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Phosphorylation of *Deinococcus radiodurans* RecA Regulates Its Activity and May Contribute to Radioresistance*[S]

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Deinococcus radiodurans **has a remarkable capacity to survive exposure to extreme levels of radiation that cause hundreds of DNA double strand breaks (DSBs). DSB repair in this bacterium depends on its recombinase A protein (DrRecA). DrRecA plays a pivotal role in both extended synthesis-dependent strand annealing and slow crossover events of DSB repair during the organism's recovery from DNA damage. The mechanisms that control DrRecA activity during the *D. radiodurans* response to γ radiation exposure are unknown. Here, we show that DrRecA undergoes phosphorylation at Tyr-77 and Thr-318 by a DNA damage-responsive serine threonine/tyrosine protein kinase (RqkA). Phosphorylation modifies the activity of DrRecA in several ways, including increasing its affinity for dsDNA and its preference for dATP over ATP. Strand exchange reactions catalyzed by phosphorylated *versus* unphosphorylated DrRecA also differ. *In silico* analysis of DrRecA structure support the idea that phosphorylation can modulate crucial functions of this protein. Collectively, our findings suggest that phosphorylation of DrRecA enables the recombinase to selectively use abundant dsDNA substrate present during post-irradiation recovery for efficient DSB repair, thereby promoting the extraordinary radioresistance of *D. radiodurans*.**

Deinococcus radiodurans has a remarkable capacity to survive extreme doses of radiations and other DNA-damaging agents. Studies aimed at unraveling the molecular bases for these unusual properties have revealed that *D. radiodurans* encodes mechanisms for highly efficient DNA double strand break (DSB)[2] repair and oxidative stress management (1–3). DSB repair in this Gram-positive bacterium is accomplished in two phases during post-irradiation recovery (PIR); phase I is dominated by extended synthesis-dependent strand annealing (ESDSA) processes, whereas phase II involves slow crossover events in homologous recombination leading to the repair and re-establishment of the multipartite *D. radiodurans* genome structure (4). Despite the fact that the two phases of PIR have DNA substrates of different structures and topologies, *D. radiodurans* RecA (DrRecA) is required throughout DSB repair during PIR (5). Biochemical characterization of recombinant DrRecA revealed that it can form a filament on single-stranded DNA (ssDNA), exhibit co-protease activity, and utilize ATP or dATP for its energy requirements, akin to other bacterial RecA proteins (6), but it also has unusual properties. In contrast to most bacterial RecA proteins, DrRecA promotes inverse strand exchange reactions (7). Also, DrRecA promotes DNA degradation during the early phase of ESDSA repair (5), which is opposite to the function observed with *Escherichia coli* RecA. Transcription of DrRecA is induced in response to γ radiation (8, 9). However, the mechanisms by which γ radiation induces DrRecA expression are unusual. Inactivation of both *D. radiodurans lexA* genes does not attenuate γ radiation induction of DrRecA expression (10, 11). Thus, in contrast to many bacteria, LexA and the widespread DNA damage-induced SOS response do not control *recA* expression in *D. radiodurans*. Two *D. radiodurans* regulators, PprI and DrRRA, are positive regulators of DrRecA expression (12, 13), but additional controls of DrRecA expression and activity are likely.

In eukaryotes, different mechanisms control recombination. For example, the activity of Rad51, the yeast RecA homologue involved in DSB repair through homologous recombination, is regulated by phoshorylation. Both Rad51 and eukaryotic single strand-binding protein (SSB) are phosphorylated by DNA damage-responsive protein kinases (14, 15). Rad51 phosphorylation by Mec1, an ATR homologue in *Saccharomyces cerevisiae*, regulates its functions *in vitro* and *in vivo*. Although involvement of Ser/Thr/Tyr phosphorylation of DNA repair proteins in DNA repair and cell cycle regulation is widespread in eukaryotes, it is unusual in bacteria. In *Bacillus subtilis*, the tyrosine phosphorylation of SSB enhances its affinity for ssDNA (16), and phosphorylation of *B. subtilis* recombinase by a DNA damage-inducible serine/threonine protein kinase was recently reported (17). We characterized RqkA, a eukaryotic type DNA damage-responsive Ser/Thr protein kinase (eSTPK) in *D. radiodurans* and demonstrated its involvement in γ radiation resistance and DSB repair (18). RqkA phosphorylates PprA, a *D. radiodurans* pleiotropic protein involved in DNA repair. PprA phosphorylation modifies its *in vitro* functions and is required for its role in *D. radiodurans* radioresistance (19).

* The authors declare that they have no conflicts of interest with the contents of this article.

[S] This article contains supplemental Figs. 1–4.

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[2] The abbreviations used are: DSB, double strand break; PIR, post-irradiation recovery; eSTPK, eukaryotic type DNA damage-responsive Ser/Thr protein kinase; ESDSA, extended synthesis-dependent strand annealing; ssDNA, single-stranded DNA; SSB, single strand-binding protein; SER, strand exchange reaction(s); P-, phospho-; ATPγS, adenosine 5′-*O*-(thiotriphosphate); IPTG, isopropyl β-D-1-thiogalactopyranoside.

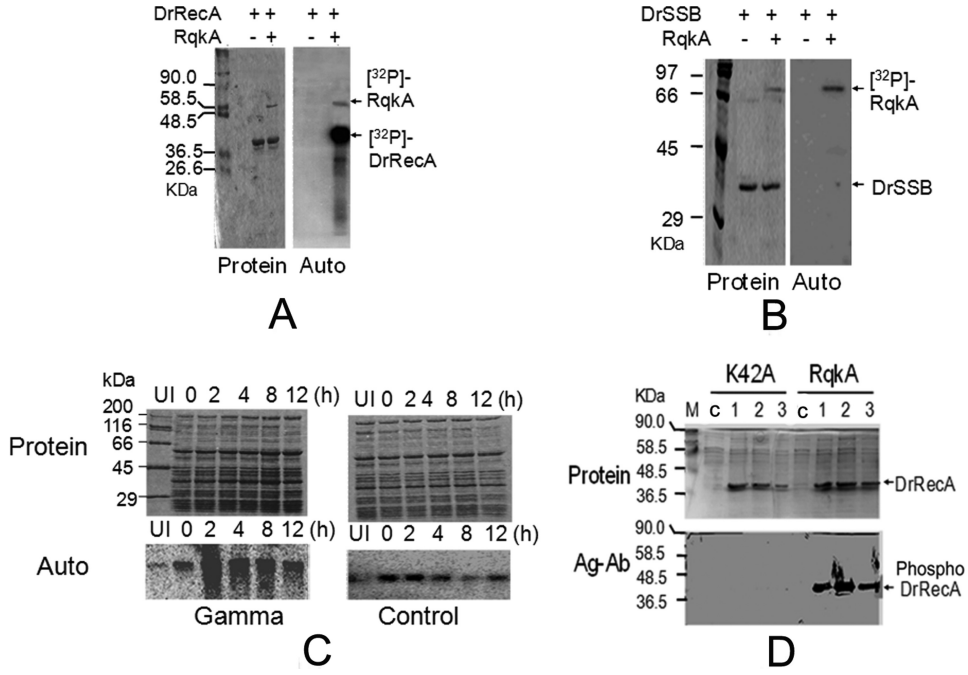

FIGURE 1. **DrRecA phosphorylation by RqkA kinase.** Purified DrRecA (*Protein*) was incubated with purified RqkA in the presence of [γ-^{32}P]ATP, and phosphorylation of RqkA ([^{32}P]RqkA) and DrRecA ([^{32}P]-DrRecA) (*Auto*) was detected (*A*), whereas DrSSB incubated under similar conditions did not become phosphorylated (*B*). DrRecA phosphorylation during post-irradiation recovery (*Gamma*) in *D. radiodurans* was monitored in cell-free extract (*Protein*) by immunoprecipitation using RecA antibodies followed by autoradiography (*Auto*) and compared with untreated cells processed identically (*Control*) (*C*). Three independent samples (*1–3*) of *E. coli* cells co-expressing DrRecA with RqkA or its null mutant K42A as well as cells expressing kinase without DrRecA (*C*) were taken (*Protein*) for monitoring DrRecA phosphorylation by RqkA *in vivo* by immunoblotting using phosphothreonine antibody (*Ag-Ab*) (*D*). Data presented are representative of reproducible experiments repeated three times. *UI,* unirradiated.

Mechanisms underlying the regulation of DrRecA functions during ESDSA and classical homologous recombination have not been described but would deepen our understanding of the molecular bases of *D. radiodurans* radioresistance. Here, we report that DrRecA is a phosphoprotein. Phosphoacceptor sites on DrRecA were identified as tyrosine 77 and threonine 318. DrRecA is phosphorylated by the RqkA kinase, and phosphorylation increases its preference for dATP and dsDNA, thereby enhancing DNA strand exchange reactions. Y77F and T318A single mutants, even after phosphorylation by RqkA, lose their preference for dATP and dsDNA. A DrRecA Y77F/T318A double mutant does not become phosphorylated, and its capacity to complement the radiation-sensitive *recA* mutant in *D. radiodurans* was highly impaired, suggesting that RecA phosphorylation may play a role in the radioresistance of this bacterium. Structural comparisons of DrRecA with homologues from other bacteria are consistent with the idea that phosphorylation of Thr-318 and Tyr-77 could modify DrRecA activity. Collectively, our findings suggest that DrRecA phosphorylation by a DNA damage-responsive protein kinase enhances its recombinogenic activity for substrates that are likely to be abundant following irradiation and thereby promotes *D. radiodurans* radioresistance.

Results

DrRecA Is Phosphorylated by RqkA Kinase—We previously found that RqkA, a γ radiation-responsive eSTPK of *D. radiodurans*, contributes to the organism's resistance to γ radiation and DSB repair (18). Among the few *D. radiodurans* proteins RqkA was found to phosphorylate *in vitro* was PprA, a pleiotropic protein involved in DNA repair. PprA phosphorylation modulates its function *in vitro* and *in vivo* (19). Proteome-wide searches for potential RqkA phosphorylation targets revealed that DrRecA contains a putative phosphorylation motif (VNTDELLV) for this eSTPK (19, 20). This prompted us to check the phosphorylation of DrRecA with RqkA kinase. Using [γ-^{32}P]ATP and purified recombinant proteins, we observed that DrRecA was phosphorylated in solution by RqkA but not in a corresponding control reaction lacking this kinase (Fig. 1*A*). The specificity of RqkA phosphorylation is supported by the absence of RqkA phosphorylation of purified deinococcal SSB, which lacks the eSTPK phosphorylation motif (Fig. 1*B*).

