Crystal structure and mutational study of RecOR provide insight into its mode of DNA binding

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The crystal structure of the complex formed between *Deinococcus radiodurans* RecR and RecO (drRecOR) has been determined. In accordance with previous biochemical characterisation, the drRecOR complex displays a RecR:RecO molecular ratio of 2:1. The biologically relevant drRecOR entity consists of a heterohexamer in the form of two drRecO molecules positioned on either side of the tetrameric ring of drRecR, with their OB (oligonucleotide/oligosaccharide-binding) domains pointing towards the interior of the ring. Mutagenesis studies validated the protein–protein interactions observed in the crystal structure and allowed mapping of the residues in the drRecOR complex required for DNA binding. Furthermore, the preferred DNA substrate of drRecOR was identified as being 3'-overhanging DNA, as encountered at ssDNA–dsDNA junctions. Together these results suggest a possible mechanism for drRecOR recognition of stalled replication forks.

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Introduction

Homologous recombination, in addition to its fundamental role in genetic diversification of bacterial genomes, plays an essential role in the repair of a variety of DNA damage, including double-strand breaks (Kuzminov, 1999; Michel *et al*, 2004). In *Escherichia coli*, the initiation of homologous recombination can be carried out by either the RecBCD or the RecFOR proteins; in both cases, these proteins act as mediators for RecA binding to single-stranded DNA (ssDNA) to allow for homologous strand invasion (Kowalczykowski *et al*, 1994). Comparative studies of bacterial genomes have revealed that many genomes display an incomplete set of DNA repair systems. Whereas RecBCD has been shown to be

the major DNA recombination pathway in *E. coli*, the RecFOR pathway actually appears to be the more frequent pathway in bacterial genomes (Rocha *et al*, 2005). RecR, along with RecA and the resolvases, has been found to be nearly ubiquitous in bacteria, suggesting they must be playing essential roles. RecO and RecF are less well conserved and appear to be missing in a number of species (Rocha *et al*, 2005).

The eubacterium *Deinococcus radiodurans* displays an outstanding resistance to radiation and other DNA-damaging agents (Anderson *et al*, 1956; Battista, 1997). Whereas the exact molecular mechanisms underlying this phenotype have yet to be identified, an ability to withstand several hundred DNA double-strand breaks in its genome strongly suggests that a highly efficient DNA repair machinery is critical (Minton, 1994; Battista *et al*, 1999). Genome sequence analysis of *D. radiodurans* has revealed that although all DNA repair pathways are represented in its genome, most of the genes encoding proteins from the RecBCD pathway appear to be missing (White *et al*, 1999) with only the gene encoding RecD having been identified (Makarova *et al*, 2001). However, all the genes encoding members of the RecFOR pathway (RecQ, RecJ, RecO, RecF and RecR) are present in *D. radiodurans*. A recent study has revealed that inhibition of the RecFOR pathway in *D. radiodurans* by overexpression of *E. coli* exonuclease I increases the sensitivity of *D. radiodurans* cells to gamma-radiation, suggesting that RecFOR is essential for repair of double-strand breaks (Misra *et al*, 2006).

In contrast to the (*E. coli*) RecBCD pathway the mechanism of recombinational repair mediated by the RecFOR pathway is still only poorly understood. The major difference between these two recombination pathways lies in the initiation step, also known as presynapsis (Kowalczykowski, 2000). RecFOR displaces ssDNA-binding protein (SSB) before loading of RecA onto the ssDNA, whereas RecBCD directly catalyses the latter step (Umezū *et al*, 1993; Umezū and Kolodner, 1994; Anderson and Kowalczykowski, 1997). In addition, RecBCD is known to function as a single holoenzyme (Taylor and Smith, 1995; Singleton *et al*, 2004), whereas it is still unclear whether RecFOR exists as a functional complex *in vivo* and how it mediates presynapsis. It has been shown that RecF, RecO and RecR are all required for protecting the nascent lagging strand when replication forks are stalled on UV-radiation-induced damage sites. The absence of any of the RecFOR genes causes *E. coli* to become both hypersensitive to UV radiation and to display extensive degradation in the nascent lagging strand, suggesting that they form an epistatic group (Chow and Courcelle, 2004). So far, there is evidence that *E. coli* and *D. radiodurans* RecO and RecR form a stable complex (Umezū and Kolodner, 1994; Leiros *et al*, 2005) and that purified RecO, RecR and RecF can associate *in vitro* together with SSB (Hegde *et al*, 1996). In *E. coli*, RecO and RecR can successfully remove SSB and allow RecA loading in the absence of RecF, suggesting that RecF is (at least partially) dispensable for this process (Umezū *et al*, 1993; Umezū and

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Kolodner, 1994). Members of the RecFOR pathway have been shown to recover the viability of certain RecBCD mutants in *E. coli*, implying that the machineries of the two pathways are, at least in part, interchangeable (Amundsen and Smith, 2003; Ivancic-Bace *et al*, 2003, 2005).

An increasing amount of structural information is becoming available for these proteins, which together with the biochemical and genetic data will no doubt improve our current understanding of the initial steps in homologous recombination. The structure of the *E. coli* RecBCD complex bound to a DNA substrate revealed the detailed architecture of this multifunctional enzyme and suggested a mechanism for its involvement in recombination (Singleton *et al*, 2004). The individual crystal structures of RecF, RecO and RecR from *D. radiodurans* have also been solved recently (Lee *et al*, 2004; Makharashvili *et al*, 2004; Leiros *et al*, 2005; Koroleva *et al*, 2007) and have indicated possible regions involved in protein–protein and protein–DNA interactions. To gain a better understanding of the detailed mechanisms involved in the RecFOR-mediated recombination events, we have determined the crystal structure of the RecOR complex from *D. radiodurans* (drRecOR). Knowledge of the three-dimensional arrangement of the drRecOR heterohexameric complex, together with mutagenesis and biochemical analysis, reveal a possible mode of binding to stalled DNA replication forks, and suggest a mechanism for its involvement in the early steps of DNA repair.

