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The DNA Replication Protein PriA and the Recombination Protein RecG Bind D-loops

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The PriA protein of *Escherichia coli* provides a vital link between recombination and DNA replication. To establish the molecular basis for this link, we investigated the ability of PriA to target DNA substrates modelled on D-loops, the intermediates formed during the early stages of RecA-mediated recombination. We show that PriA binds D-loops and unwinds the DNA in reactions that rely on its ability to function as a helicase. The minimal structure that binds PriA is a duplex DNA molecule with unpaired single strands at one end, an arrangement likely to occur at a D-loop. It resembles features of the stem-loop formed by primosome assembly site (PAS) sequences in the DNA of bacteriophage ϕX174 and plasmid ColE1, and which enable PriA to assemble active primosomes for the initiation of lagging strand synthesis. We suggest that PAS sequences may have evolved to mimic the natural D-loop target for PriA formed in the chromosome of *E. coli* during recombination and DNA repair. Genetic studies have revealed an interaction between PriA and RecG, a DNA helicase that drives branch migration of recombination intermediates. We therefore compared PriA and RecG for their ability to bind and unwind DNA. RecG, like PriA, binds D-loops and unwinds the DNA. However, it prefers branched structures with at least two duplex components. The possibility that it competes with PriA for binding recombination intermediates is discussed.

Keywords: helicase; D-loops; recombination; DNA repair

Introduction

Recent studies in *Escherichia coli* have implicated extensive DNA replication in the formation of recombinants in genetic crosses and highlighted close parallels between recombinant formation, the repair of DNA double-strand breaks, and the restoration of collapsed replication forks (Asai *et al.*, 1994; Kogoma, 1996; Kuzminov, 1995). These processes have in common the ability of a DNA end to provoke recombination through the sequential action of RecBCD enzyme and RecA protein (Kowalczykowski *et al.*, 1994). RecBCD unwinds and degrades DNA from a duplex end to expose a single-strand tail that can be recruited by RecA to initiate pairing and strand exchange with a homologous duplex, thus creating a D-loop (Figure 1). An invading strand ending 3′ could initiate DNA synthesis, extend the D-loop, and allow lagging-strand synthesis to be primed on the displaced strand, and when coupled with branch migration of the three-strand junction into regions of duplex:duplex DNA pairing and resolution of the resulting Holliday junction, could link the invading duplex to the recipient duplex *via* a replication fork. A single event of this type has been proposed as a mechanism for restoring collapsed replication forks (Kuzminov, 1995). Co-ordination of two such events provides plausible models for the repair of DNA double-strand breaks (Asai *et al.*, 1994) and for integrating a linear DNA fragment into the chromosome during conjugation or transduction (Smith, 1991). These models are supported by the recent discovery that the replication protein PriA is required for efficient recombination, for repair of double-strand breaks, and to maintain high cell viability. It is also required to initiate a novel form of DNA replication induced by chromosome breaks that relies on the activities of RecA protein and RecBCD enzyme (Asai *et al.*, 1993, 1994; Kogoma *et al.*, 1996; Nurse *et al.*, 1991; Sandler, 1996).

Abbreviation used: PAS, primosome assembly site.

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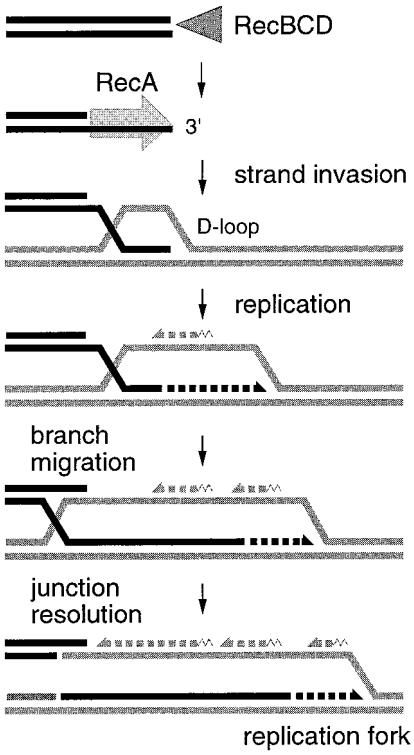

Figure 1. Diagram showing a model for initiation of recombination from a duplex DNA end by the sequential action of RecBCD enzyme (shaded triangle) and RecA protein (shaded arrow), and the priming of leading and lagging-strand DNA synthesis. The polarity of RecA polymerisation and strand exchange is indicated by the arrowhead.

PriA was first identified as an essential component of the primosome responsible for priming complementary strand ϕX174 DNA synthesis (Wickner & Hurwitz, 1975). It has two distinct biochemical activities (a) it catalyses assembly of the primosome needed to initiate lagging-strand DNA synthesis from a specific primosome assembly site (PAS) in ϕX174 DNA (Shlomai & Kornberg, 1980b); and (b) it unwinds partial duplex DNA, with a 3′ to 5′ polarity (Lee & Marians, 1987). However, PriA is apparently not required for normal chromosomal DNA replication from *oriC* since *priA* null mutants are viable, though the cells are quite sick and chronically induced for the SOS response (Nurse *et al*., 1991). Also, no role has been identified yet for the helicase function, since mutant proteins devoid of this activity can still initiate primosome assembly and promote cell viability (Sandler, 1996; Zavitz & Marians, 1992). It has been suggested that PriA is a repair protein promoting initiation of DNA replication whenever replication initiated from *oriC* fails to replicate the entire chromosome (Nurse *et al*., 1991).