We also tested whether γ irradiation of *D. radiodurans* altered the amount of DrRecA phosphorylation *in vivo*. As observed previously (8, 9), exposure to γ radiation led to an increase in total DrRecA. Changes in DrRecA phosphorylation were also observed during *D. radiodurans* recovery from irradiation (Fig. 1*C*), suggesting that γ radiation induced DrRecA phosphorylation. Because *D. radiodurans* contains a large number of Ser/Thr protein kinases and these eSTPKs can phosphorylate proteins in a promiscuous fashion, the specificity of RqkA phosphorylation of DrRecA was also tested in a surrogate host, *E. coli* co-expressing wild type RqkA and DrRecA. A kinase-deficient RqkA mutant K42A and DrRecA were also co-expressed in *E. coli* as a negative control for these reactions (Fig. 1*D*). A protein corresponding to phosphorylated DrRecA was only detected in cells co-expressing wild type RqkA kinase and not the K42A mutant, although DrRecA is present in both of the strains. Interestingly, expression of RqkA in *E. coli* did not

TABLE 1

Effect of phosphorylation on ssDNA and dsDNA affinity of DrRecA and its derivatives

The K_d values of were determined for non-phosphorylated and phosphorylated forms of wild type, Y77F, T318A, and Y77F/T318A mutants, as shown in Fig. 9.

Derivatives of DrRecA	K_d values for dsDNA		K_d values for ssDNA	
	Non-phosphorylated	Phosphorylated	Non-phosphorylated	Phosphorylated
	μM		μM	
Wild type	0. 90 ± 0.12	0.21 ± 0.02	0.52 ± 0.11	0.10 ± 0.01
Y77F	4.48 ± 0.23	0.84 ± 0.10	1.55 ± 0.17	0.28 ± 0.02
T318A	1.97 ± 0.20	3.31 ± 0.47	0.37 ± 0.05	0.44 ± 0.05
Y77FT318A	5.49 ± 0.68	5.52 ± 0.63	4.37 ± 0.73	4.03 ± 0.52

appear to phosphorylate endogenous proteins, at least not at motifs that could be detected by anti-phosphothreonine epitope antibody. Together, these results suggest that DrRecA is a phosphoprotein in *D. radiodurans* and that RqkA kinase phosphorylates DrRecA both *in vitro* and *in vivo*, at least in the surrogate host *E. coli*.

DrRecA Phosphorylation Affects Its Binding to ssDNA and dsDNA Substrates—RecA proteins are known for their differential binding affinity for ssDNA *versus* dsDNA substrates. Here we purified both the unphosphorylated form (DrRecA) and phosphorylated form (P-DrRecA) of DrRecA from *E. coli* co-expressing DrRecA with RqkA kinase (supplemental Fig. S1). The effect of phosphorylation on DNA binding activity of DrRecA was monitored for DrRecA and P-DrRecA (Table 1). Phosphorylation increased the affinity of DrRecA for both ssDNA (DrRecA *versus* P-DrRecA, K_d 0.52 ± 0.11 and 0.10 ± 0.01 μM, respectively) and dsDNA (K_d = 0.90 ± 0.12 and 0.21 ± 0.02 μM) (Fig. 2, *A* and *B*). The increased affinity of P-DrRecA for both ssDNA and dsDNA suggests that phosphorylation enhances the affinity of DrRecA for DNA substrates. *E. coli* RecA binds ssDNA with greater affinity than it does dsDNA, and this differential substrate binding modulates the different steps of strand exchange reactions (21). Therefore, the effects of DrRecA phosphorylation on its preferential binding between dsDNA and ssDNA were monitored by competition assay. We observed that when protein bound first to dsDNA and then competed with ssDNA, the log equilibrium dissociation constants (log K_i) were 6.52 ± 0.89 and 2.32 ± 0.14 for P-DrRecA and DrRecA, respectively (Fig. 3*A*). Similarly, when dsDNA binding was challenged with higher concentration of similar unlabeled dsDNA, the log K_i values were 10.0 ± 0.40 and 1.36 ± 0.58 for P-DrRecA and DrRecA, respectively (Fig. 3*B*). On the other hand, the phosphorylation of DrRecA did not affect the competition of ssDNA binding with ssDNA, and the log K_i values were 1.60 ± 0.13 and 1.46 ± 0.16 for P-DrRecA and DrRecA, respectively (Fig. 3*C*). However, when ssDNA binding was challenged with increasing concentration of dsDNA, a new DNA species was generated, which migrated more slowly than the ssDNA substrate but faster than the ssDNA-protein complex (Fig. 3*D*). Although the identity of this product is not known, the possibility of this being a recombination product between labeled ssDNA and cold dsDNA cannot be ruled out. However, we noted that the lowest concentration of dsDNA required for the appearance of this product (*P** in Fig. 3*D*) in the case of DrRecA was nearly 8-fold lower (2.5 μM) than P-DrRecA (~20 μM). These results suggested that DrRecA phosphorylation improves its binding with both ssDNA and dsDNA.

P-DrRecA Prefers dATP in Strand Exchange Reaction and dATP Inhibits ATP Hydrolysis—DrRecA promotes DNA strand exchange reactions (SER) in the presence of both ATP and dATP, and it hydrolyzes dATP more efficiently than ATP but prefers ATP for its SER activity (6). Therefore, the effect of DrRecA phosphorylation on its preference for dATP *versus* ATP in SER was monitored. Interestingly, in comparison with DrRecA, P-DrRecA showed ~2-fold lower efficiency in SER activity in the presence of ATP (Fig. 4*A*) but ~2.5-fold higher activity in the presence of dATP (Fig. 4*B*). These results suggest that phosphorylation could alter the preference of DrRecA for ATP *versus* dATP during SER. When the effect of phosphorylation on hydrolysis of these nucleotide triphosphates was monitored, only minimal differences in the dATPase and ATPase activities of P-DrRecA *versus* DrRecA were detected, indicating that both forms of the protein can hydrolyze both nucleotides (Fig. 5*A*). dATP inhibition of ATP hydrolysis was observed in both forms of DrRecA and was relatively greater with P-DrRecA. As a result, the amount of dATP required for 50% inhibition of ATPase activity was 10-fold less in the case of P-DrRecA (1 mM) *versus* DrRecA (10 mM) (Fig. 5*B*). Reduction of ATPase activity in the presence of dATP could be explained either by inactivation of enzyme or by reduced ATP binding to the enzyme. Collectively, these results suggested that phosphorylation of DrRecA increases its preference for dATP over ATP in SER, perhaps by reducing ATP hydrolysis.

RqkA Phosphorylates DrRecA Predominantly at Tyr-77 and Thr-318—DrRecA was phosphorylated by RqkA *in vitro*, and the phosphorylation sites were identified by mass spectrometry. DrRecA, incubated with RqkA in the presence of ATP, showed multiple sites of phosphorylation. Mutational studies confirmed the contribution of only five sites, Ser-19, Ser-27, Tyr-77, Thr-254, and Thr-318 residues (supplemental Fig. S2), in the total phosphorylation of DrRecA. However, none of these sites were the one that was predicted theoretically. Given the promiscuity of phosphorylation by Ser/Thr protein kinases *in vitro*, the specificity of RqkA phosphorylation of DrRecA and its functional significance were further established by creating substitution mutants, where the phosphorylation sites in DrRecA were replaced with alanine or phenylalanine residues, yielding S19A, S27A, Y77F, T254A, and T318A DrRecA mutants. Unlike the wild type and the S19A, S27A, and T254A mutants, the levels of phosphorylation in Y77F and T318A proteins were significantly reduced. Furthermore, RqkA kinase failed to transfer γ-^{32}P from [γ-^{32}P]ATP to Y77A/T318A double mutant (Fig. 6*A*). Thus, Tyr-77 and Thr-318 appear to be the two major RqkA phosphorylation sites in DrRecA. There-

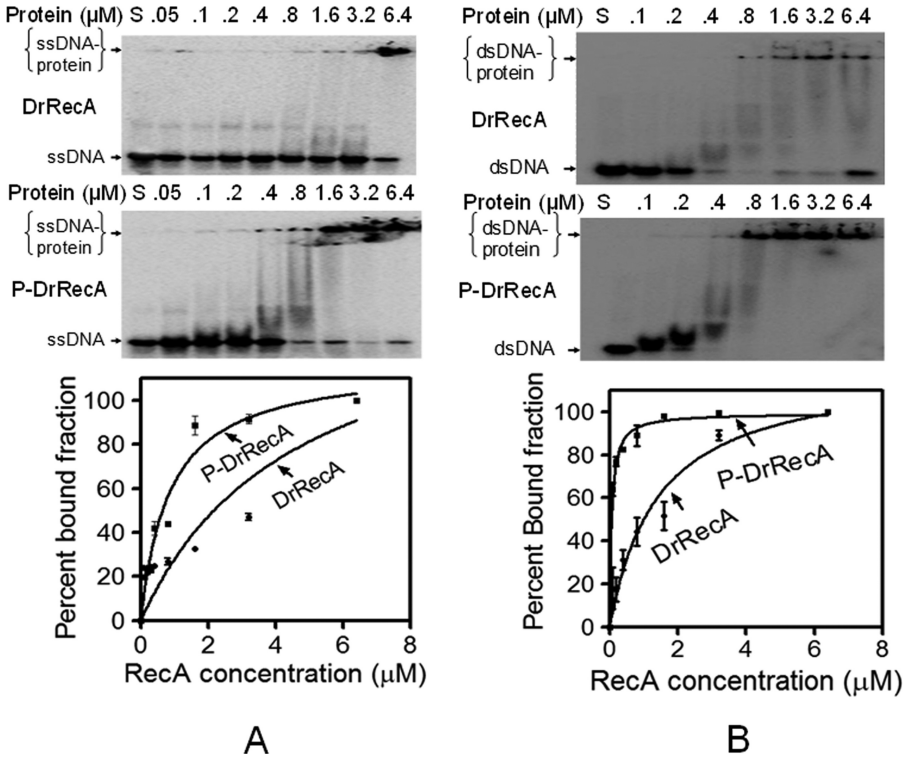

FIGURE 2. **Effect of phosphorylation on DNA binding activity of DrRecA.** Different concentrations of DrRecA purified from *E. coli* expressing wild type RqkA (*P-DrRecA*) and null mutant (*DrRecA*) were incubated with ^{32}P-labeled ssDNA (*A*) and dsDNA (*B*). Mixtures were separated on native PAGE as described under "Experimental Procedures," and autoradiograms were developed. Data from a reproducible representative experiment are shown (*top*). Both free DNA and DNA bound to protein were quantified densitometrically from three independent experiments and validated statistically. The percentage of bound fractions was calculated and plotted as a function of protein concentrations and analyzed using GraphPad Prism version 6 (*bottom graphs*). *Error bars*, S.E.