Results

The structure of the RecOR complex

The crystal structure of the drRecOR complex was determined to 3.8 Å resolution by molecular replacement (MR). The crystal structures of the individual drRecO (Makharashvili *et al*, 2004; Leiros *et al*, 2005) and drRecR (Lee *et al*, 2004) proteins, previously determined by experimental phasing methods at atomic resolution were used as search models. Due to the observation-to-parameter ratio of the collected data being less than unity, refinement was stopped after a single round of rigid-body refinement and manual rebuilding of some residues according to conventional and composite-omit electron density maps (see Table I for data collection statistics). Although the drRecOR complex structure is limited to 3.8 Å resolution, the corresponding maps are of overall good quality for the protein backbone (Supplementary Figure S1), also revealing regions (mainly in drRecR) that had to be remodelled due to the protein–protein complex formation.

Table I Data collection statistics

Data collection	
Unit cell (Å/deg)	$a = 144.0, b = 83.2, c = 66.6, \beta = 106.9$
Resolution range (Å)	45–3.8 (4.0–3.8)
Number of unique reflections	7194
Redundancy	3.1 (2.1)
R_{merge} (%) ^a	13.6 (63.7)
Completeness (%)	95.9 (95.9)
Mean $I/\sigma(I)$	6.5 (1.4)
Wilson B-value (Å ²)	96.0

^a $R_{\text{merge}} = (\sum_h \sum_i |I_i(h) - \langle I(h) \rangle|) / (\sum_h \sum_i I_i(h))$, where $I_i(h)$ is the i th measurement of reflection h and $\langle I(h) \rangle$ is the weighted mean of all measurements of h .

The drRecOR complex has a 2:1 molecular ratio of drRecR to drRecO, where the content of the crystallographic asymmetric unit is a heterotrimer (Figure 1A and B). The C2 space group symmetry operator ($-X, Y, -Z$) generates the most probable biologically relevant heterohexameric molecular unit. By this symmetry operator, the tetrameric structure previously described for drRecR (Lee *et al*, 2004) is reconstructed, and the estimated molecular weight of the according drRecOR complex is consistent with its size-exclusion chromatography elution profile (Leiros *et al*, 2005). This entity thus consists of a heterohexamer of two drRecO molecules and four drRecR molecules and forms the basis for the following analysis (Figure 1C). In the drRecOR complex, the drRecO molecules are rather unexpectedly positioned on either side of the tetrameric ring of drRecR, obstructing access to the interior of the ring. In this complex, the N-terminal OB (oligonucleotide/oligosaccharide-binding) fold (Murzin, 1993) domains of each of the two drRecO molecules point towards the interior of the drRecR ring (Figure 1B). The accessible surface area lost for one monomer of drRecO when binding to the tetramer of drRecR was calculated using ArealMol (CCP4, 1994) to be around 1500 Å², or 14%

Figure 1 Crystal structure of drRecOR complex. (A, B) Side view and top view of a ribbon representation of the heterotrimeric drRecOR assembly present in the asymmetric unit. drRecO is coloured in blue and the two drRecR molecules are coloured in gold. (C) Ribbon representation of the heterohexameric drRecOR complex, as reconstituted by symmetry. (D) Top view of the overlay of the C α trace of drRecR crystallised alone (grey) and in complex with drRecO (red). (E) Surface representation of drRecOR, illustrating the displaced loop region of drRecR (residues 109–124) in red. The structure of drRecR crystallised alone is illustrated in ribbon representation. The arrows indicate the movement of these loops upon drRecO binding.

of the total accessible surface area for a monomer of drRecO, thus well within the representative range found for protein–protein complexes (Chakrabarti and Janin, 2002; Bahadur *et al*, 2004).

Comparison of the structures of drRecO and drRecR crystallised alone and as a complex reveals that, in both cases, the overall protein architecture is maintained with root-mean-square deviations (r.m.s.d.) of 0.79 and 1.07 Å, respectively (Figure 1D). Whereas the overall structure of drRecO is highly conserved, distinct changes are observed in drRecR upon complex formation. In particular, a loop region of drRecR (comprising residues 109–124) moves by up to 6.5 Å (distance between C α positions of Met114 in the two structures) to accommodate a drRecO molecule (Figure 1E). As no positional refinement was performed, the size of this loop movement is nonetheless approximate.

The drRecO–drRecR interface

The electrostatic surface potentials of drRecO and drRecR reveal that drRecO possesses two regions of pronounced positive charge and that drRecR displays a negatively charged surface within its ring-like structure. The binding of the overall positively charged OB-fold domain of drRecO within the ring of drRecR thus indicates that electrostatic interactions are important in complex formation in addition to a pronounced molecular surface complementarity (Figure 2A–C).

Most of the drRecR residues contributing to complex formation are located around its central hole (Figure 2D), whereas those of drRecO are clustered on one side of the OB-fold domain and its neighbouring α -helix (Figure 2E). The OB-fold domain of drRecO contacts residues from both the N-terminal Helix-hairpin-Helix (HhH) motif (Thayer *et al*, 1995) and the C-terminal region, including the Walker B motif (residues 167–182) of drRecR (Figure 2F). Both these regions in drRecR participate in domain swapping with neighbouring molecules and are thus also critical for stabilisation of the tetrameric structure of drRecR. In addition to this interaction area, drRecO also contacts the Toprim (Aravind *et al*, 1998) domain (residues 78–166) of drRecR (Figure 2F). drRecO residues in the loop and α -helix following the OB-fold (residues 80–97) are in the proximity of the drRecR residues 143–156 and the C-terminal drRecO residues (residues 230–235) contact drRecR residues 112–116, located in the loop, which is most significantly shifted upon RecO binding.

The interactions formed between a monomer of drRecO and the tetramer of drRecR are mostly of hydrophobic character. Relatively few intermolecular hydrogen bonds or potential ion pairs can be found. Only two of the latter were identified between drRecO and the drRecR tetramer: RecO-His93 to RecR-Glu146 (MolA) and RecO-His65 to RecR-Asp182 (MolD). All the regions of drRecR responsible for interacting with drRecO are strongly conserved in RecR proteins, including Glu146 and Asp182 (Figure 2G). In addition, ionic interactions between residues Asp22 and Arg39 of symmetry-related drRecO molecules also contribute to the overall stability of the biological complex, as they form drRecO–drRecO interactions across the interior of the ring. Furthermore, a total of 12 potential drRecO–drRecR hydrogen bonds were identified, where most interactions were found between drRecO and either molecule A or molecule D (the symmetry-related copy of molecule A) of drRecR.

