classical inchworm method of translocation was used. This finding demands an alternative model of translocation. Given this poor binding, the ∆wedge protein displays surprisingly robust unwinding activity with J12 and RF, although concentrations far in excess of those required for the wild-type protein are required to see substantial unwinding of the substrate. It is likely that the unwinding is simply caused by trying to pull a branched molecule through a DNA translocation channel that is only large enough to take a single DNA duplex, in a similar manner to that reported for the hexameric DnaB helicase (4, 5). This suggests that the translocation activity is relatively unaffected by the loss of the wedge domain in a manner similar to substitutions at Phe96 and Phe97. While bound to the DNA the protein can translocate and does so efficiently. This suggests a pivotal role of the wedge domain interactions in achieving the high processivity required for RecG activity. Thus, in addition to providing specificity for branched DNA molecules, the role of the wedge domain is to stop dissociation of the RecG-DNA complex.

The role of the wedge in Holliday junction binding is emphasized by our studies of the isolated domain. This small domain shows striking structural similarity to SSB proteins, suggesting the possibility RecG has evolved from the fusion of an SSB-like protein and a helicase motor. It binds a Holliday junction substrate (J12) with high affinity. Indeed, its affinity is almost identical to that of the GVG motif mutants of the full-length protein. This would imply that with loss of binding at the GVG motif, the only remaining interactions between native RecG and J12 are at the wedge domain. The high rate of unwinding of J12 by these GVG mutants reinforces our suggestion that the wedge in important for processivity. We have identified the key interactions at the branch point of a fork, but the poor binding of the RF substrates points to other stabilizing interactions when the four strands of an open Holliday junction structure encircle the wedge. The relatively high unwinding activity of the RF substrate indicates that there are significant stabilizing interactions between residues in the wedge and the lagging strand displaced by unwinding. Once RF unwinding has started, it becomes a three-armed molecule. The displaced strand may wrap around the wedge domain in a manner analogous to the binding of J12. The role of such interactions at a
damaged replication fork may be to bring displaced leading and lagging strands into close proximity, thus promoting the annealing of these strands and thus the formation of a Holliday junction from a replication fork (23, 26).

This study of RecG suggests that the wedge can be regarded both as a strand separation module and a processivity factor. Furthermore, it reveals how it might be possible to couple a dsDNA translocase motor such as the one in RecG with any protein domain that can secure the motor to the DNA, either directly or via protein-protein interaction. This DNA binding domain could be a site-specific and immovable, providing a direct or via protein-protein interaction. This DNA binding domain that can secure the motor to the DNA, either 

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DNA Binding Activity of RecG Helicase

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