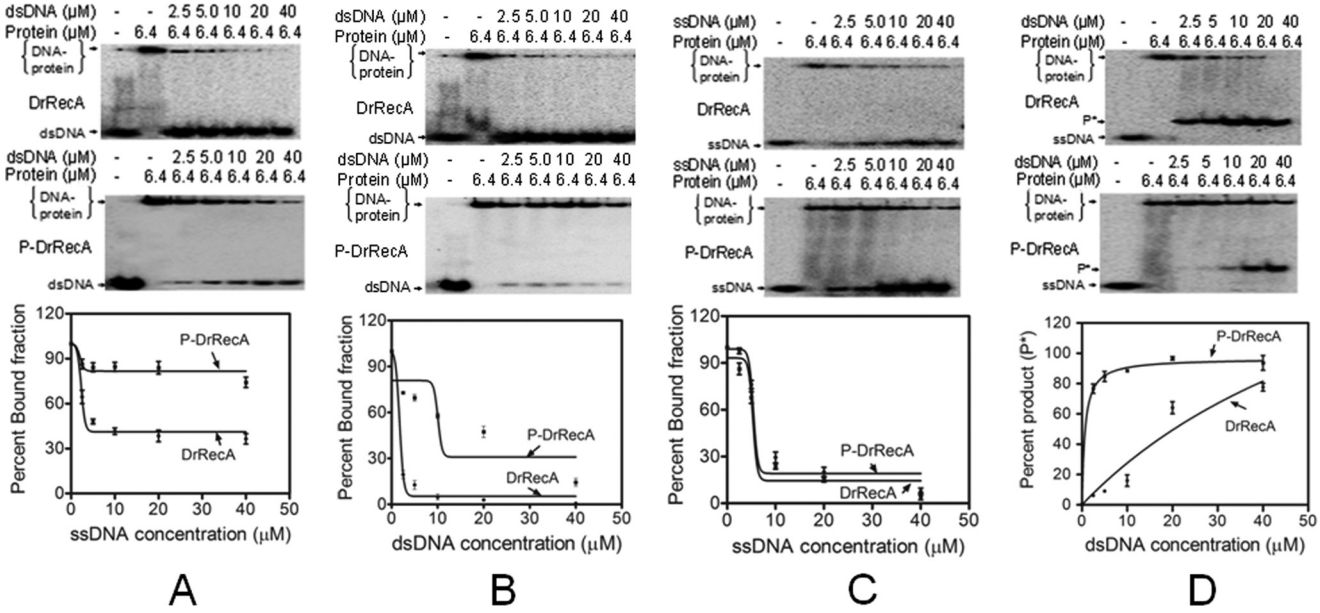

FIGURE 3. **Effect of phosphorylation on DNA binding preference between ssDNA and dsDNA substrates.** P-DrRecA and DrRecA were purified, and a ~6.4 μM concentration of each was first incubated with ^{32}P-labeled dsDNA (*A* and *B*) and ssDNA (*C* and *D*). Protein bound to these substrates was chased with increasing concentrations of unlabeled ssDNA (*A* and *C*) and dsDNA (*B* and *D*). Mixtures were analyzed on native PAGE, and autoradiograms from a reproducible representative experiment are shown (*top panels*). Both free DNA and DNA bound to protein were quantified densitometrically from three independent experiments, and the percentage of bound fractions was calculated as described under "Experimental Procedures." Results were plotted as function of unlabeled DNA concentration and analyzed using GraphPad Prism (*bottom graphs*). *Error bars*, S.E.

fore, Tyr-77 and Thr-318 were also replaced with glutamic acid, yielding Y77E and T318E.

In vivo phosphorylation of these mutants was confirmed using a surrogate *E. coli* host, and the results were very similar to the *in vitro* findings. When expressed in *E. coli* along with RqkA, the T318A and Y77A single mutants showed reduced phosphorylation compared with the other single mutants, and the Y77A/T318A double mutant did not become phosphorylated

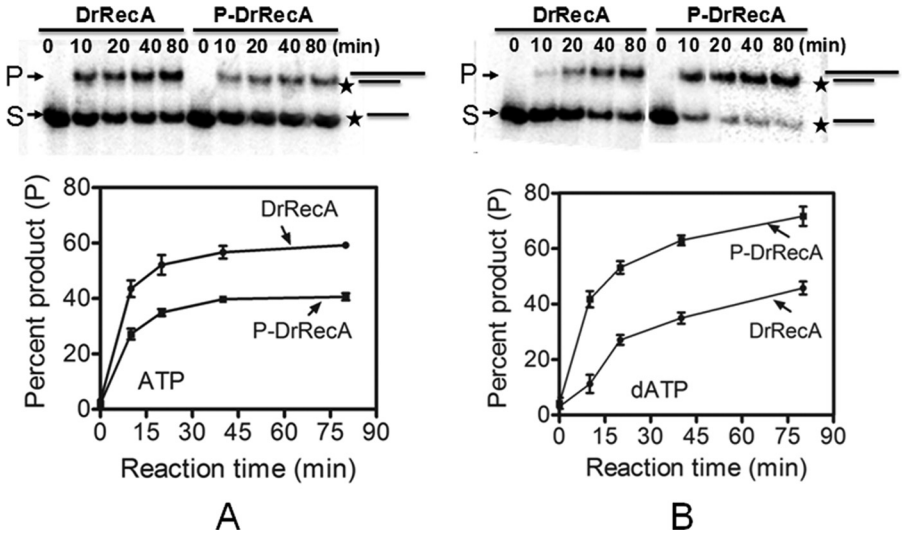

FIGURE 4. **Effect of phoshorylation on DNA strand exchange activity of DrRecA.** Purified P-DrRecA and DrRecA (~6.4 μM) were used for a DNA strand exchange activity assay in the presence of ATP (*A*) and dATP (*B*) using standard protocols as described under "Experimental Procedures." Reaction mixtures were separated on a 10% polyacrylamide gel, and autoradiograms were developed. Data shown are representative of reproducible experiment repeated three times (*top panels*). The amounts of strand exchange reaction products (*P*) were estimated densitometrically and converted into percentages of total labeled substrate (*S*) used in the respective experiments. Data were plotted, and results were analyzed using GraphPad Prism (*bottom graphs*). *Error bars*, S.E.

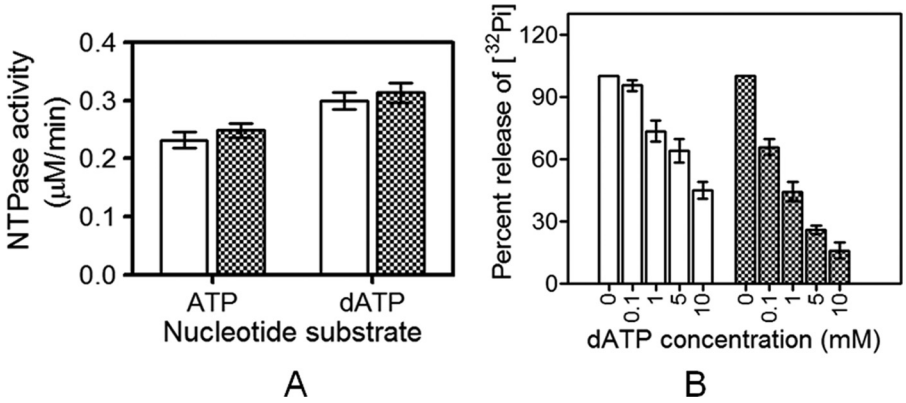

FIGURE 5. **Protein phosphorylation effect on NTPase activity regulation in DrRecA.** DrRecA (*open bars*) and P-DrRecA (*filled bars*) were incubated for different time intervals in the presence of ATP and dATP, and the release of inorganic phosphate was measured. Using these data, specific NTPase activity was determined (*A*). Similarly, the ATPase activity was measured using [γ-^{32}P]ATP at varying concentrations (0.1, 1, 5, and 10 mM) of dATP. Release of $^{32}P_i$ was monitored in a liquid scintillation counter. The percentage release of P_i was calculated against control without dATP (*B*). *Error bars*, S.E.

(Fig. 6*B*). The reduced cross-reactivity with anti-phosphothreonine antibodies in the Y77F mutant suggests that the Y77F mutation possibly affects the phosphorylation of Thr-318 or some other threonine. These observations provided strong support for the possibility that DrRecA residues Tyr-77 and Thr-318 are phosphorylated by RqkA kinase and that the double mutant provides a non-phosphorylatable derivative of DrRecA.

DrRecA Tyr-77 Is Involved in Determining dATP/ATP Preference—Because P-DrRecA has greater preference for dATP *versus* ATP, and dATP could inhibit the ATPase activity of DrRecA, the effect of dATP on the ATPase activities of the phosphoacceptor site mutants was compared with the effect on the wild type. In the absence of dATP, all of the mutants tested showed reduced ATPase activity compared with the wild type (Fig. 7*A*). However, in the presence of dATP, the mutants exhibited different degrees of ATPase inhibition (Fig. 7*B*). After incubation with RqkA, the T318A mutant (referred to as P-T318A) had dATP inhibition of ATPase activity similar to that of P-DrRecA. On the other hand, after the Y77F and Y77F/T318A mutants were incubated with RqkA kinase (referred as P-Y77F and P-Y77F/T318A, respectively), they had ATPase activity as well as dATP inhibition similar to that of DrRecA (compare Figs. 7*B* and 5*B*). These observations suggest that Tyr-77 phosphorylation, which would occur in the T318A mutant and wild type DrRecA proteins, modulates ATP hydrolysis in the presence of dATP, possibly through competition at the binding site.

Thr-318 in DrRecA Is Involved in DNA Binding Activity—DNA binding activity of Y77F, Y77E, T318A, T318E, and Y77F/T318A were monitored with both ssDNA and dsDNA substrates. Interestingly, all of the mutants except T318E showed reduced DNA binding activity as compared with DrRecA (Fig. 8). For instance, the DNA binding activity of Y77F and Y77E was nearly similar, whereas T318A showed lesser binding to dsDNA as compared with T318E. The ssDNA binding activity of all of the mutants was less than that of wild type controls. Further, the affinities of Y77F, T318A, and Y77F/T318A mutants with both types of DNA substrates were monitored

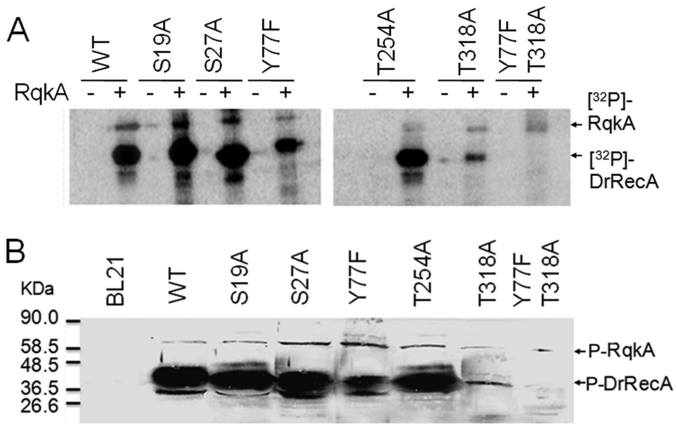

FIGURE 6. **RqkA phosphorylation to different putative phosphosite mutants of DrRecA.** Five phosphosites were detected in DrRecA incubated with RqkA *in vitro*. These were mutagenized, and S19A, S27A, Y77F, T254A, and T318A single and Y77F/T318A double mutants were generated. Recombinant proteins were purified and incubated with RqkA in the presence of [γ-^{32}P]ATP. Mixtures were separated on SDS-PAGE, and autoradiograms were developed (*A*). Similarly, these proteins were expressed in *E. coli* along with wild type RqkA on plasmids, and total proteins were separated on SDS-PAGE. Blots were probed with antiphosphothreonine epitope antibodies, and signals were developed colorimetrically (*B*). Data shown are representative of reproducible experiments repeated two times.