Mutagenesis study of the drRecOR complex

To examine the significance of protein–protein interactions suggested by the 3.8 Å crystal structure of drRecOR, single and multiple mutants were designed (Figure 3A and B and Table II). In addition, previous studies of drRecO and drRecR DNA binding (Lee *et al*, 2004; Leiros *et al*, 2005), together with the structure of the complex and DNA modelling allowed us to locate possible DNA-binding sites within the complex and thus design additional mutants to further investigate the DNA-binding ability of drRecOR. For the purposes of studying both protein–protein and protein–DNA interactions, a number of charged residues from both drRecO and drRecR were mutated to either Ala or the opposite charge (positively charged residues were mutated into Glu and negatively charged residues were mutated into Arg). Whereas mutations to the opposite charge were expected to be more efficient in disrupting protein–protein and protein–DNA interactions, mutations to Ala were prepared to maintain structural integrity. This was particularly important for drRecR, which forms a higher order quaternary structure. Several of the drRecR residues potentially involved in interactions with drRecO and/or DNA are also involved in contacts within the drRecR tetramer itself (Figure 3A). Sixteen drRecOR complexes (named C1–C16) were produced by combining the mutants as shown in Table II (see Supplementary Data for more details). Complex C1 corresponds to the wild-type drRecOR complex. Of these 16 complexes, C8 and C9 were largely insoluble and were not used for further study and C7 was only weakly soluble.

Disruption of protein–protein interactions within the complex

The crystal structure of drRecOR identified residues in both drRecO and drRecR along the complex-forming interface that may be important for interprotein ionic interactions and relevant mutants were prepared. To study complex formation, the wild-type and mutant drRecOR proteins obtained from the anion-exchange column were further purified by size-exclusion chromatography, allowing for efficient separation of the complex from the individual proteins (Figure 3C). The elution profile exhibits three distinct peaks, which are known (Leiros *et al*, 2005) to correspond respectively to the drRecOR complex (peak 1), and the individual drRecR (peak 2) and drRecO (peak 3) proteins, in agreement with their molecular weights (Figure 3C). For each mutant complex, the peak fractions of peaks 1, 2 and 3 were analysed by Western blot using anti-drRecO and anti-drRecR antibodies (Figure 3D).

For complexes C1, C3–C5, C7 and C10–C16, peak 1 is the major form of the protein and both drRecO and drRecR were detected in peak 1 fraction (Figure 3D), strongly suggesting that these complexes are intact. In some cases, a small additional peak 2 was observed corresponding to an excess of drRecR, which probably results from an incomplete separation of drRecR from drRecOR during the anion-exchange chromatography step. In the case of C14 (and to a lesser extent C12 and C16), all three peaks were observed (peaks 2 and 3 being only minor) and the two individual proteins were detected in peaks 2 and 3 in addition to the presence of the complex in peak 1. These results suggest that these complexes may not be as stable as the wild-type complex for which only a slight excess of drRecR was observed.

Figure 2 Analysis of the drRecO-drRecR interface. (A–C) Electrostatic surface potentials of drRecO, drRecR and drRecOR respectively, contoured at ± 6 kT/e, where red describes a negative and blue a positive potential. These figures were prepared with Swiss-PdbViewer (Guex and Peitsch, 1997). (D) Surface representation of drRecR indicating in blue the residues involved in protein–protein interactions with drRecO. Selected residues from the interface are labelled on one of the drRecR monomers. (E) Surface representation of drRecR indicating in gold the residues involved in protein–protein interactions with drRecR. Selected residues from the interface are labelled. (F) Ribbon representation of the heterotrimeric drRecOR assembly with individual domains coloured and labelled as follows: drRecO OB-fold (dark blue), helical bundle (light blue) and Zinc-finger (purple), and drRecR HhH (orange), Toprim domain (gold) and Walker B (red). (G) Amino-acid sequence alignment of RecR proteins from *D. radiodurans*, *B. subtilis*, *S. aureus*, *T. thermophilus* and *E. coli*. The secondary structure of drRecR is shown above the sequences and the conserved residues are coloured in red. Residues involved in complex formation with drRecO are underlined in blue.

Anion-exchange followed by size-exclusion chromatography clearly revealed that complexes C2 and C6 were disrupted (Figure 3C–D). In both cases, peak 1 was very small and peaks 2 and 3 became the major forms. Western blot analysis of these fractions confirmed the presence of the individual drRecR and drRecO proteins in these two peaks and the absence of drRecO at the expected elution volume for the complex (peak 1). Some drRecR was detected in peak 1, most likely corresponding to a concentration-dependent octameric form of drRecR previously observed by Lee *et al* (2004), eluting at a similar volume to the drRecOR complex. C2 and C6 both correspond to drRecO mutants; C2 is a single point mutant of drRecO in which His93 is replaced by Glu and C6 is a triple mutant in which Lys72 is mutated to Glu, Gln73 to Arg and His93 to Glu. The single and double drRecO K72E

and K72E/Q73R mutants were not expressed in *E. coli* and as a result, we cannot firmly conclude regarding the effect of the single K72E and Q73R mutations in the triple mutant C6. These results suggest, however, that disruption of the ionic interaction observed between drRecO His93 and drRecR Glu146 in the drRecOR crystal structure is sufficient to prevent complex formation (Figure 3E), thereby substantiating that the crystal structure of the drRecOR complex presented herein is valid, although resolved only at relatively low resolution.

drRecOR complex binding to plasmid DNA

Both drRecO and drRecR have been shown to interact with plasmid DNA (Lee *et al*, 2004; Leiros *et al*, 2005). In both cases, protein–DNA interaction was studied using