The discovery that PriA is essential for replication primed by recombination has led to models whereby PriA targets an intermediate in recombination to initiate lagging-strand synthesis (Al-Deib *et al*., 1996; Kogoma *et al*., 1996; Sandler *et al*., 1996). Binding of PriA to DNA is reported to require PAS sequences (Shlomai & Kornberg, 1980a; Zipursky & Marians, 1980). These sequences have little homology but all possess the ability to form stable stem-loop structures, to which PriA binds (Greenbaum & Marians, 1984; Ng & Marians, 1996a). However, searches of the available *Escherichia coli* chromosomal sequence did not reveal any putative PAS sequences (Masai *et al*., 1994), and so direct binding of recombination intermediates by PriA was questioned (Liu *et al*., 1996; Ng & Marians, 1996b). An alternative suggestion, based on *in vitro* data demonstrating that preprimosomes remain bound to replicated ϕX174 DNA, was that these complexes translocate through duplex DNA on the *E. coli* chromosome allowing delivery of primosomes to recombination intermediates without a need for PAS sequences throughout the chromosome (Ng & Marians, 1996b). However, although replication of ColE1-based plasmid replicons shows an absolute requirement for PriA, deletion of the PAS sequences in the plasmid does not block replication; it merely lowers the copy number (Nurse *et al*., 1991; van der Ende *et al*., 1983). This suggests PriA can recognise sequences other than PAS and so the possible initiation of PriA-dependent DNA replication from D-loops cannot be discounted (Kogoma *et al*., 1996).

A direct interaction between PriA and recombination intermediates is supported by the discovery that certain mutations in *priA* suppress the DNA repair and recombination defects associated with *recG* mutations (Al-Deib *et al*., 1996). These mutations appear to affect the helicase activity of PriA and do not confer the reduced viability associated with *priA* null mutations. This is significant as RecG is also a 3′ to 5′ DNA helicase that targets strand exchange intermediates in recombination and catalyses their branch migration (Lloyd & Sharples, 1993a; Whitby *et al*., 1994). Although necessary for efficient recombination and repair RecG seems to counter strand exchange mediated by RecA *in vitro* (Whitby & Lloyd, 1995; Whitby *et al*., 1993), which has led to a model in which RecG targets the three-strand junction at a D-loop formed by 3′ strand invasion, and drives the junction into duplex:duplex regions to form a Holliday junction, thus overcoming the 5′–3′ polarities of RecA polymerisation and strand exchange (Figure 1) (Whitby & Lloyd, 1995). The junction could then be resolved, possibly by the RuvABC proteins (West, 1996). The recent discovery that RecG is also targeted to R-loops supports this model (Fukuoh *et al*., 1997; Vincent *et al*., 1996). Additionally, multicopy expression of PriA exacerbates the deficiencies in recombination and DNA repair in *recG* strains (Al-Deib *et al*., 1996). This effect was not seen with a helicase-deficient PriA and so it was suggested that the helicase functions of PriA and RecG, nominally both 3′ to 5′, are antagonistic and that the balance between the two is

critical during the early stages of recombination (Al-Deib *et al*., 1996).

We have tested the hypothesis that PriA targets recombination intermediates by examining its ability to bind and unwind D-loop structures. The ability of RecG to interact with these structures was also investigated to ascertain whether PriA and RecG compete for the same substrates during the early stages of recombination.

Results

PriA binds and unwinds D-loops

To investigate the ability of PriA to target D-loops, we designed the two DNA substrates shown in Figure 2a. These were made by annealing in each case three partially complementary oligonucleotides (Table 1, substrates C and D) that base-pair as shown. They differ with respect to the polarity of the invading single strand. Band-shift assays performed in the presence of EDTA revealed that PriA binds both substrates to form well-defined protein-DNA complexes (Figure 2b). Two distinct bandshifts can be seen with the 3′ D-loop (lanes b to h), which suggests PriA can bind as a dimer or higher multimer, or that the substrate provides more than one binding site. A similar result is seen with the 5′ D-loop (lanes j to p), although the complex detected at the lower concentrations of PriA migrates more rapidly. Also, the complexes detected at higher concentrations of protein are less well defined. No bandshifts were detected with a control linear duplex or single-stranded DNA, although some smearing of the substrate consistent with non-specific binding was detectable, especially at 100 nM protein (Figure 4 (below), substrates I and K and data not shown). We conclude that the well-defined bandshifts detected with D-loops are due to structure-specific binding of the DNA.