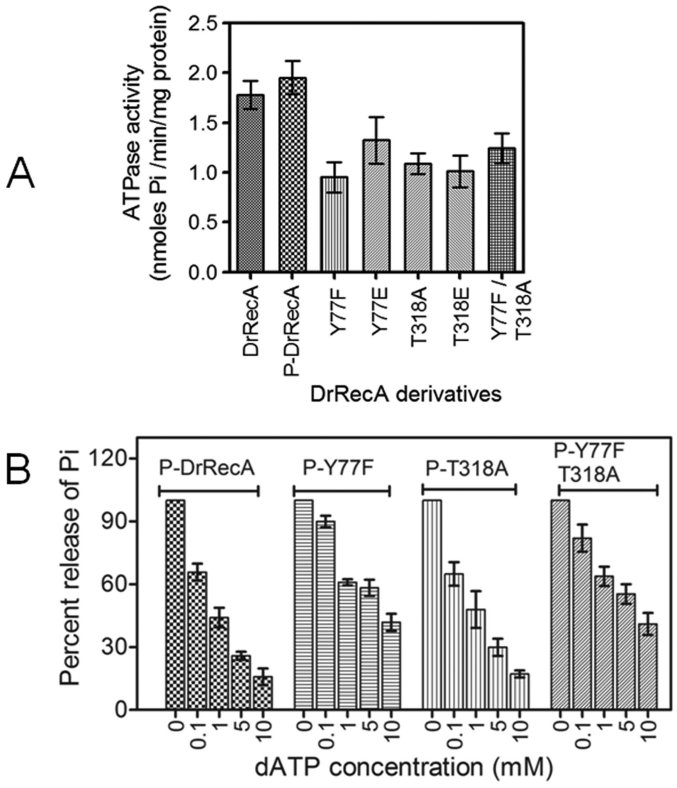

FIGURE 7. **ATPase activity of phosphosite mutants purified from RqkA-expressing *E. coli* cells.** Recombinant DrRecA, P-DrRecA, Y77F, Y77E, P-Y77F, T318A, T318E, P-T318A, Y77F/T318A, and P-Y77F/T318A proteins were made by co-expressing them with RqkA, and ATP hydrolysis was measured (*A*). P-DrRecA, P-Y77F, P-T318A, and P-Y77F/T318A were incubated with [γ-^{32}P]ATP in the presence of increasing concentrations of dATP, and release of $^{32}P_i$ from [γ-^{32}P]ATP was measured by liquid scintillation counting (*B*). *Error bars*, S.E.

(Table 1). The affinity of P-Y77F for ssDNA (K_d = 0.28 ± 0.02 μM) and dsDNA (K_d = 0.84 ± 0.12 μM) was ~6.0 and ~5.0 times higher than that of Y77F with corresponding K_d values of 1.55 ± 0.17 and 4.48 ± 0.23 μM, respectively (Fig. 9). However, T318A phosphorylation did not change the DNA binding affinity of this mutant. The K_d values for ssDNA and dsDNA in the case of P-T318A were 0.44 ± 0.05 and 3.31 ± 0.47 μM, whereas the K_d values for ssDNA and dsDNA for T318A protein were 0.37 ± 0.05 and 1.97 ± 0.20 μM, respectively (Fig. 9). Both ssDNA and dsDNA binding affinity of Y77F/T318A mutant decreased more than 10-fold, which did not change when this protein was incubated with RqkA kinase (Table 1). These results suggested that besides a major role of Thr-318 and its phosphorylation, Tyr-77 also contributes to the DNA binding activity of DrRecA.

Tyr-77 and Thr-318 Contribute to DrRecA SER Activity in the Presence of dATP—The magnitude of ATPase activity inhibition by dATP on the strand exchange activity of DrRecA in the phosphoacceptor mutants was compared with the wild type. The SER activity of all three mutants was significantly less than that of the wild type protein regardless of its phosphorylation state and the presence of ATP or dATP (Fig 10). However, upon phosphorylation, both P-DrRecA and P-T318A proteins promoted SER with ~2-fold higher efficiency in the presence of dATP as compared with ATP (Fig. 10, *B* and *D*). The SER activity of P-Y77F and P-Y77F/T318A proteins was lower than that of P-DrRecA in the presence of dATP but higher than the corresponding protein activity in the presence of ATP. Because Tyr-77 phosphorylation would be expected to be the same in wild type and T318A mutant and the phosphorylated forms of these proteins show nearly 2.5-fold higher SER activity in the presence of dATP, the involvement of Tyr-77 in discrimination between dATP and ATP and thus inhibition of ATPase activity and enhanced SER activity in the presence of dATP could be suggested. These findings suggest that Tyr-77 phosphorylation seems to be responsible for determining the nucleotide specificity and regulating the ATPase role of DrRecA in its strand exchange activity.

Potential Structural Changes Induced by Phosphorylation of Tyr-77 and Thr-318 May Explain Phosphorylation Effects on DrRecA Activity—The locations and environments of the DrRecA Tyr-77 and Thr-318 amino acids in the protein's crystal structure (Protein Data Bank entry 1XP8) were analyzed to further understand how phosphorylation of these residues might explain changes in the protein's SER activity. Tyr-77 is located at the end of the β-strand (β1) just before the P-loop (Fig. 11*A*). The P-loop, which runs from residue 78 to 85, is an important structural feature associated with ATP binding. The β-strand in DrRecA is part of rigid β sheet architecture, which forms the core of the protein. Tyr-77 is conserved in all of the members of the Deinococcaceae family (supplemental Fig. S3*A*), whereas in some of the non-Deinococcaceae, the corresponding amino acid is phenylalanine (supplemental Fig. S3*B*). The Tyr-77 residue is closely packed against neighboring residues, including Arg-234, Asp-236, and Val-260, in this protein (Fig. 11, *A* and *C*), and there is no steric space for a large phosphor group. In addition to steric clashes, the phosphorylation of Tyr-77 would also introduce negative charge creating repulsion with Asp-236 and attraction with Arg-234, probably resulting in a conformational change. In such a situation, the possibility of the other residues that matter for dATP/ATP discrimination

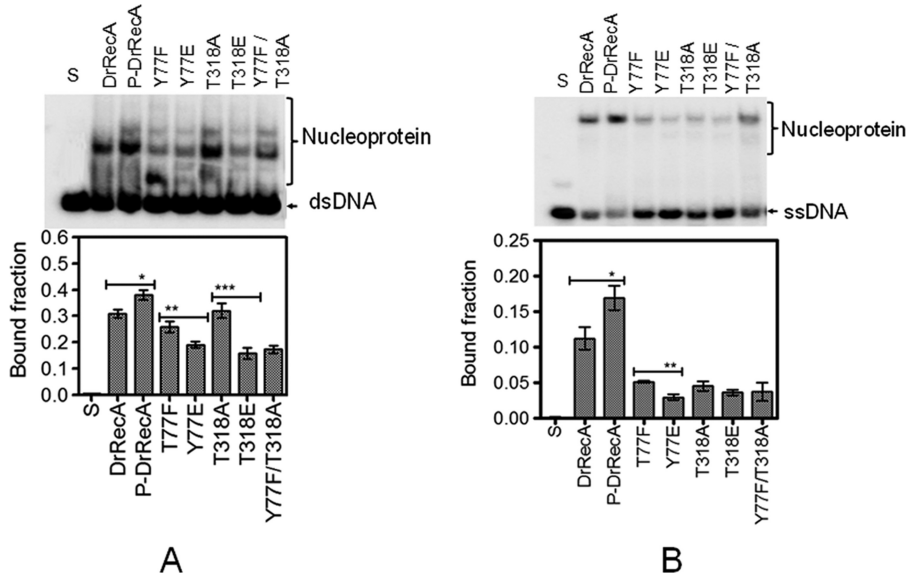

FIGURE 8. **DNA binding activity of DrRecA and its mutant derivatives.** Recombinant DrRecA, P-DrRecA, Y77F, Y77E, T318A, T318E, and Y77F/T318A proteins were purified and incubated with ^{32}P-labeled double-stranded DNA (*A*) and single-stranded DNA (*B*) in a reaction buffer as described under "Experimental Procedures." Reaction mixtures were separated on native PAGE, gels were dried, and autoradiograms were developed (*top panels*). DNA bound to protein (*Nucleoprotein*) and free form (*dsDNA/ssDNA*) was quantified densitometrically and shown (*bottom graphs*). Experiments were repeated two times and were reproducible. *Error bars*, S.E.