Figure 3 Study of the ionic interactions between drRecO and drRecR. (A) Ribbon illustration of a monomer and a tetramer of drRecR with ball-and-sticks representation of the mutated residues. (B) Ribbon illustration of drRecO with ball-and-sticks representation of the mutated residues. Residues coloured in blue were mutated in order to disrupt protein–protein interactions, whereas residues in red were predicted to be involved in protein–DNA contacts. (C) Overlay of the chromatograms obtained when purifying wild-type drRecOR (C1) and mutant drRecOR (C2) on a Superdex 200 size-exclusion column. The three peaks are labelled from 1 to 3. (D) Western blot analysis of fractions corresponding to peaks 1, 2 and 3 from the gel filtration runs of each of the drRecOR complexes (C1–C7 and C10–C16). The Western blots were duplicated and were stained for either drRecO (upper bands) or drRecR (lower bands). (E) Illustration of the drRecR (gold)—drRecO (blue) interface with a ball-and-sticks representation of drRecO-His93 and drRecR-Glu146, displaying the 2mFo-DFc sigmaA-weighted electron density map contoured at 1.3σ.

electrophoretic mobility shift assays (EMSA) in which the plasmid DNA alone and in complex with protein was separated by agarose gel electrophoresis. The same method was therefore maintained to study the DNA-binding ability of drRecOR (Figure 4A and Supplementary Data). The binding assays were carried out in both the presence and absence of 40 mM MgCl₂. The plasmid DNA runs as two bands: the lower form being the supercoiled DNA and the upper the relaxed DNA. Addition of increasing amounts of drRecO leads to a concentration-dependent shift of the plasmid DNA into the well and Mg²⁺ appears to enhance this shifting (Figure 4A, lanes 7–9). In the case of drRecR, increasing amounts of protein shifts the plasmid upwards to the relaxed form (Figure 4A, lanes 10 and 11) in the presence of Mg²⁺,

whereas in the absence of Mg²⁺, the DNA shift is greater resulting in two bands (Figure 4A, lane 12). Addition of drRecOR, in contrast, causes a significant retardation of the plasmid DNA, which is further enhanced in the absence of Mg²⁺ (Figure 4A, lanes 13 to 15). In all cases, however, DNA binding does not require Mg²⁺, although the presence of 40 mM Mg²⁺ certainly affects the binding, particularly for drRecR and drRecOR. drRecO, drRecR and drRecOR binding to linear plasmid DNA (Supplementary Figure S2A) is very similar to that observed with supercoiled DNA: drRecO and drRecOR display a clear binding to the linear DNA, whereas drRecR only causes a minor shift of the plasmid DNA. The drRecOR-induced band shifts are clearly distinct from those caused by either drRecO or drRecR alone.

Table II drRecOR mutants

No.	RecO	RecR
C1	Wild-type	Wild-type
C2	H93E	Wild-type
C3	Wild-type	K23E
C4	Wild-type	R27E
C5	Wild-type	K23E/R27E
C6	K72E/Q73R/H93E	Wild-type
C7	Wild-type	D182R/E183A
C8	K72E/Q73R/H93E	D182R/E183A
C9	Wild-type	K23E/R27E/ D182A/E183A
C10	Wild-type	K23A/R27A
C11	Wild-type	D182A/E183A
C12	R16E	Wild-type
C13	R39E	Wild-type
C14	K35E/R39E	Wild-type
C15	K35E/R195E/R196E	Wild-type
C16	R195E/R196E	Wild-type

Mutagenesis study of drRecOR binding to plasmid DNA

Ten mutant complexes of drRecOR were used for DNA binding studies. Five of these (C3, C4, C5, C10 and C11) correspond to wild-type drRecO combined with drRecR mutants and five others (C12–C16) correspond to wild-type drRecR combined with drRecO mutants. The purified drRecOR samples were subjected to limited proteolysis (Supplementary Figure S2B). Very similar degradation patterns were obtained for all the treated complexes, indicating that the mutant complexes had maintained a native fold and were suitable for comparative DNA-binding studies.

In the case of drRecR, two potential DNA-binding sites were mutated: (i) K23 and R27 located in the N-terminal HhH motif and (ii) D182 and E183 at the C-terminus of the Walker B motif. Single and double mutations of K23 and R27 to Glu (complexes C3–C5) interfere with DNA binding, but only in the absence of Mg^{2+} (Figure 4B, lanes 3–8). The smearing of the plasmid bands for mutant C3 (Figure 4B, lane 4) in the absence of Mg^{2+} suggests that weak DNA binding may be retained in the single K23E mutant. For all three of these complexes, however, DNA binding is maintained in the presence of Mg^{2+} (Figure 4B, lanes 3, 5 and 7). The double mutation of K23 and R27 to Ala in complex C10, however, fully disrupts DNA binding even in the presence of Mg^{2+} (Figure 4B, lanes 9 and 10). This finding suggests that these two residues are essential for drRecOR DNA binding, but that Mg^{2+} coordination by the introduced Glu residues is compensating, at least in part, for the K23E/R27E mutations. The second DNA-binding site (D182/E183) was proposed by Lee *et al* (2004) to be involved in Mg^{2+} coordination, and therefore mutations to both Arg and Ala were prepared. However, due to the poor solubility of complexes C7–C9, only the double mutant D182A/E183A (C11) could be used for the DNA-binding study. This mutant was found to retain DNA binding, leading to a significant band shift (Figure 4B, lanes 11 and 12) that is very similar to that obtained for wild-type drRecOR complex (Figure 4B, lanes 23 and 24).

Mutant complexes C12–C16 were prepared by combining five of the most disrupting DNA-binding drRecO mutants identified previously (Leiros *et al*, 2005) with wild-type drRecR (Table II). These include mutations in both previously proposed DNA-binding regions of drRecO: the OB-fold domain and the C-terminal positively charged ridge. Unlike the

drRecR mutants, DNA binding of complexes C12–C16 is strongly affected by all the mutations in drRecO, particularly in the presence of Mg^{2+} (Figure 4B, Lanes 13, 15, 17, 19 and 21). Mutations in the OB-barrel (mutant complexes C12–C14) completely abolish DNA binding in the presence of Mg^{2+} . In the absence of divalent cations, incubation of complexes C13 and C15 with plasmid DNA leads to a shift of both the supercoiled and relaxed forms of the DNA (Figure 4B, lanes 16 and 20). Regardless of the presence or not of Mg^{2+} , the double OB-barrel (K35E/R39E) drRecO mutant complex C14 no longer binds DNA (Figure 4B, lanes 17 and 18), whereas the drRecO double mutant (R195E/R196E), complex C16, seems to retain some DNA binding ability (smearing in Figure 4B, lanes 21 and 22).