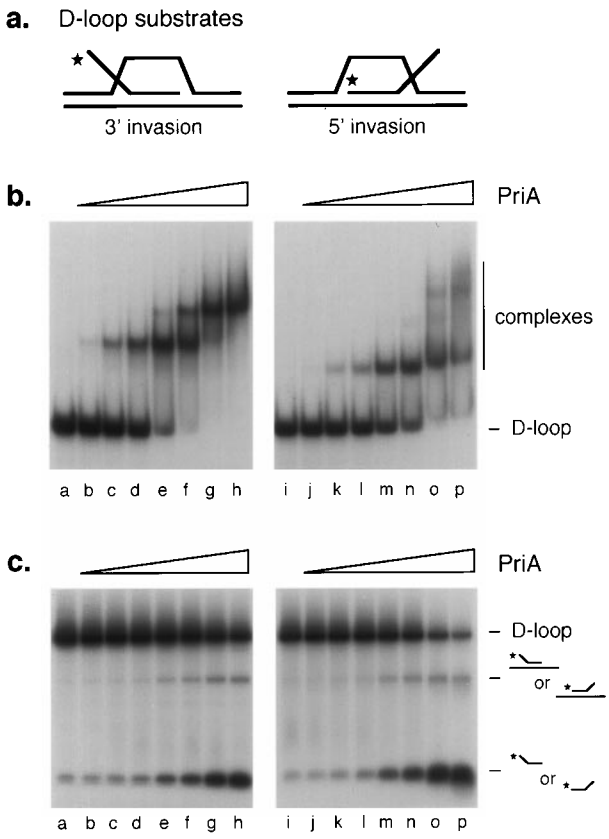

Figure 2. Binding and unwinding of D-loops by PriA. (a) Schematic structure of D-loop substrates C and D with an invading strand ending either 3′ (left) or 5′ (right), respectively, and ^{32}P-labelled at the 5′ end as indicated by the asterisk. (b) Band-shift assays showing binding of PriA to D-loops. Reactions were conducted as described in Materials and Methods and contained PriA at 0, 0.1, 0.5, 1, 5, 10, 50, or 100 nM (lanes a to h and i to p) with D-loops at 0.8 nM. (c) Helicase assays showing unwinding of D-loops by PriA. Reactions were conducted as described in Materials and Methods with PriA concentrations as in (b). Gels in (b) and (c) are aligned below the substrates depicted in (a).

PriA has been reported to function as a 3′–5′ DNA helicase on partial duplex substrates (Lee & Marians, 1987). To see if this activity could unwind D-loops, the same two substrates were incubated with PriA in the presence of ATP and Mg^{2+}. After deproteinisation, the products were analysed by electrophoresis on non-denaturing polyacrylamide gels (Figure 2c). PriA clearly unwinds both substrates to produce in each case two labelled products (lanes b to h and j to p). Reference to appropriate control constructs (Figure 6 (below), and data not shown) identified the faster-migrating major product as the invading oligonucleotide and the slower-migrating minor species as the partial duplex remaining after unwinding both ends of the D-loop (upper strand in Figure 2a).

To see if the dissociation of D-loops was due to a specific unwinding activity, the substrates were incubated with a mutant PriA protein, in which the lysine 230 residue was altered to an arginine. The K230R mutation is situated in the consensus nucleotide binding motif and abolishes the helicase activity of PriA without disrupting its ability to assemble active primosomes (Zavitz & Marians, 1992). PriA K230R is able to bind a D-loop as well as the wild-type protein (Figure 3a, compare lane c with lane b). However, as predicted from the absence of helicase activity on partial duplex substrates (Zavitz & Marians, 1992), it failed to unwind the DNA (Figure 3b, compare lane c with lanes b and a). We conclude that PriA is able to form a specific protein-DNA complex with D-loops that enables it to unwind the structure by means of its 3′–5′ helicase activity.

PriA targets duplex DNA with single strand tails at one end

To identify the precise structure recognised by PriA, a series of substrates were designed to mimic features present within D-loops (Table 1). The ability of PriA to bind these substrates was assessed in

Table 1. Dissociation of DNA substrates by PriA and RecG

Substrate	Oligonucleotide composition	DNA structure[a]	Substrate dissociation (% of total)[b]	
			PriA	RecG
A	1 + 2 + 3 + 4		2 ± 2	50 ± 17
B	1 + 2 + 3		74 ± 5	66 ± 4
C	7 + 10 + 12		41 ± 12	33 ± 7
D	7 + 10 + 13		83 ± 3	78 ± 0
E	7 + 10 + 11		18 ± 5	44 ± 6
F	7 + 10		1 ± 1	0 ± 0
G	1 + 4 or 1 + 2 or 2 + 3		26 ± 17	1 ± 1
H	7 + 12		42 ± 7	1 ± 1
I	1 + 5		0 ± 1	0 ± 1
J	7 + 11		15 ± 8	1 ± 1
K	1		NA	NA
L	1 + 4 + 6		0 ± 0	12 ± 2
M	1 + 4 + 8		60 ± 3	59 ± 8
N	1 + 4 + 9		3 ± 0	16 ± 2
O	4 + 8		15 ± 1	0 ± 0
P	1 + 9		0 ± 1	0 ± 0

[a] The asterisk denotes the position of the 5′ end-label.

[b] Some of the substrates were unwound in different orientations to yield mixtures containing more than one labelled product. In these cases, no significant differences were observed between the PriA and RecG reactions in the ratios of these products, except with substrate M; PriA produced approximately equal ratios of the labelled partial duplex (equivalent to substrate O) and free oligonucleotide products, whereas RecG produced almost exclusively the free oligonucleotide. The values given are means plus standard errors of between two and ten assays. Values for substrate G are based on three preparations of the DNA as shown. NA, not applicable.

bandshift assays using a range of PriA concentrations from 0.1 nM to 100 nM. A D-loop (substrate C) was included for comparison. Figure 4a shows the results obtained with 10 nM PriA. Significant bandshifts were obtained with substrates B, E, G and H. All four are branched DNAs with a single-stranded component. Partial duplex DNA (J) was retarded to some extent but failed to form a complex with a well defined mobility shift under the conditions used. No specific bandshifts were observed with duplex (I) or single-stranded (K) DNA, which indicates that stable binding of B, E, G, and H is structure specific.