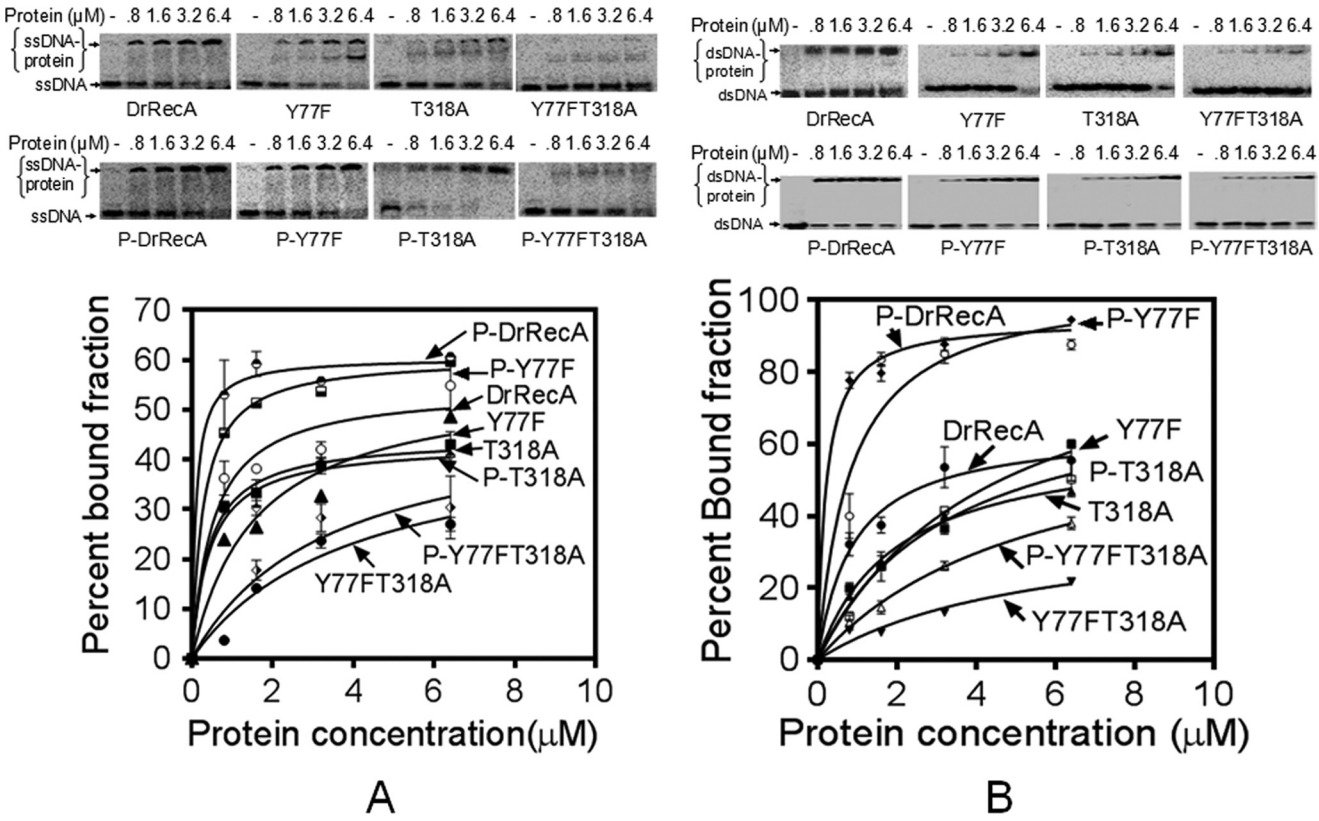

FIGURE 9. **DNA binding activity of different phosphosite mutants of DrRecA.** Different concentrations of purified proteins were incubated with ^{32}P-labeled ssDNA (*A*) and dsDNA (*B*). Mixtures were separated on native PAGE, autoradiograms were developed, and representative data of a reproducible experiment repeated three times are shown (*top panels*). DNA band intensities were quantified densitometrically, and percentage of bound fractions was calculated. Results were plotted and analyzed using Sigma Plot (*bottom graphs*). *Error bars*, S.E.

and dATP binding/hydrolysis in the active site becoming reoriented seems plausible. *Mycobacterium tuberculosis* RecA is another enzyme that prefers dATP over ATP (22). Comparing DrRecA with *M. tuberculosis* RecA, we noted that Lys-239 and Asn-253 of DrRecA are analogous to Arg-228 and Asn-241 in *M. tuberculosis* RecA. Arg-228 and Asn-241 in *M. tuberculosis* RecA potentially interact with the O3′ hydroxyl of ATPγS and are thought to relay information from the nucleotide-binding site to the dsDNA-binding C-terminal domain (23). Also, Gly-82, a unique residue in DrRecA, could possibly make the P-loop

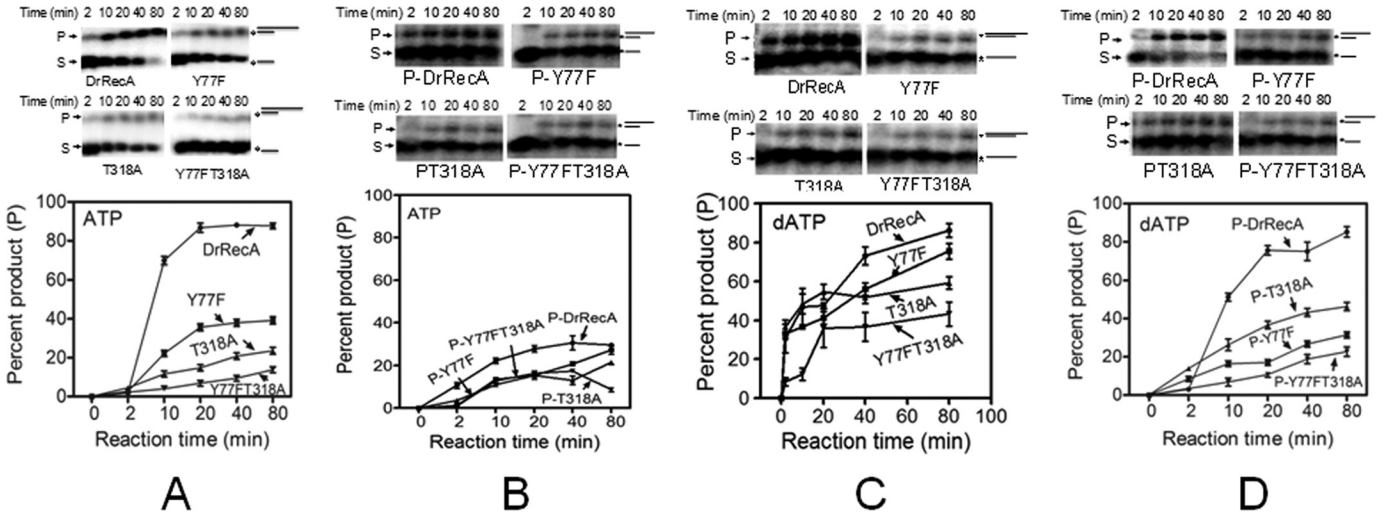

FIGURE 10. **DNA strand exchange activity of phosphosite mutants of DrRecA.** Both DrRecA (*A* and *D*) and P-DrRecA (*B* and *C*) derivatives of wild type and phosphosite mutants were purified, and ~6.4 μM concentration of each protein was used for strand exchange activity in the presence of ATP (*A* and *B*) and dATP (*C* and *D*). Products were analyzed on 10% polyacrylamide gel, and autoradiograms were developed. Results shown are representative data of reproducible experiments repeated three times (*top panels*). Both labeled substrate (*S*) and products (*P*) were quantified densitometrically. The amount of products made was calculated as a percentage, plotted, and analyzed using Sigma Plot (*bottom graphs*). *Error bars*, S.E.

more flexible to accommodate potential conformational changes.

The alignment of DrRecA with RecA homologues of other bacteria also showed that Thr-318 is unique to *D. radiodurans.* In most other bacterial species, including Deinococcaceae, the corresponding position has alanine or valine (supplemental Fig. S4*A*), whereas RecA of *Geobacter bemidjiens* and *Psychrobacter* are found to have serine instead of threonine at the position that corresponds with Thr-318 in DrRecA (supplemental Fig. S4*B*). Thr-318 is found in the middle of helix I in the C-terminal domain of DrRecA, part of a predominantly hydrophobic environment (23) just outside the primary dsDNA-binding motif 314–317 (Fig. 11*B*). Phosphorylation of Thr-318 will not only result in steric clashes with neighboring residues; it would also add a negatively charged group in a hydrophobic environment. It is likely that this might lead to the distortion of local structure and induce conformational changes in surrounding dsDNA-binding residues. The exact means by which phosphorylation of Tyr-77 and Thr-318 modulate DrRecA activity remain to be elucidated. However, the locations of these phosphoacceptor residues within the DrRecA structure strongly suggest that their phosphorylation induces conformational changes that are likely to have important functional consequences.

DrRecA Tyr-77 and Thr-318 Phospho-minus Mutant Decrease Radioresistance—Inactivation of the *D. radiodurans recA* gene renders the organism hypersensitive to γ radiation (24). We used complementation to investigate the effect of DrRecA phosphorylation on *D. radiodurans* γ radiation resistance. In these experiments, wild type DrRecA as well as its Y77F, Y77E, T318E, T318A, and Y77F/T318A mutant derivatives of DrRecA were expressed in the *D. radiodurans recA* mutant, and γ radiation survival was measured. Wild type DrRecA nearly completely complemented the loss of γ radiation resistance in the *recA* mutant. In contrast, when the Y77F, T318A, and Y77F/T318A, as well as phosphomimetic mutants like Y77E and T318E of DrRecA, were expressed in the *recA* strain, they showed partial complementation to the loss of γ radiation resistance in *recA* mutant. For instance, the Y77F, T318A, and Y77F/T318A phosphoacceptor mutants exhibited 0.8, 1.5, and 2.5 log cycles and higher sensitivity to γ radiation, respectively, as compared with mutant cells expressing DrRecA *in trans* at 9 kilograys (Fig. 12). Interestingly, T318E replacement showed less support to *recA* mutant as compared with its phosphoablative mutant, T318A. These results suggested that the Tyr-77 and Thr-318 amino acids play the important roles in the regulation of DrRecA functions, and Tyr-77 and Thr-318 replacement could affect the role of DrRecA in radioresistance of *D. radiodurans*. Because phosphomimetic mutants did not mimic phosphorylation, the involvement of Tyr-77 and Thr-318 phosphorylation in DrRecA functions still remains obscure.

Phosphomimetic Mutant of Tyr-77 and Thr-318 Did Not Mimic Phosphorylation Effect in DrRecA—Y77A and T318A mutants showed defects in ATPase and DNA binding activity, respectively. On the other hand, incubation of these mutants with RqkA affected their counterfunctions like DNA binding and ATPase, respectively. This indicated the influence of phosphorylation on the roles of these residues in DrRecA functions. In order to obtain further insight, the phosphomimetic mutants of these residues were generated with the anticipation that Y77E and T318E would mimic the phosphorylation effect in DrRecA. DNA binding and ATPase activity of purified recombinant Y77E and T318E were checked and compared with corresponding phosphoablative mutants. Results showed that phosphomimetic mutants do not mimic phosphorylation effects on ATPase (Fig. 7) and DNA binding (Fig. 8) activities of DrRecA *in vitro* as well as functional complementation in *recA30* mutant *in vivo* (Fig 12). Earlier, the phosphomimetic mutants mimicking the phosphorylation effect have been reported in some proteins only. For example, these are observed in two-component system proteins (25) but not in other proteins, including RqkA and