Identification of the preferred DNA substrate of drRecOR

EMSA studies were also carried out with various DNA oligonucleotides, forming ssDNA, double-stranded DNA (dsDNA), 3'overhanging DNA (OH1 and OH2 with 7 base and 15 base overhangs, respectively) or 5'overhanging DNA (15 base overhang) substrates. drRecO alone shows preferential binding to ssDNA (and thus also binds to a lesser extent to both 3' and 5' overhanging DNA) and drRecR shows no detectable binding to these short oligonucleotides (Supplementary Figure S2C). Wild-type drRecOR displays a preference for binding to 3'overhanging DNA compared to 5'overhanging, ssDNA or dsDNA. The highest affinity is seen for the longer 15-mer 3'overhang (Figure 4C). This binding was unaffected by the addition of the divalent cation, Mg^{2+} and ATP. Four of the mutant drRecOR complexes (C5, C10, C14 and C16) were tested for binding to single-stranded and double-stranded oligonucleotides and to the 15-mer 3'overhanging DNA substrate (OH2). Mutants C5, C10 and C14 display very weak binding to all three of these substrates, whereas C16 exhibits an increased binding relative to wild-type drRecOR (Figure 4D). None of the mutant complexes display a clear preference for one of the DNA substrates.

Structural rearrangement of drRecOR

Evidence for a clear change in the architecture of drRecOR upon DNA binding comes from chemical crosslinking and native gel electrophoresis studies (Figure 5A and Supplementary Figure S3). Use of a lysine-specific chemical crosslinker, BS³, allowed us to determine the various oligomeric structures, and their relative abundance, formed in solution by drRecR and drRecOR in the presence and absence of 3'overhanging DNA (Figure 5A). The crosslinking pattern of drRecR reveals broad bands corresponding to dimeric, trimeric and tetrameric drRecR. Additional minor bands corresponding to higher molecular weight oligomers are also visible. The major form is dimeric drRecR that represents the stable building block for the tetramer. In the case of drRecOR, the pattern is more distinct with seven different forms corresponding to intermediate assemblies of the complex (Figure 5A, bands a–e), the heterohexameric complex (band f) and most likely its dimer (band g). A Western blot analysis of the crosslinking pattern using anti-drRecO and anti-drRecR antibodies facilitated the interpretation of the various forms observed (Supplementary Figure S3A and B). At the higher concentration of crosslinking agent, the major form of drRecOR is band f—the heterohexameric

Figure 4 drRecOR-DNA binding. (A) EMSA analysis of drRecO, drRecR and drRecOR binding to supercoiled plasmid DNA. Lanes 1–4 correspond to the proteins alone in the order drRecO, drRecR, drRecOR and BSA. Lanes 5 and 6 correspond to plasmid DNA alone in the presence and absence of 40 mM $MgCl_2$. 1 and 5 μ g of each of the proteins were added to the DNA in the presence of 40 mM $MgCl_2$ and separated by EMSA (lanes 7–8 for drRecO, lanes 10–11 for drRecR, lanes 13–14 for drRecOR and lanes 16–17 for BSA). In addition, in lanes 9, 12, 15 and 18, 5 μ g of each of the proteins was mixed with plasmid in the absence of $MgCl_2$. In the lower panel, the agarose gel was stained with Coomassie blue to locate the protein. (B) Mutant drRecOR-plasmid binding. Lanes 1 and 2 correspond to supercoiled plasmid DNA alone in the presence and absence of 40 mM $MgCl_2$. Lanes 3–22 correspond to the analysis of the binding of drRecOR mutants (C3–C5, C10–C16) to plasmid DNA, whereas lanes 23 and 24 correspond to the wild-type complex. In all cases, the reactions were prepared with (lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 and 23) and without $MgCl_2$ (lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24). (C) Four types of DNA (dsDNA, two 3'overhanging DNA, 3'-OH1 (+7 nt) and 3'-OH2 (+15 nt), and ssDNA) were mixed with wild-type drRecOR at various protein:DNA ratios (0.5:1, 1:1 and 2:1) and were analysed by EMSA. In the second panel, drRecOR was mixed with 3'-OH2 at a 2:1 ratio in the absence and presence of either $MgCl_2$ alone or $MgCl_2$ and ATP. In the third panel, drRecOR was mixed with 3'-OH2 and 5'-OH2 at various protein:DNA ratios (0.5:1, 1:1 and 2:1) (D) EMSA analysis of dsDNA, ssDNA and 3'-OH2 DNA alone and after addition of a 2:1 ratio of mutant complexes C5, C10, C14 and C16 and wild-type (wt) complex.

complex as observed in our crystal structure. Upon addition of DNA, a number of changes are observed: (i) the intensity of the higher molecular weight bands reduces, (ii) the amount of monomeric drRecO and dimeric drRecR assemblies increases, (iii) an additional form (band c) appears (corresponding to trimeric drRecR) and (iv) band d, most likely corresponding to an assembly of two drRecR and one drRecO molecules (as observed in the asymmetric unit of our

crystals), remains unchanged. Addition of increasing amounts of DNA to drRecOR also led to a clear reduction of the amount of drRecO associated with the complex as observed by native gel and Western blot analysis (Supplementary Figure S3C). Together these results support the idea that the heterohexameric complex of drRecOR undergoes a significant structural rearrangement upon binding to DNA.

Figure 5 Models of the drRecOR complex bound to 3'-overhanging DNA. (A) SDS-PAGE analysis of the crosslinking pattern of drRecR alone (lanes 1–3) and drRecOR alone (lanes 4–6) and after addition of a 0.5:1 (lanes 7–8) and 2.5:1 (lanes 9–10) DNA:protein ratio of 3'-OH2 DNA. No crosslinker was added to the protein in lanes 1 and 4, 0.4 mM BS³ was added in lanes 2, 5, 7 and 9, and 1 mM BS³ was added in lanes 3, 6, 8 and 10. Molecular weight markers (kDa) are shown on the left of the gel and the different bands observed are labelled from a to g. (B) Ribbon representation of the drRecOR complex with one drRecO molecule replaced by a dsDNA fragment with a 3'overhanging strand. (C) Close-up view of the contacts between drRecOR and the modelled DNA. (D) Surface representation of the drRecOR complex after removal of one drRecO molecule (blue). Residues of drRecOR required for DNA binding are shown in red. (E) Illustration of the modelled complex formed between drRecOR (surface representation) and DNA (ribbon). The crucial residues of drRecOR are illustrated in ball-and-sticks and labelled. (F) Illustration of the electrostatic surface potential of the heterotrimeric drRecOR assembly contoured at ± 6 kT/e bound to 3'-overhanging DNA (dark red).