The minimal requirement for stable binding of PriA appears to be provided by substrate G, a duplex with unpaired single-strand tails at one end (flayed duplex). The poorly defined retardation of substrate J indicates that a transition point from single-stranded DNA to duplex DNA is not sufficient to form a stable complex under the conditions used. Substrate F does not give a strong bandshift with PriA despite being designed to have a branched structure with a single-stranded component. A retarded complex is visible, but most of the DNA is unbound (Figure 4a). This result could be explained by F failing to adopt a branched structure in solution due to the presence of two duplex regions and the lack of a third strand to physically impose a looped-out structure.

PriA binds poorly to a Holliday junction

The only other branched structure that was not shifted to a substantial extent by PriA was a four-way duplex junction designed to mimic the structure of a Holliday intermediate (Figure 4a,

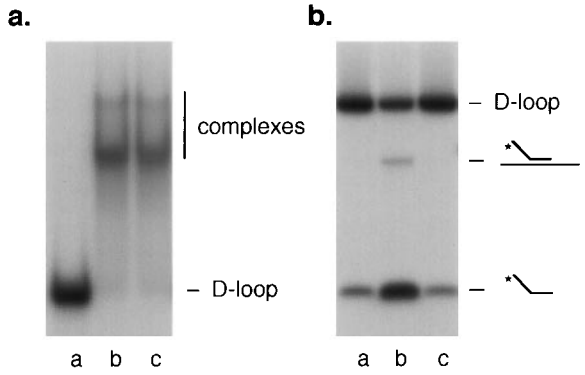

Figure 3. Effect of a K230R substitution in PriA on binding and unwinding of a D-loop. Band-shift (a) and helicase reactions (b) contained D-loop substrate C at 0.8 nM and were incubated without protein (lanes a), or with PriA at 10 nM for the bandshift assays and at 100 nM for helicase assays ($PriA^+$, lanes b; PriA K230R, lanes c).

substrate A). This lack of binding was seen at PriA concentrations of up to 500 nM (data not shown). The junction in substrate A is located in a homologous core of 12 bp within which it is free to branch migrate. Similar results were obtained with junctions containing homologous cores of 11 bp or 2 bp, or a static X-junction (data not shown). The absence of a single-stranded component in these Holliday junctions supports the minimal binding requirement suggested above.

RecG targets structures recognised by PriA

Genetic observations suggest there is an antagonistic interplay between PriA and the branch migration enzyme RecG during recombination and DNA repair, and it has been proposed that this is the result of both proteins being able to target strand exchange intermediates (Al-Deib *et al.*, 1996). To ascertain whether RecG can bind structures similar to those bound by PriA, bandshift assays were performed on the same substrates (Figure 4b). RecG clearly binds the D-loop substrates C and D, and also structures B, E and G, although with apparently lower affinity than PriA, but fails to bind substrate H. However, substrate A, the Holliday junction, was shifted completely by RecG at the concentration shown, which is in marked contrast to the result obtained with PriA. Therefore, although RecG clearly binds other branched DNA structures, its highest affinity is for a four-way duplex junction.

The requirements for DNA binding by PriA and RecG were further compared using substrates L to P, of which M and N were designed to contain structural elements expected to be found at the boundaries of D-loops. Figure 5a shows that PriA