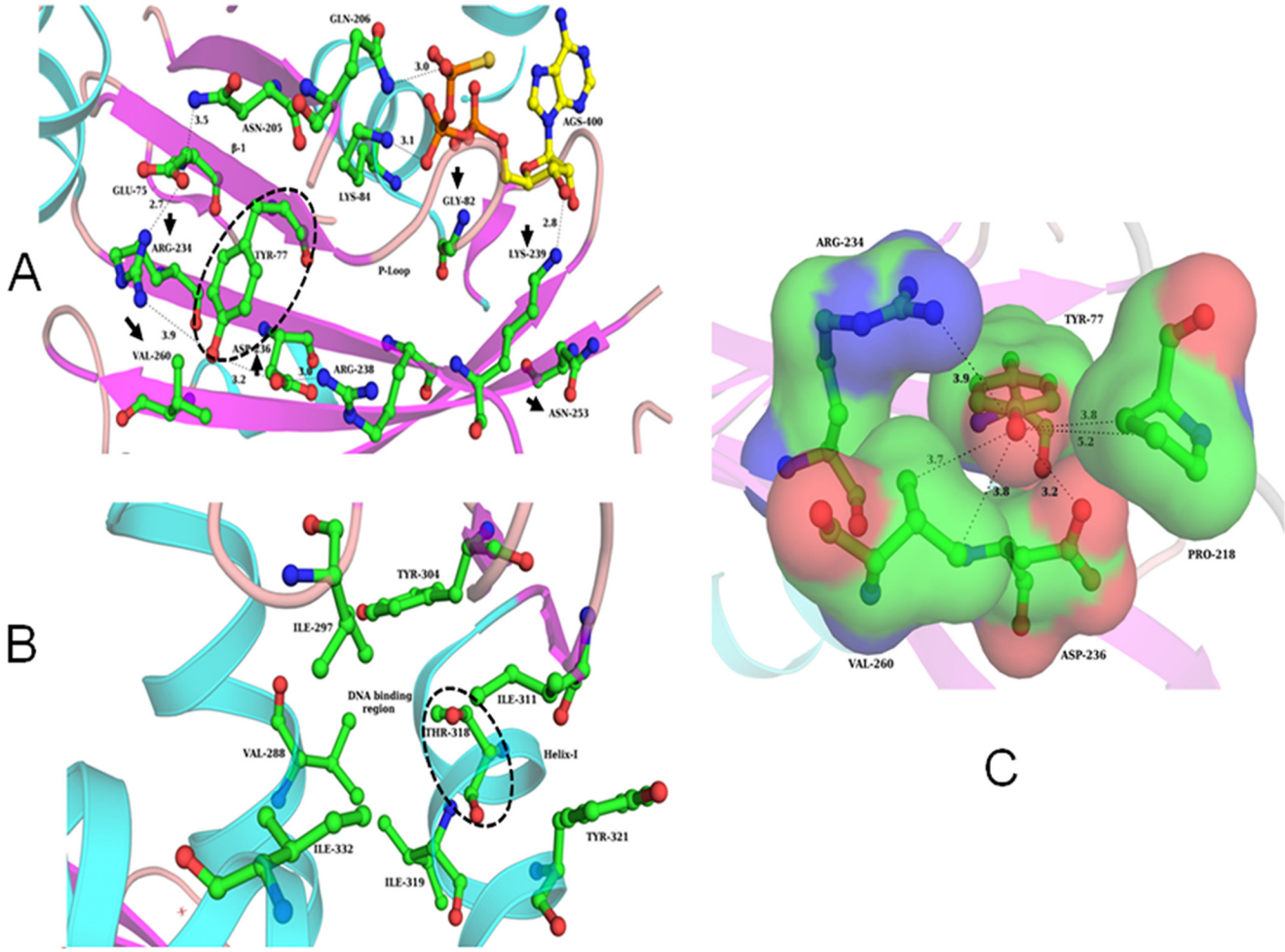

FIGURE 11. **Structural analyses of phosphosite residues in the three-dimensional structure of DrRecA using PyMOL.** The position of Tyr-77 shown within the *dotted circle* was mapped in the DrRecA structure (Protein Data Bank entry 1XP8), and some of the other corresponding residues that could be important either for their roles in other bacteria or for sterically supporting the functions of Tyr-77 upon phosphorylation are marked with a *solid arrow* (*A*). Similarly, the position of Thr-318, as shown within the *dotted line*, is mapped in helix I of the C-terminal domain along with other neighboring important residues that could contribute to the structural stability of this protein upon Thr-318 phosphorylation (*B*). Residues surrounding Tyr-77 were shown closely packed in the three-dimensional structure of DrRecA (Protein Data Bank entry 1XP8). Because the Pro-218 residue is not visible in the DrRecA structure, the position of Pro-218 is modeled using the crystal structure of *M. tuberculosis* (Protein Data Bank entry 1MO4) (*C*).

PprA of *D. radiodurans* (19). DrRecA seems to be another protein where phosphomimetic mutants do not mimic the phosphorylation effect on its functions.

Discussion

RecA is known to play an essential role in the extraordinary radioresistance of *D. radiodurans* (24). However, *D. radiodurans* lacks the widespread LexA- and RecA-mediated SOS response, and the pathways that control RecA activation following this organism's exposure to radiation are unknown. Our work here has suggested a new posttranslational pathway for modulating RecA-mediated DNA double strand break repair. RqkA, a eukaryotic-like serine/threonine/tyrosine protein kinase, phosphorylates DrRecA primarily at its Tyr-77 and Thr-318 residues. Phosphorylation of these amino acids alters the preference of DrRecA for DNA and nucleotide substrates and thereby probably promotes DSB repair during recovery of *D. radiodurans* from radiation damage of its genome. Linking RecA activity to a post-translational process like phosphorylation may represent a mechanism to circumvent the potential problems created when massive DNA damage could inhibit transcriptional/translational processes from elevating *recA* expression during the PIR period.

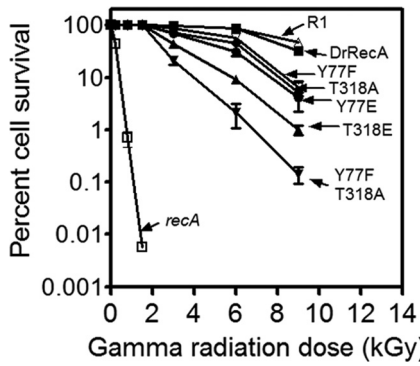

FIGURE 12. **Functional complementation of DrRecA loss in *D. radiodurans recA30* mutant by phosphosite mutants of DrRecA.** DrRecA and its phosphosite mutants, such as Y77F, Y77E, T318A, T318E, and Y77F/T318A, were expressed in the *recA30* mutant of *D. radiodurans* (*recA*), and cell survival was monitored at different doses of γ radiation and compared with wild type (*R1*). *Error bars*, S.E.

The biphasic DSB repair in *D. radiodurans* (26) includes a rapid assembly of shattered genome fragments by a unique mechanism of ESDSA and a second phase involving slow crossover events of homologous recombination (4). RecA is essential in both phases of DSB repair (2, 5). Increased demand for RecA in bacteria is met by increasing its synthesis through the SOS response and/or its activity by an array of other proteins (27). In *D. radiodurans*, increased *recA* transcription occurs independently of LexA-mediated SOS regulation (10, 11). Protein phosphorylation and dephosphorylation provide a dynamic and reversible process that regulates the physico-chemical properties and cellular location of proteins (28). Linking RecA activity to a post-translational process like phosphorylation rather than to changes in its expression could represent a mechanism to circumvent the potential problems created when massive DNA damage could potentially prevent transcriptional/translational processes from elevating *recA* expression and maintaining it at the high levels during the entire PIR period.

Phosphorylation is an unusual but not unprecedented means to regulate DNA repair processes in prokaryotes. Phosphorylation of *B. subtilis* RecA at serine 2 by the YabT Hanks type serine/threonine kinase modulates spore development (17). *B. subtilis* also encodes a tyrosine kinase that phosphorylates a tyrosine in RecA (29). However, the functional/biochemical significance of *B. subtilis* RecA phosphorylation by either Ser/Thr protein kinase or tyrosine kinase has not been reported. The SSB of *B. subtilis* undergoes phosphorylation at tyrosine 82 by its cognate tyrosine-protein kinase YabT, elevating its ssDNA binding activity *in vitro* (16). PprA, a novel multifunctional DNA repair protein that is found only in the Deinococcaceae, is phosphorylated at threonines 72 and 144 and serine 112 by RqkA kinase, and phosphorylations of these sites as well as RqkA kinase activity are important for the roles of PprA and RqkA in *D. radiodurans* radioresistance (19).

To investigate how DrRecA phosphorylation affects its functions, the sites of RqkA phosphorylation in DrRecA were identified by mass spectrometry and confirmed by site-directed mutagenesis. Tyr-77 phosphorylation influenced the dATP hydrolysis of DrRecA and increased dATP inhibition of its ATPase activity. The effects of the Y77F and T318A mutations on DrRecA nucleotide discrimination and DNA substrate preferences for strand exchange reaction activity differed. For instance, the dATP effect on strand exchange activity of the Y77F mutant was the same as that of DrRecA but different from those of P-DrRecA and T318A, suggesting the involvement of Tyr-77 in nucleotide discrimination. Determination of how exactly Tyr-77 contributes to nucleotide discrimination requires further investigation. However, it was observed that Tyr-77 is found at the end of the β-strand (β1) and just before the ATP binding pocket that runs between amino acids 78 and 85 in the P-loop (Walker A) motif. Because the P-loop region is largely conserved in RecA homologues, we analyzed the corresponding and neighboring amino acids in RecA proteins of other bacteria. *E. coli* RecA has a serine at position 69, again just outside the P-loop, and its replacement with glycine (S69G) altered the rates of hydrolysis of ATP, dATP, and ddATP nucleotides (30). Also, the Arg-228 and Asn-241 residues in *M. tuberculosis* RecA, which are juxtaposed to the P-loop region, regulate the protein's interaction with dATP (22). When we compared the structure of *M. tuberculosis* RecA with that of DrRecA, we noticed that Lys-239 and Asn-253 of DrRecA are analogous to Arg-228 and Asn-241 in *M. tuberculosis* RecA. Lys-239 of DrRecA is found interacting with the O3′ hydroxyl of ATPγS and helping in information relay from the nucleotide-binding site to the dsDNA-binding C-terminal domain (23). These findings suggest that Tyr-77 phosphorylation may create conformational change in DrRecA that, along with the residues surrounding the P-loop region of the ATP binding pocket, affects the nucleotide preference of DrRecA. ATP binding-mediated conformational change in the RecA filament has been demonstrated in other bacteria (31). It has been shown that DNA synthesis during PIR is exceptionally high (5), which would require higher levels of dNTP, including dATP, and the levels of adenine nucleotide triphosphate measured through HPLC had also increased at 2 h PIR (32). Therefore, the preference of dATP by DrRecA upon phosphorylation is understandable and could be one reason why DrRecA is highly efficient in DSB repair processes throughout PIR phases. The requirement for ATP or dATP in *E. coli* RecA functions is controversial because it does not require ATP for promoting a strand exchange reaction (33). However, ATP hydrolysis is important for subsequent energy-driven steps during homologous recombination (27), including the bypass of structural barriers in the DNA substrates (34), and in the facilitation of four DNA strand exchange reactions (35, 36).