Discussion

In the present study, the crystal structure of the RecOR heterohexameric complex from *D. radiodurans*, which consists of two molecules of drRecO pointing towards the inside of a tetrameric ring of drRecR molecules has been described. The heterohexamer is consistent with the complex observed in solution (Leiros *et al*, 2005), but differs from the 1:1 RecO-RecR heterotetrameric complex described in *E. coli* (Umezue and Kolodner, 1994).

Examination of the drRecO-drRecR interface reveals that the buried surface area, fraction of nonpolar residues, number of interface residues and number of estimated hydrogen bonds (approximate estimation due to the difficulty to precisely model the orientation of certain side chains) are well within the range of what has been described in the literature for protein-protein interfaces (Chakrabarti and Janin, 2002; Bahadur *et al*, 2004). Furthermore, the correctness of the crystallographic drRecOR model was confirmed by mutagenesis analysis in which some of the mutants were designed to

disrupt potential ionic interactions required for stabilisation of protein–protein interactions within the complex. This study revealed that the interaction between drRecO His93 and drRecR Glu146 was essential in maintaining the heterohexameric assembly. Glu146 is highly conserved in RecR proteins and contributes to a characteristic acidic surface patch found in Toprim (Aravind *et al*, 1998) domains, previously proposed to be involved in magnesium coordination (Lee *et al*, 2004). Recent work by Honda *et al* (2006) also identified the equivalent glutamate residue (Glu144) of *T. thermophilus* RecR (ttRecR) as being critical for its interaction with *T. thermophilus* RecO. In this study, mutation of ttRecR Glu144 to Ala resulted in a nonfunctional RecR protein that could no longer facilitate the loading of RecA onto SSB-coated ssDNA. Two independent studies thus show the importance of this ionic interaction for the stability of the RecOR complex, and consequently for its function in RecA loading onto ssDNA. Whereas most of the contacts between drRecR and drRecO are of hydrophobic nature, the ionic interactions appear to contribute significantly to the overall stability of the complex. RecR, unlike RecO, is highly conserved throughout bacteria and the observation that the residues responsible for binding to RecO are also strongly conserved suggests that this protein–protein interface may well be observed in other bacteria.

Whereas the structure observed is consistent with the drRecOR complex observed in solution, it is also unexpected. Lee *et al* proposed that drRecR may act as a nonsliding DNA clamp, which could accommodate dsDNA within its central hole (Lee *et al*, 2004). In the drRecOR complex, drRecO reduces access to the interior of the ring, suggesting that such a complex, if functional, must adopt a different mode of DNA binding. Extensive mutagenesis studies have identified the key residues in drRecOR responsible for protein–DNA interactions and thus allow a model to be proposed for how such a complex may interact with DNA in the initial steps of homologous recombination. Both drRecR and drRecO provide key residues involved in binding to DNA; in drRecR, two positively charged amino acids, K23 and R27, located in the HhH motif are essential for drRecOR DNA interactions, as are two residues, K35 and R39, found in the OB-fold domain of drRecO. In both cases, these residues had been shown to be critical for the binding of the individual drRecO and drRecR proteins to dsDNA (Lee *et al*, 2004; Leiros *et al*, 2005). Studies of the individual proteins had also revealed additional regions of drRecR (such as Asp182) and drRecO (positively charged ridge at the C-terminus; residues Arg195 and Arg196) believed to be involved in DNA binding (Lee *et al*, 2004; Leiros *et al*, 2005). Both these regions have been shown in this study of the drRecOR complex to be only minor contributors to DNA binding. In the context of the drRecOR complex, only the two sites consisting of the HhH of drRecR and the OB-fold domain of drRecO are essential for DNA binding. Interestingly, mutating one of these two sites was sufficient to fully disrupt plasmid DNA binding, suggesting that both sites are needed for stable association of drRecOR with DNA.

The preferred DNA substrate of the complex was identified as being dsDNA with a 3'overhang, such as those encountered at ssDNA–dsDNA junctions in the lagging strand of stalled replication forks. These junctions are believed to be the sites of action of RecF and RecOR *in vivo* (Morimatsu and

Kowalczykowski, 2003). The individual drRecO and drRecR proteins show a very different DNA-binding pattern to that observed for drRecOR. drRecO alone displays preferential binding to ssDNA and drRecR shows no detectable interaction with either ssDNA and dsDNA oligonucleotides under these conditions. In addition, mutating the DNA-binding sites of either drRecO or drRecR also resulted in mutant complexes that no longer displayed preferential binding to 3'overhanging DNA and retained weak binding to short fragments of both ssDNA and dsDNA. Selective binding to 3'overhanging DNA thus requires the assembly of drRecO and drRecR as a complex. Recent studies have shown that specific binding to ssDNA–dsDNA junctions requires the presence of RecF, which recognises and forms a high affinity complex with such junctions (Hegde *et al*, 1996; Morimatsu and Kowalczykowski, 2003). Here, we present evidence that the drRecOR complex by itself displays a clear preference for binding to 3'overhanging DNA in the absence of RecF. However, this does not exclude a role for RecF in stabilising the assembly on ssDNA–dsDNA junctions through its interaction with RecR *in vivo*.