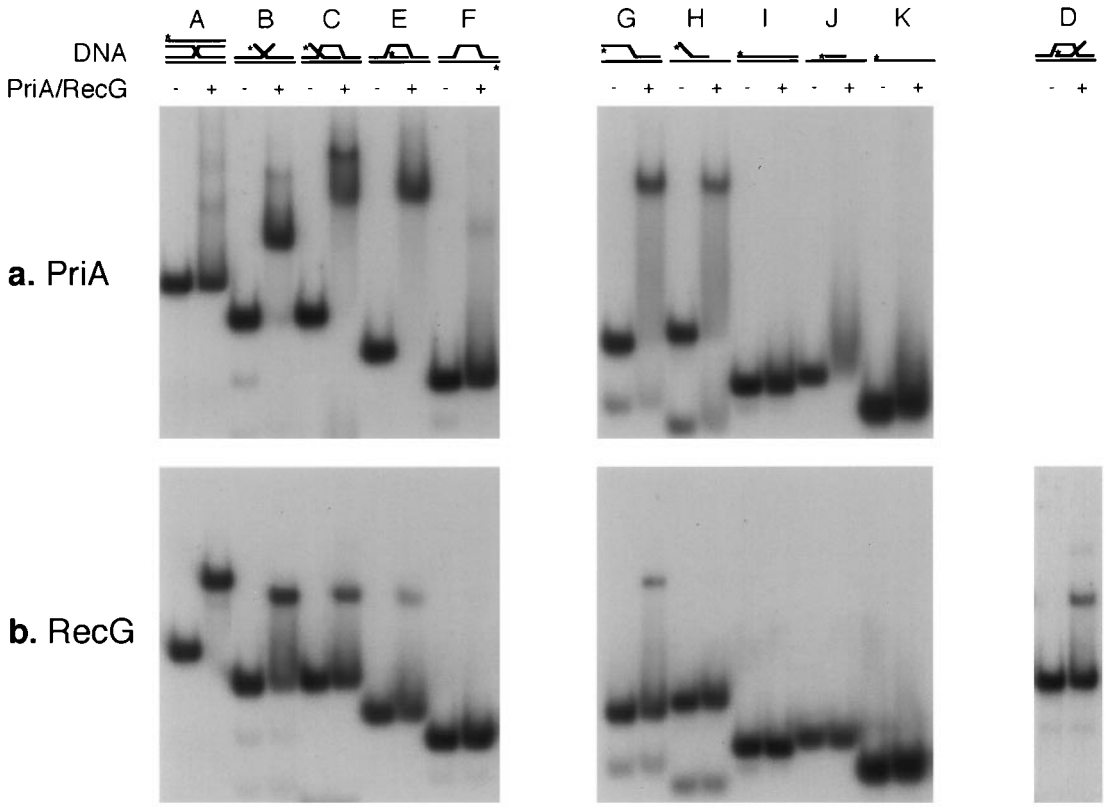

Figure 4. Bandshift assays showing formation of protein-DNA complexes with DNA substrates A to K. (a) PriA protein; (b) RecG protein. Parallel reactions were conducted with and without proteins as indicated (10 nM final concentration).

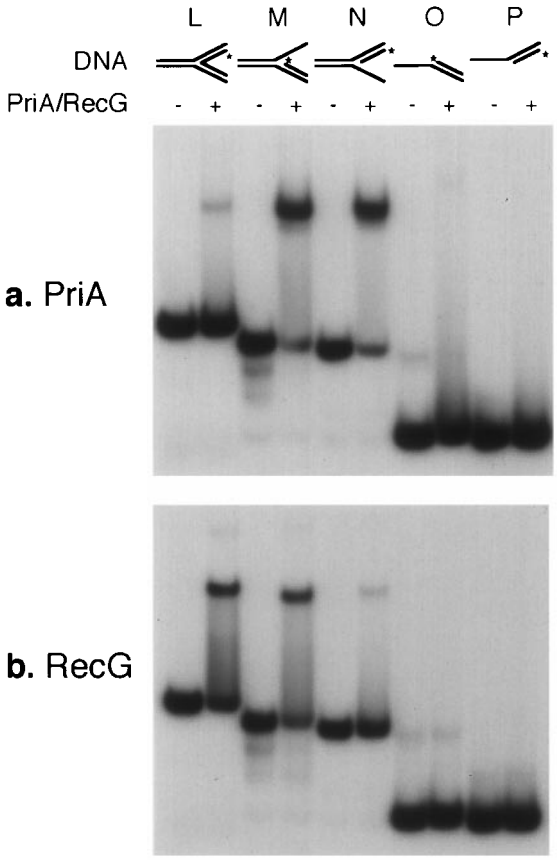

Figure 5. Bandshift assays showing formation of protein-DNA complexes with DNA substrates L to P. (a) PriA protein; (b) RecG protein. Parallel reactions were conducted with and without proteins as indicated (10 nM final concentration).

binds M and N with high affinity. Substrate L, which contains a three-way duplex junction not expected to be present at D-loops is also bound, but only very weakly. No stable bandshifts were detected with substrates O and P. These data support the conclusion that stable binding by PriA requires a branched DNA structure with a single-stranded component. The binding of both M and N also demonstrates that the polarity of the single-stranded component does not affect the binding. RecG displays a significantly different pattern of bandshifts (Figure 5b). It binds quite strongly to substrate M, but less so to N, which may indicate the importance in this case of the polarity of the single-stranded component. However, DNA sequence-dependent effects cannot be ruled out. No bandshifts were observed with RecG and substrates O or P. RecG also binds substrate L much more strongly than PriA. These findings reinforce the fact that a branched DNA structure with at least two duplex components is required for efficient binding of RecG.

In the absence of RecG, PriA interferes with recombination and DNA repair; RecG normally counters this effect (Al-Deib *et al.*, 1996). This observation could mean the two proteins interact by binding simultaneously to the same DNA structure, possibly making protein-protein contacts. To investigate this possibility, bandshift assays were performed with mixtures of PriA and RecG on substrates A to C, G and H. No novel bandshifts indicative of PriA-RecG-DNA complexes were observed (data not shown). The only complexes detected corresponded to those obtained with PriA or RecG alone. However, we cannot rule out the possibility that the substrates used are too small to accommodate both proteins.

Structure-specificity of PriA and RecG helicase activities

The inhibitory effect of PriA in the absence of RecG is abolished by mutations that eliminate its helicase activity (Al-Deib *et al.*, 1996). Given that both proteins target structures present at a D-loop, the balance between the two could be crucial *in vivo*, especially if they were also required to unwind or branch migrate these structures. The ability of PriA and RecG to unwind structures present within D-loops was therefore compared on substrates A to P over a range of protein concentrations from 0.1 to 100 nM. Figure 6 shows the results obtained with substrates A to C and E to J at 100 nM protein. Table 1 summarises the maximal yields of dissociation products for all the substrates. Both PriA and RecG unwind the DNA structures to which they bind, which supports the bandshift analyses shown in Figures 4 and 5, although substrate N is unwound rather poorly by PriA (see below). Of particular note is that PriA does not unwind substrate A, the four-way duplex (Holliday) junction. PriA also failed to unwind X-junctions with 11 bp or 2 bp homologous cores, or a static X-junction. These three junctions are unwound very efficiently by RecG (data not shown). This supports the conclusion that PriA does not target Holliday junctions.