Interestingly, the Y77F mutant, which should undergo phosphorylation at Thr-318, did not exhibit altered DNA substrate preference. Thr-318 is situated in the middle of helix I in the DrRecA C-terminal domain, just outside the primary dsDNA-binding motif 314–317, and is part of a predominantly hydrophobic environment in DrRecA (Fig. 10, *A* and *B*) (23). Multiple-sequence alignment revealed that Thr-318 is unique to DrRecA; even other members of the Deinococcaceae family as well as most other bacteria have either an alanine or valine at this position. Interestingly, in a very few cases, like *G. bemidjiens* and *Psychrobacter*, the corresponding position of Thr-318 is serine (supplemental Fig. S4*B*). Therefore, the possibility that RecA activity is also regulated by Ser/Thr phosphorylation in some other bacteria cannot be ruled out. Mazin and Kowalczykowski (21) have shown that *E. coli* RecA possesses distinct DNA binding sites, with primary sites occupied by ssDNA and secondary sites occupied by dsDNA during the strand exchange reaction. DrRecA does not show discrimination for ssDNA and dsDNA binding (37). However, the possibility of phosphorylation introducing such discrimination for different types of DNA substrate is possible. Our results support this idea because we found that DrRecA phosphorylation enhances its preference for dsDNA (Fig. 3*A*). The fact that T318A protein and its kinased derivative showed dsDNA binding similar to that of nonphosphorylated wild type DrRecA indicated the involvement of Thr-318 phosphorylation in dsDNA binding activity of DrRecA. The presence of Thr-318 in the C-terminal domain, which in the RecA of other bacteria is known to interact with dsDNA during homologous pairing of DNA molecules (38), supports the argument.

TABLE 2
List of primers used

Serial no.	Primers	Nucleotide sequences of primers	Purpose
1	RecA-F	5′-CATGCCATGGTGAGCAAGGACGCCACCA-3′	pET28a, recA
2	RecA-R	5′-CCGCTCGAGCGGTTCGGCGGCTTCGGG-3′	pET28a, recA
3	S19F	5′-GACGCCAAGGAACGCGCCAAGGCCATCGAAAC-3′	S19A
4	S19R	5′-GTTTCGATGGCCTTGGCGCGTTCCTTGGCGTC-3′	S19A
5	S27F	5′-CATCGAAACAGCCATGGCCCAGATCGAAAAGGC-3′	S27A
6	S27R	5′-GCCTTTTCGATCTGGGCCATGGCTGTTTCGATG-3′	S27A
7	Y77F	5′-CATCACCGAGATCTTCGGCCCCGAGTCG-3′	Y77F
8	Y77R	5′-CGACTCGGGGCCGAAGATCTCGGTGATG-3′	Y77F
9	Y77EF	5′-CGCATCACCGAGATCGAGGGCCCCGAGTCGGGC-3′	Y77E
10	Y77ER	5′-GCCCGACTCGGGGCCCTCGATCTCGGTGATGCG-3′	Y77E
11	T254F	5′-CGCGGTCGCCAACGCCGTCAAGATCAAG-3′	T254A
12	T254R	5′-CTTGATCTTGACGGCGTTGGCGACCGCG-3′	T254A
15	T318F	5′-CCAGGGCAAGGAAAAGGCCATCGCCTACATCGC-3′	T318A
16	T318R	5′-GCGATGTAGGCGATGGCCTTTTCCTTGCCCTGG-3′	T318A
19	T318EF	5′-CAGGGCAAGGAAAAGGAGATCGCCTACATCGCC-3′	T318E
20	T318ER	5′-GGCGATGTAGGCGATCTCCTTTTCCTTGCCCTG-3′	T318E
11	T7-F	5′-TAATACGACTCACTATAGGG-3′	p11559, recA
12	T7-R	5′-CTAGTTATTGCTCAGCGGTG-3′	p11559, recA
13	40F	5′-GAATTCGGTGCGCATAATGTATATTATGTTAAATCATGTC-3′	EMSA
14	40R	5′-GATCGAAGCTTACTGCATACGCTTGGTTTATATTGGGGCA-3′	EMSA
15	82F	5′-GAATTCGGTGCGCATAATGTATATTATGTTAAATCATGTCCCTGCCCCAATATAAACCAAGCGTATGCA GTAAGCTTCGATC-3′	EMSA
16	82R	5′-GATCGAAGCTTACTGCATACGCTTGGTTTATATTGGGGCAGGGACATGATTTAACATAATATACATTAT GCGCACCGAATTC-3′	EMSA

Because DrRecA phosphorylation had significantly affected the efficiency of its DNA strand exchange activity, a contribution of Tyr-77 and Thr-318 to DrRecA roles in the radioresistance of *D. radiodurans* could be suggested. We noticed that single Y77F and T318A proteins partly complemented *recA* loss of radioresistance. However, as expected, the Y77F/T318A double mutant, which is phosphonegative by RqkA, failed to fully complement the functional loss of DrRecA in the *recA* mutant. The phosphomimetic mutants like Y77E and T318E did not mimic the phosphorylation effects in DrRecA. Because phosphomimetic mutants do not always mimic the phosphorylation effect in the protein, the possibility that Tyr-77 and Thr-318 phosphorylation would affect DrRecA functions could not be fully ascertained. However, these results suggested that Tyr-77 and Thr-318 contribute significantly to the DrRecA role(s) in the radioresistance of this bacterium.

Collectively, our findings suggest that a phosphorelay-based signaling pathway modulates *D. radiodurans* radioresistance. DrRecA is a target of RqkA, a eukaryotic type DNA damage-responsive serine/threonine/tyrosine protein kinase. DrRecA phosphorylation affects its ATP/dATP discrimination and DNA substrate preference, leading to enhanced DNA strand exchange activity in the presence of dATP *in vitro*. DrRecA Tyr-77 and Thr-318, the predominant phosphoacceptor residues, are found in the protein's central core and C-terminal domain, respectively. These domains have roles in nucleotide and DNA substrate discrimination in other RecA proteins. The inability of the Y77F, Y77E, T318A, T318E, and Y77F/T318A mutants to fully complement DrRecA functions in a *D. radiodurans recA30* mutant suggests the possibility of Tyr-77 and Thr-318 phosphorylation contributing to DrRecA functions in the extraordinary radioresistance of this bacterium. Because it is known that not all phospho-mimic mutants actually recapitulate the effect of phosphorylation, it remains unclear whether the observed effects are due to phosphorylation of these amino acids or other consequences of mutations on protein function. Future studies to delineate how DrRecA phosphorylation during PIR helps to partition this protein's role between ESDSA and the second phase of DSB repair will further illuminate this novel regulatory scheme. Extrapolating from our current results, we speculate that DrRecA phosphorylation enables this protein to work efficiently on preferred dsDNA and dATP substrates, perhaps for carrying out DNA strand exchange activity during early PIR.

Experimental Procedures

Bacterial Strains and Materials—The wild type *D. radiodurans* R1 (ATCC13939) was a generous gift from Prof. J. Ortner (20), and *recA30* mutant (39) was a generous gift from Professor Yuijin Hua (Zhijiang University, Hangzhou, China). Wild type and their respective derivatives were grown aerobically in TGY (0.5% Bacto-tryptone, 0.3% Bacto-yeast extract, and 0.1% glucose) broth or on an agar plate at 32 °C in presence of antibiotics as required. Shuttle expression vectors pRADgro (40) and pVHS559 (41) and their derivatives were maintained in *E. coli* strain DH5α as described earlier. Molecular biology grade chemicals and enzymes were procured from Sigma, Roche Applied Science, New England Biolabs, Cell Signaling, and Merck.

Cloning and Site-directed Mutagenesis—Genomic DNA of *D. radiodurans* was prepared as described (42). The DrRecA coding sequence was PCR-amplified using gene-specific primers RecA-F and RecA-R (Table 2) and cloned at NcoI and XbaI sites in pET28a+. The resultant plasmid pETrecA was used for site-directed mutagenesis of DrRecA. Ser-19, Ser-27, and Thr-254 were replaced with alanine, whereas Thr-318 and Tyr-77 were replaced with alanine and glutamic acid and with phenylalanine and glutamic acid, respectively, using the respective site-specific mutagenic primers (Table 2) and a site-directed mutagenesis kit (New England Biolabs) following the kit manufacturer's protocols. The *in vitro* mutagenesis was confirmed by sequencing. The resultant plasmids expressing S19A, S27A, Y77F, Y77E, T254A, T318E, T318A, and Y77F/T318A mutants of DrRecA were named as pETS19, pETS27, pETY77F, pET77E, pETT254, pETT318E, pETT318A, and pETY77T318, respectively. These plasmids were transformed into *E. coli*

BL21 (DE3) pLysS for expression of recombinant proteins. For functional complementation studies, the coding sequences of wild type *recA* and its Y77F, Y77E, T318A, T318E, and Y77F/T318A mutant versions were PCR-amplified from respective pET derivatives using T7-F and T7-R primers (Table 2) and cloned at NdeI and XhoI sites in deinococcal shuttle expression vector pVHS559 (41), resulting in pVHSRecA, pVHSY77F, pVHSY77E, pVHST318A, pVHST318E, and pVHSY77T318. These recombinant plasmids were transformed to the *recA30* mutant of *D. radiodurans*, the recombinant clones were scored on TGY agar plates supplemented with spectinomycin (75 μg/ml), and cloning was confirmed by restriction analysis.

Protein Purification—E. coli BL21 (DE3) pLysS harboring pETrecA, pETS19, pETS27, pETY77F, pETY77E, pETT254, pETT318E, pETT318A, and pETY77T318 was induced with 500 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 20 °C, and the expression of recombinant proteins was confirmed on SDS-PAGE. The phosphorylated and unphosphorylated forms of wild type DrRecA and its mutant derivatives were purified from the *E. coli* BL21 (DE3) pLysS cells co-expressing these proteins with wild type RqkA and its kinase mutant on plasmid pRAD2518 and pRADK42A, respectively (19). In brief, *E. coli* transformants were selected in the presence of both ampicillin (100 μg/ml) and kanamycin (25 μg/ml). These cells were induced with 500 μM IPTG at 20 °C, and the expression of recombinant proteins was confirmed on SDS-PAGE. The cells expressing these proteins were sonicated for 5 min on ice with 30-s pulses at 1-min intervals in buffer containing 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, protease inhibitor mixture (Sigma, catalog no. S8820), and phosphatase inhibitor mixture (Sigma, catalog no. P0044). The supernatant containing soluble protein was collected by centrifugation at 12,000 × *g*, and the recombinant proteins containing a hexahistidine tag were purified using nickel affinity chromatography as described (19). The RqkA kinase was purified as described earlier (18). The histidine tag was cleaved with thrombin and removed passing through an Ni^{2+} column, followed by dialysis in buffer containing 50% glycerol, and purified proteins were stored at −20 °C for further use.

In Vitro and in Vivo Protein Phosphorylation—In vivo phosphorylation of DrRecA was monitored during PIR in *D. radiodurans* cells exposed to 6.5 kilograys of γ radiation using a protocol similar to that described previously for RqkA (18). In brief, overnight-grown culture was subcultured and allowed to grow for 3 h under normal growth conditions. Cells were harvested, resuspended in sterile PBS, and divided into two sets. One set was exposed to 6.5 kilograys of γ radiation at a dose rate of 3.87 kilograys/h (Gamma Cell 5000, ^{60}CO, Board of Radiation and Isotopes Technology, Department of Atomic Energy, Navi Mumbai, India). The other set was used as unirradiated SHAM control. Aliquots were withdrawn periodically, cells were lysed by several rounds of freezing/thawing, and cell-free extracts were obtained by centrifugation at 12,000 × *g*. Equal amounts of total proteins were used for immunoprecipitation of DrRecA using RecA polyclonal antibodies and protocols as described earlier. Immunoprecipitates were separated on 10% SDS-PAGE and dried, and autoradiograms were developed.