The mutational analysis performed taken together with the three-dimensional structure of drRecOR allows us to propose a model for drRecOR binding to gapped DNA. In order for DNA to interact with residues located both inside the ring of drRecR and in the OB-fold domain of drRecO, a structural rearrangement of the complex needs to take place. drRecOR most likely undergoes both local conformational changes and a larger architectural reorganisation upon addition of DNA as evidenced by our crosslinking and native gel electrophoresis studies. We propose that the displacement of one of the two drRecO molecules is a prerequisite for allowing dsDNA to bind to the drRecR tetrameric ring, in the same orientation as the drRecO molecule (Figures 1C and 5B). This leaves enough space for a 3'overhanging strand of DNA to pass through the drRecR ring and to interact with the OB-fold of the second drRecO molecule (Figure 5C and D). In this model, the ssDNA–dsDNA junction interacts with the crucial residues from both drRecO and drRecR (Figure 5D and E). Further rearrangement of the complex may involve opening of the drRecR ring and release of the two noninteracting drRecR molecules. Such an opening mechanism for drRecR has been proposed previously based on the crystal structure of two interlocked tetrameric rings of drRecR (Lee *et al*, 2004). Binding of the resulting heterotrimeric drRecOR complex to 3'overhanging DNA would allow for residues of the OB-fold of drRecO and the HhH motif of drRecR to contact the DNA. Disruption of the tetrameric ring of drRecR would also affect the electrostatic surface potential of drRecR, by freeing the positively charged faces of the HhH motifs from the dimer–dimer interface and allowing them to interact with the negatively charged DNA (Figure 5F).

The steps involved in RecOR-mediated assembly of RecA onto ssDNA are not known in great detail. Whereas there is no clear evidence for the existence *in vivo* of a complex containing all three proteins (RecO, RecR and RecF), there is convincing evidence for protein–protein interactions between RecR and RecF (Webb *et al*, 1995) and between RecR and RecO (Umezumi and Kolodner, 1994; Bork *et al*, 2001; Leiros *et al*, 2005). Of these three proteins, RecR is by far the most highly conserved and widespread throughout bacterial genomes and always occurs along with either RecO or RecF,

suggesting it must be the key player in this pathway (Rocha *et al*, 2005). RecF, on the contrary, is missing in many genomes and appears to be dispensable in the RecOR-mediated displacement of SSB (Umezumi *et al*, 1993; Bork *et al*, 2001). The *E. coli* RecF exhibits a weak DNA-dependent ATPase activity (Webb *et al*, 1995, 1999) and has been proposed to be an accessory protein, perhaps playing a regulatory role in this process. RecR is known to form a stable complex with RecO, whereas its interaction with RecF is both ATP- and DNA-dependent (Webb *et al*, 1995, 1999). Upon assembly at ssDNA-dsDNA junctions, structural rearrangements may occur as a result of protein-protein and/or protein-DNA interactions between the various partners (Figure 6). The RecOR complex assembly may be affected by the interaction of RecR with RecF and RecO with SSB upon DNA binding. This may subsequently induce a conformational change in either RecR or RecO, resulting in the displacement of a RecO molecule and the stable assembly of RecOR on stalled replication forks. In the case of ttRecR, it was recently shown that Glu144 plays an essential role in the formation of both the RecOR and the RecFR complexes (Honda *et al*, 2006). RecO and RecF may thus be competing for a common binding site on RecR in which case the relative affinity of RecO and RecF for RecR may be central to these events. This relative affinity may be species-specific, as RecO and RecF proteins are less conserved. This process may also be regulated by the nucleotide-bound state of RecF to control the assembly of RecA onto ssDNA. The ATPase activity of RecF may provide the necessary energy required for such structural rearrangements.

The high level of conservation of RecR together with its ability to interact with two essential proteins, RecO and RecF, suggests that it may be the central component of this process. Its function is most likely regulated by its successive association with RecO, RecF and/or DNA. A better understanding of the processes regulating complex formation between these partners will certainly help in establishing the detailed sequence of events leading to the assembly of RecA onto ssDNA. The three-dimensional structures of the individual proteins have served as solid frameworks to identify residues or regions potentially involved in either protein-protein or protein-DNA interactions. However, structural and biochemical studies of the protein complexes, such as that of drRecOR, will now provide us with more valuable information regarding the functions of these proteins *in vivo*.

Materials and methods

Expression, purification and crystallisation of drRecOR

drRecO (SwissProt: Q9RW50) and drRecR (SwissProt: Q9ZNA2) were cloned and the drRecOR complex was expressed and purified as described previously (Leiros *et al*, 2005). Crystals of the drRecOR complex were obtained using the hanging drop method by mixing 1 μ l of protein at 12 mg/ml with 1 μ l of reservoir solution (0.1 M MES (pH 6.5), 10% dioxane, 1.6 M ammonium sulphate). The crystals were subsequently cryoprotected in a solution of the reservoir solution with 30% (v/v) glycerol added and flash-cooled at 100 K.

Data collection, structure determination and refinement

X-ray intensity data was collected on ID14-2 at the ESRF. The crystal used for data collection belonged to the monoclinic space group C2 and had unit cell dimensions of $a = 144.0$ Å, $b = 83.2$ Å, $c = 66.6$ Å, $\beta = 106.9^\circ$. Diffraction was observed to a maximum resolution of about 3.8 Å. MR searches were performed using PHASER (McCoy

Figure 6 Model for RecOR recognition of stalled replication forks. Whereas the role of RecF in this process is still unclear, it is known to associate with DNA in an ATP-dependent fashion. Upon binding of RecOR to ssDNA-dsDNA junctions (step 2), we propose that interactions with RecF, SSB and/or DNA may cause a structural rearrangement of RecOR (e.g. one RecO and two RecR molecules may dissociate from the RecOR complex). RecF-dependent ATP hydrolysis may provide the necessary energy for this reorganisation (step 3) resulting in the formation of a stable complex between RecOR and the stalled replication fork (step 4). As a consequence, the assembled RecOR complex may initiate the displacement of SSB and thus facilitate the loading of RecA onto ssDNA, allowing for homologous recombination to take place (step 5).

et al, 2005), using one monomer of drRecR (pdb1VDD) and one monomer of drRecO (pdb1W3S) as search models (but searching for two monomers of drRecR and one monomer of drRecO, according to the expected 2:1 ratio of drRecR:drRecO). PHASER identified one unique solution, having a log likelihood gain of 160 and no symmetry-generated clashes, indicative of a correct MR result (see Supplementary data for further details).