The percentage dissociation of substrates B to E is approximately equal for both PriA and RecG, despite the apparently lower affinity of RecG for these substrates. This may indicate unstable binding by RecG during the bandshift assays. PriA also unwinds partial duplexes J and O to some extent, and must therefore bind these substrates. However, PriA did not give well-defined bandshifts (Figures 4a and 5a), which again suggests the presumed binding of substrates J and O is not sufficiently stable to be detected in gel assays. The unwinding of partial duplex DNA by PriA has been described (Lee & Marians, 1987).

The three-way duplex substrate, L, was unwound to some extent by RecG, as described (Lloyd & Sharples, 1993b; Whitby *et al.*, 1994), but not at all by PriA (Table 1). This supports the conclusion that PriA requires a single-stranded component within a branched DNA structure for efficient binding, and therefore helicase activity.

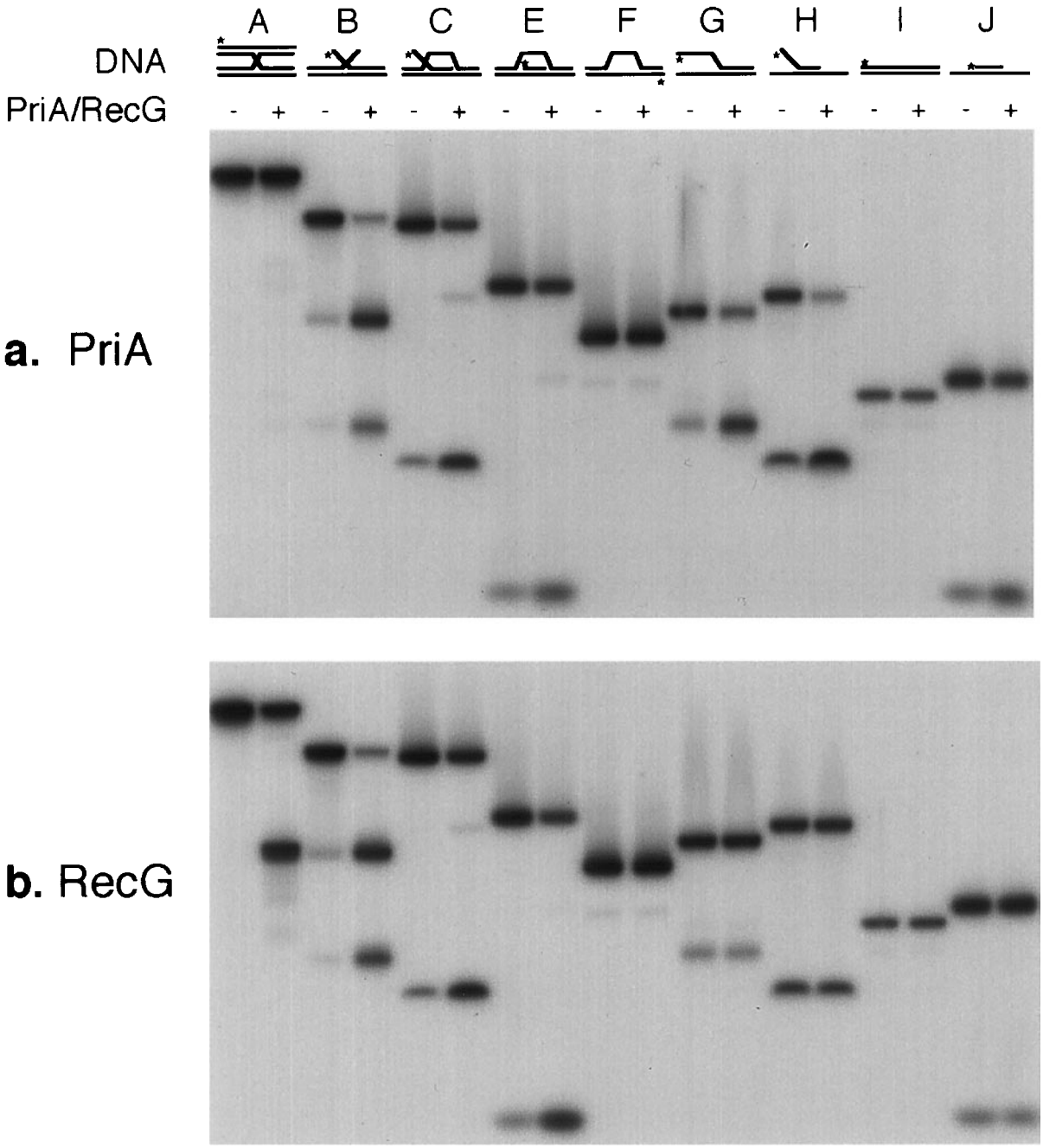

Figure 6. Dissociation assays showing DNA helicase activity of PriA and RecG on substrates A to C and E to J. (a) PriA; (b) RecG. Parallel reactions were incubated with and without proteins as indicated (100 nM final concentration).