With a limited observation-to-parameter-ratio, refinement was terminated after a single round of rigid-body refinement ($R_{\text{work}}/R_{\text{free}}$ of 45.9%/44.3%, respectively) and subsequent manual rebuilding. With the individual drRecO and drRecR crystal structures being previously determined to much higher resolution, we believe that the crystal structure of the drRecOR complex provides useful information, in particular as protein-protein interactions identified from the structure could be validated through mutational analysis. Atomic coordinates and structure factors have been deposited in the Protein Data Bank with accession numbers 2V1C and r2V1Cs respectively.

Cloning, expression and purification of mutant drRecOR complexes

Single and multiple point mutants of drRecO and drRecR were prepared using the QuikChange-single- and QuikChange-multi-site-directed

mutagenesis kits (Stratagene). The mutants were confirmed by DNA sequencing (EMBL GeneCore Facility) and were expressed in BL21 Star (DE3) pLysS cells. For all the biochemical characterisation studies, drRecO, drRecR and wild-type and mutant drRecOR complexes were purified on 2 ml Ni-loaded chelating sepharose gravity-flow columns (Amersham Biosciences), pre-equilibrated in Buffer A (50 mM Tris-HCl (pH 7.5), 100 mM NaCl and 5 mM β -mercaptoethanol). After a high salt wash (buffer A with 500 mM NaCl) to remove nucleic acids and a wash with buffer A containing 50 mM imidazole, the His-tagged proteins were eluted with buffer A supplemented with 250 mM imidazole. The protein was then dialysed overnight at 4°C into buffer A and further purified on a MonoQ column (Amersham Biosciences). If multiple forms of the protein were observed, the fractions from the major peak were pooled and further purified by size-exclusion chromatography before use in further assays. Wild-type and mutant drRecOR complexes used for the DNA-binding studies were treated with Proteinase K at a 1:4000 ratio at room temperature for 1 h and the digestion pattern was analysed by SDS-PAGE.

Size-exclusion chromatography

Wild-type and mutant drRecOR complexes were subjected to size-exclusion chromatography on a Superdex 200 column equilibrated in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl and 5 mM β -mercaptoethanol. In all cases, 0.5 ml of protein obtained from the MonoQ purification step was injected onto the column and the purification was run at 0.5 ml/min at room temperature. The peak fractions were analysed by SDS-PAGE, followed by Western blotting using a semi-dry system (Biorad). Polyclonal antibodies were raised against drRecO and drRecR (Centre Lago, France) and were used for Western blotting at a 1:25 000 dilution in 5% milk in tris-buffered saline. An anti-rabbit antibody conjugated to alkaline phosphatase (Promega) was used as secondary antibody.

DNA binding assays

DNA binding was performed in 10 μ l reactions. A 0.2 μ g portion of supercoiled or linearised plasmid DNA (pcDNA3.1; 6.4 kbp) was mixed with protein buffer A, 5 μ g bovine serum albumin (BSA; 66 kDa), 5 μ g drRecO (29 kDa), 5 μ g drRecR (26 kDa), 5 μ g wild-type drRecOR (162 kDa) or 5 μ g mutant drRecOR complexes. The reactions were prepared both in the presence and absence of 40 mM MgCl₂. Tris-HCl (0.1 M, pH 7.5) was added to all reactions to ensure that all samples were analysed at the same pH. The following oligonucleotides were used for the binding assays: (a) ssDNA, 5'-GGG GAC TTT GTA CAA GAA AGC TGG GTC TCA GTG ATG GTG ATG GTG GTG -3', (b) dsDNA, 5'-TCT AAT GCG AGC ACT GCT ATT CCC TAG CAG TGC TCG CAT TAG A-3', (c) 3'-OH1, 5'-TCT AAT GCG AGC ACT GCT ATT CCC TAG CAG TGC TCG CAT TAG ATT TTT TT-3', (d) 3'-OH2, 5'-TCT AAT GCG AGC ACT GCT ATT CCC TAG CAG TGC TCG CAT TAG ATT TTT TTT TTT TTT T-3' and (e) 5'-OH2, 5'-TTT TTT TTT TTT TTT TCT AAT GCG

AGC ACT GCT ATT CCC TAG CAG TGC TCG CAT TAG A-3'. Before use, the oligonucleotides were heated to 95°C for 10 min and placed immediately on ice. A 5 μ M portion of each oligonucleotide was mixed with drRecO, drRecR and drRecOR at various protein:DNA molar ratios (0.5:1, 1:1 and 2:1, respectively), and a single ratio (i.e. 2:1) was used for the binding to mutant drRecOR complexes. All reactions were incubated at 25°C for 1 h. One microlitre of glycerol was then added to each reaction before separation on a 0.7% agarose gel in 0.5 $\times$ TBE (50 mM Tris-borate, 0.5 mM EDTA) containing either SYBR safe DNA stain (Invitrogen) or ethidium bromide. The agarose gels were stained with Coomassie blue to visualise the protein bands.

Chemical crosslinking

Purified drRecR and drRecOR proteins were dialysed into 50 mM Hepes (pH 7.5), 100 mM NaCl and 5 mM β -mercaptoethanol. Portions (0, 0.4 and 1 mM) of BS³ crosslinker (Pierce) were added to 10 μ g of dialysed protein and incubated at 25°C for 30 min. The reaction was quenched by addition of 0.1 M Tris (pH 7.5) and the samples were analysed by SDS-PAGE and Western blotting, using anti-drRecO and anti-drRecR diluted 1:25 000 in 5% milk. The bands were revealed using a second anti-rabbit antibody conjugated to alkaline phosphatase and a colorimetric substrate (Promega).

Modelling of the Complex between drRecOR and DNA

The DNA used to create this model was extracted from the crystal structure of RecBCD in complex with blunt-ended dsDNA (pdb1W36) (Singleton *et al*, 2004). As the interaction with RecBCD caused this dsDNA to open at one end, a 3'-overhang mimicking the proposed binding site of RecFOR (Morimatsu and Kowalczykowski, 2003), could be extracted and modelled into the drRecOR complex. No alterations to the DNA backbone were necessary to obtain a good fit with the surface of the drRecOR complex or to allow for residues in drRecO and drRecR to make favourable interactions with the DNA.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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