Substrate M was efficiently unwound by both PriA and RecG. However, substrate N, which has a similar forked DNA structure but with different strand polarity, is unwound much less efficiently, especially by PriA. Therefore, the polarity of these DNA structures may be important in their catalytic unwinding by PriA and RecG and such polarity effects appear to be similar for both PriA and RecG. However, DNA sequence-dependent effects cannot be excluded. The data for substrate N also demonstrate that DNA structures can be bound without necessarily being good helicase substrates (Figure 5 and Table 1).

Substrate O is unwound by PriA whereas substrate P is not (Table 1), as predicted by the 3′ to 5′ polarity of unwinding (Lee & Marians, 1987). RecG cannot displace the labelled strand from either of these structures, which probably reflects the low processivity of RecG helicase on partial duplex substrates (Whitby *et al.*, 1994). The failure to unwind substrate O in the case of RecG, and the rather low level of unwinding in the case of PriA, are in marked contrast to the highly efficient unwinding of substrate M by both proteins (Table 1). These results demonstrate that the preferred substrates for both helicases are branched DNA structures.

The PriA K230R mutant protein was also tested for its ability to bind and unwind partial D-loop structures. As found with D-loop substrate C (Figure 3a), bandshift assays with substrates A, B, and D to K gave results identical to those obtained with the wild-type protein (data not shown), which indicates that the K230R substitution does not affect the ability of PriA to bind DNA. However, no unwinding activity was detected (data not shown).

Discussion

The data presented here show that the DNA replication protein PriA binds and unwinds D-loops. They provide the first direct evidence of how DNA replication can be linked to recombination and support models for the early stages of recombination in which PriA activity leads to the priming of lagging-strand DNA synthesis on the displaced strand of a D-loop, thereby converting the recombination intermediate to a replication fork (Figure 1; Al-Deib *et al.*, 1996; Asai *et al.*, 1994; Kogoma, 1996; Kuzminov, 1995; Sandler *et al.*, 1996; Smith, 1991). Most models of recombination initiated from DNA ends assume the invading strand of the D-loop ends 3′ so as to allow priming of leading strand synthesis. However, recombination could also initiate with an invading strand ending 5′ (Rosenberg & Hastings, 1991). Our discovery that PriA targets D-loops irrespective of the polarity of the invading strand is consistent with this view.

Previous models of the type shown in Figure 1 assumed that leading-strand synthesis primed by the invading 3′ strand extended the D-loop and exposed PAS-like sequences in the displaced strand to which PriA could bind (Asai *et al.*, 1994). However, the dearth of such sequences in the *E. coli* chromosome presented a difficulty (Masai *et al.*, 1994; Ng & Marians, 1996b). Our results eliminate this problem since they show that PriA could target D-loops directly. They also imply that PAS sequences and D-loops share a common feature recognised by PriA. PAS sequences all have the potential to form stem-loop structures and it is this feature that is thought to be essential for PriA binding (Greenbaum & Marians, 1984; Soeller *et al.*, 1984). However, a stem-loop formed in single-stranded DNA can be viewed as a branched structure with a single-stranded component. This is very similar to the flayed duplex structures used in this study (substrates G and H, Table 1), to which PriA binds. It is tempting to conclude therefore that this is the feature of D-loops targeted by PriA. No significant binding or unwinding of four-way duplex junctions was detected. This is a critical observation as it implies PriA activity is restricted to the early structures present at D-loops and cannot be targeted to the Holliday junction formed by branch migration of the initial three-strand junction into regions of duplex:duplex DNA pairing (Figure 1).

Specific PAS sequences may be required for PriA-initiated assembly of primosomes only when such branched DNA structures would otherwise be absent, for example during replication of ϕX174 single-stranded DNA. PAS sequences are also found in ColE1-based plasmid replicons but these sites are not essential for PriA-catalysed initiation of DNA replication, although their deletion lowers plasmid copy number (Nurse *et al.*, 1991; van der Ende *et al.*, 1983). Initiation of ColE1 replication involves the formation of an R-loop, a structure closely resembling a D-loop, which may allow alternative PriA binding. However, a PAS sequence may provide the means to maximise the efficiency of plasmid replication, a situation in which binding of PriA is required at the same location repeatedly. In contrast, recombination intermediates may arise anywhere within the chromosome and so direct recognition of D-loops by PriA would allow primosome assembly to occur regardless of the chromosomal location. Indeed, PAS sequences may have evolved in ϕX174 and ColE1 to provide structures that mimic the natural DNA binding site for *E. coli* PriA protein.

Our analysis of PriA shows that it not only binds D-loops but also unwinds the structure. This observation may explain why PriA inhibits recombination and DNA repair in strains lacking RecG (Al-Deib *et al.*, 1996). PriA helicase activity is clearly responsible for this effect since no inhibition is observed with a mutant protein (K230R) deficient in DNA unwinding (Al-Deib *et al.*, 1996). We have shown that PriA K230R binds D-loop structures in a manner identical with the wild-type protein, but cannot unwind the DNA, which means the inhibitory effect of the wild-type protein cannot be due to DNA binding alone. Since wild-type PriA binds poorly to a synthetic X-junction, and cannot unwind the structure, we can also eliminate the possibility that it interferes with the processing of Holliday junctions. Given its affinity for D-loops, it seems more likely, therefore, that it prevents junctions from being set up in the first place, as suggested by Al-Deib *et al.* (1996). Presumably, RecG, which we have shown to bind D-loop structures targeted by PriA, normally counters this effect by driving branch migration of the three-strand junction formed at the D-loop into regions of duplex:duplex DNA pairing (Whitby & Lloyd, 1995), or by blocking some activity of PriA that prevents branch migration by RecA or RuvAB.

To conclude, we have shown that D-loops are targeted by both PriA and RecG proteins to link together the processes of DNA replication and recombination. The precise catalytic activities of these proteins at such structures remain to be determined.

Materials and Methods

Proteins

RecG was purified as detailed by Vincent *et al.* (1996). Wild-type and mutant PriA were purified as described (Zavitz & Marians, 1992).

DNA substrates

DNA substrates were made by annealing combinations of the following oligonucleotides: 1, 5′-GACGCTGCCGAATTCTGGCTTGCTAGGACATCTTTGCCCACGTTGACCC-3′; 2, 5′-TGGGTCAACGTGGGCAAAGATGTCCTAGCAATGTAATCGTCTATGACGTT-3′; 3, 5′-CAACGTCATAGACGATTACATTGCTAGGACATGCTGTCTAGAGACTATCGA-3′; 4, 5′-ATCGATAGTCTCTAGACAGCATGTCCTAGCAAGCCAGAATTCGGCAGCGT-3′; 5, 5′-GGGTCAACGTGGGCAAAGATGTCCTAGCAAGCCAGAATTCGGCAGCGTC-3′; 6, 5′-TGGGTCAACGTGGGCAAAGATGTCCGGACATGCTGTCTAGAGACTATCGA-3′; 7, 5′-GACGCTGCCGAATTCTACCAGTGCCTTGCTAGGACATCTTTGCCCACCTGCAGGTTCACCC-3′; 8, 5′-GGACATGCTGTCTAGAGACTATCGA-3′; 9, 5′-TGGGTCAACGTGGGCAAAGATGTCC-3′; 10, 5′-GGGTGAACCTGCAGGTGGGCGGCTGCTCATCGTAGGTTAGTTGGTAGAATTCGGCAGCGTC-3′; 11, 5′-AAAGATGTCCTAGCAAGGCAC-3′; 12, 5′-TAAGAGCAAGATGTTCTATAAAAGATGTCCTAGCAAGGCAC-3′; 13, 5′-AAAGATGTCCTAGCAAGGCACGATCGACCGGATATCTATGA-3′. The oligonucleotide composition of each structure is identified in Table 1. Substrate A is a four-way duplex (X) junction designed to mimic a Holliday intermediate. It has a homologous core of 12 bp within which the junction point is free to branch migrate. Similar junctions with homologous cores of 2 bp or 11 bp, and a static junction with no homology, were made by annealing in each case four oligonucleotides of ~50 bp in length whose sequences have been described (Saito *et al*., 1995; Shah *et al*., 1994). Prior to annealing, the oligonucleotides marked with an asterisk in Table 1 were labelled at the 5′ end with [γ-^{32}P]ATP and polynucleotide kinase. Annealed substrates were purified by non-denaturing electrophoresis on 10% (w/v) polyacrylamide gels followed by electroelution. Appropriate markers for identification of the required construct were provided by annealing combinations of the oligonucleotides used. The concentrations of DNA substrates were estimated by monitoring the specific activity of each labelled oligonucleotide after end-labelling and the final activity of the purified substrate. DNA concentrations are in moles of DNA substrate.

Binding assays

Bandshift reactions were performed in 20 µl volumes in 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 1 mM dithiothreitol, 100 µg/ml bovine serum albumin and 6% (v/v) glycerol, with labelled DNA at 0.5 to 1.5 nM and PriA or RecG protein in the range of 0.1 to 100 nM. Reactions were incubated on ice for 15 minutes before loading 12 µl on 4% polyacrylamide gels in 6.7 mM Tris-HCl (pH 8.0), 3.3 mM sodium acetate and 2 mM EDTA. Electrophoresis was at 160 volts for 90 minutes at room temperature, with buffer recirculation. Gels were then dried and autoradiographed.

Helicase assays

Reactions were performed in 20 µl volumes in 20 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol, 5 mM $MgCl_2$, 5 mM ATP and 100 µg/ml bovine serum albumin, with labelled DNA at 0.5 to 1.5 nM and PriA or RecG protein in the range of 0.1 to 100 nM. After incubation at 37°C for 30 minutes, reactions were stopped by adding 5 µl of 100 mM Tris-HCl (pH 7.5), 2.5% (w/v) SDS, 200 mM EDTA, 10 mg/ml Proteinase K, and incubating for a further ten minutes at 37°C; 5 µl of each reaction was electrophoresed at room temperature on 10% polyacrylamide gels in 90 mM Tris-borate, 2 mM EDTA at 190 volts for 90 minutes. Gels were dried, autoradiographed, and the amount of dissociation quantified by phosphorimaging (Molecular Dynamics).

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