In vivo phosphorylation of DrRecA and its mutant derivatives were also checked using *E. coli* surrogate host co-expressing these proteins with wild type RqkA kinase on plasmids. For that, *E. coli* BL21 (DE3) pLysS cells were co-transformed with pETrecA, pETS19, pETS27, pETY77, pETT254, pETT318, and pETY77T318 plasmids with pRAD2518, separately. The recombinant proteins were induced with IPTG and were purified as described above. Equal amounts of wild type and mutant proteins were separated on SDS-PAGE and transferred to PVDF membrane and probed with polyclonal phosphothreonine antibody (Cell Signaling Technology) as described earlier (18). For *in vitro* phosphorylation studies, ~20 ng of purified recombinant RqkA kinase and ~200 ng of wild type DrRecA and its mutant derivatives were incubated in a 25-μl kinase reaction mixture (70 mM Tris-HCl, pH 7.6, 5 mM DTT, 10 mM $MgCl_2$, 100 μM ATP, 1 μCi of [γ-^{32}P]ATP) for 30 min at 37 °C. The reactions were stopped by adding 25 μl of 2× SDS-PAGE dye (100 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 0.2% (w/v) bromphenol blue, 20% (v/v) glycerol, and 200 mM DTT) and heated at 95 °C. The reaction mixtures were separated on SDS-PAGE, and protein phosphorylation was detected by autoradiography. Phosphosites in DrRecA incubated with purified RqkA in the presence of unlabeled ATP were detected at the Taplin Biological Mass Spectrometry Facility at Harvard Medical School.

Cell Survival Studies—Cell survival studies were carried out as described earlier (40). In brief, *D. radiodurans* R1 and its *recA* mutant were transformed with pVHS559 vector and recombinant plasmids pVHSRecA, pVHSY77F, pVHSY77E, pVHST318A, pVHST318E, and pVHSY77T318 to express DrRecA and its respective mutant derivatives. These cells were grown in TGY medium at 32 °C in the presence of 10 mM IPTG, and the expression of recombinant protein was confirmed by immunoblotting against RecA antibodies as described earlier (40). Cells expressing these proteins were washed, suspended in sterile PBS, and exposed to different doses of γ radiation at a dose rate of 3.87 kilograys/h (Gamma Cell 5000, ^{60}CO, Board of Radiation and Isotopes Technology). The colony-forming units were recorded after 40 h of incubation at 32 °C on TGY agar plates supplemented with spectinomycin (75 μg/ml) and 500 μM IPTG as required.

DNA Binding Assay—The DNA binding activities of DrRecA and its mutant derivatives were checked using EMSA as described earlier (19). In brief, a 40-nucleotide-long random sequence oligonucleotide (Table 2) was used as ssDNA substrate, and dsDNA substrate was made by annealing it with its complementary strand (Table 2). Both ssDNA and dsDNA were labeled with [γ-^{32}P]ATP using polynucleotide kinase and purified by a G-25 column. 0.2 pmol of labeled probe (ssDNA and dsDNA) was incubated with increasing concentrations of both phosphorylated and nonphosphorylated DrRecA in 10 μl of reaction mixture containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM ATP/dATP, and 1 mM DTT for 10 min at 37 °C. Products were analyzed on a 12.5% native polyacrylamide gel and dried, and signals were recorded by autoradiography. DNA band intensity either in free form or bound to protein was quantified using ImageJ. The amount of DNA bound to protein was divided by total DNA, and the bound DNA fraction was obtained. The percentage of bound fraction of DNA was plot-

ted against protein concentration using GraphPad Prism version 5. The K_d for curve fitting of individual plots was determined by the software working on the principle of the least squares method, applying the formula, $Y = B_{max} \times [X]/K_d + [X]$, where $[X]$ is the protein concentration, and Y is the bound fraction as described earlier (43). To determine the log equilibrium dissociation constant (log K_i) of nonphosphorylated and phosphorylated DrRecA, the competition assay was performed, where the binding of 0.2 pmol of ssDNA/dsDNA was challenged with either unlabeled homologous ssDNA and dsDNA (2.5–40 μM) or unlabeled heterologous dsDNA and ssDNA (2.5–40 μM), respectively. Log K_i was calculated by curve fitting using nonlinear regression of the competition binding equation of one-site fit K_i in GraphPad Prism. The DNA fraction bound to protein was plotted as a function of protein concentration using GraphPad Prism version 5.

ATPase Assay—ATP and dATP hydrolysis was monitored as the release of inorganic phosphate estimated by the malachite green assay using a protocol modified from Ref. 44 as described (45). In brief, 3 μM DrRecA was preincubated in 25 μl of assay buffer (25 mM Tris acetate (80% cation, pH 7.5), 1 mM DTT, 5% glycerol, 3 mM potassium glutamate, and 10 mM magnesium acetate) at 37 °C for 10 min before the reaction was initiated by adding ATP/dATP. The reaction mixture was incubated for different time intervals (10–80 min) and terminated using 200 μl of freshly prepared malachite green reagent. The final volume of the sample was increased to 1 ml with distilled water and incubated at room temperature for 15 min. Absorbance at 630 nm was measured relative to a buffer control and normalized with protein control without ATP/dATP and ATP/dATP control without protein. To check the dATP effect on ATPase activity of DrRecA, the competition assay was done using a modified protocol as given in Ref. 46. In brief, 500 μM unlabeled ATP and 0.2 μCi of [γ-^{32}P]ATP were incubated with increasing concentrations of dATP (0.1–10 mM) in 50 μl of assay buffer (25 mM Tris acetate (80% cation, pH 7.5), 1 mM DTT, 5% glycerol, 3 mM potassium glutamate, and 10 mM magnesium acetate) at 37 °C. The reaction was initiated with 3 μM DrRecA, and the reaction was stopped after 10 min by adding 50 μl of stop solution (5% TCA, 0.14% ammonium heptamolybdate, 3% sulfuric acid, and 1 mM orthophosphoric acid). Further, 300 μl of ice-cold isobutyl alcohol and ethyl acetate (1:1) was added into the reaction and mixed. Samples were centrifuged for 10 min at 12,000 rpm, and 100 μl of upper, organic phase was mixed with 200 μl of AquaLight (PerkinElmer Life Sciences) for scintillation counting. A control without DrRecA was processed identically, and control values were subtracted from the experimental values. The percentage of ATPase activity was plotted as a function of the dATP concentration.

DNA Strand Exchange Reaction—Unless stated otherwise, all reactions were carried out at 37 °C. The ^{32}P-labeled 40-mer oligonucleotide (2.5 μM nucleotides) (Table 2) was incubated with pure wild type DrRecA or its mutant derivatives (1.6 μM) in 10 μl of buffer (35 mM Tris-HCl, pH 7.5, 1 mM DTT, 2.5 mM $MgCl_2$, 0.1 mM ATP, 25 mM KCl) containing 1 mM ATP for 5 min. The reaction was initiated by adding homologous 82-mer duplex (1.25 μM nucleotides) (Table 2) to 12.5 μl of final volume. At the indicated times, a 2.5-μl aliquot was removed and mixed with an equal volume of 1% SDS containing proteinase K (1 mg/ml) and incubated at 37 °C for 20 min. The samples were analyzed on 10% PAGE, and the results were imaged on a PhosphorImager and analyzed using GraphPad Prism version 6.

Author Contributions—Y. S. R. conducted experiments, analyzed and discussed results, and wrote the draft of the manuscript. S. C. B. contributed to computational biology and conducted experiments. M. K. W. performed mass spectrometry of phosphoproteins using the MS facility at Harvard Medical School and contributed to data analysis and writing of the paper. H. S. M. was project leader, conceived the idea and hypothesis, analyzed and discussed results, wrote the paper, and conducted communication for publication.

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Phosphorylation of *Deinococcus radiodurans* RecA regulates its activity and may contribute to radioresistance

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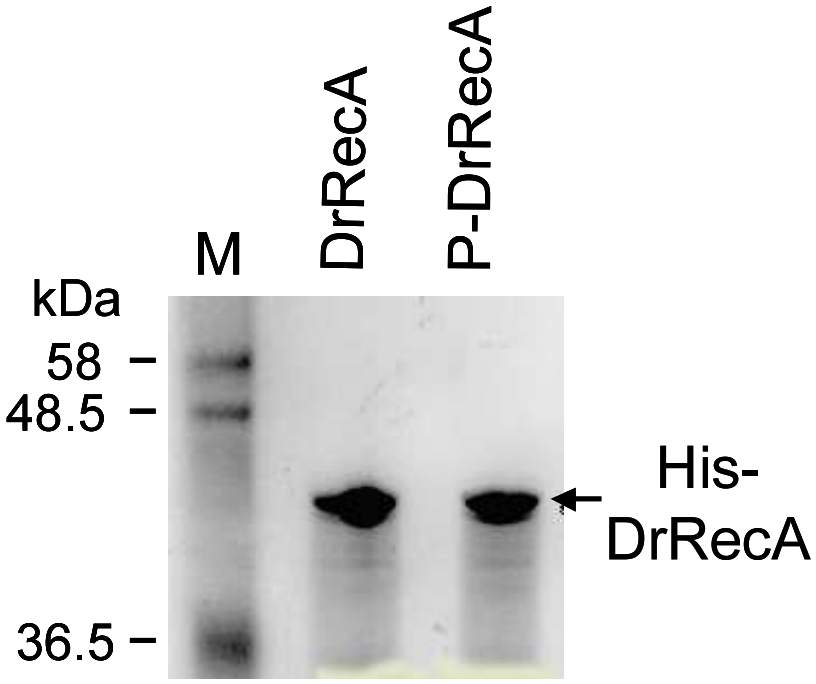

Figure S1. SDS-PAGE analysis of DrRecA purified from recombinant *E. coli.* Recombinant DrRecA purified from *E. coli* harbouring vector (DrRecA), and co-expressing RqkA and getting phosphorylated (P-DrRecA) were analysed on SDS-PAGE.

# indicates site of phosphorylation. The site shown in the html link may not be confidently assigned. See below for site assignment information.

Sample: RecA-P (tracking number 38944)

Database search results: Data link (login-"msguest" and passward is "spectrum"

1. S#KAIETAMSQIEK – site confidently assigned
2. ITEIYGPES#GGKTTLALAIVAQAQK – site not confidently assigned. 5 possible sites for 1 phosphate: ITEIYGPESGGKTTLALAIVAQAQK
3. ITEIY#GPES#GGKTTLALAIVAQAQK – site not confidently assigned. 5 possible sites for 2 phosphates: ITEIYGPESGGKTTLALAIVAQAQK
4. VGNDAVANT#VK – site confidently assigned
5. EKT#IAYIAERPEM*EQEIR – site confidently assigned
6. DAT#KEISAPTDAK – site confidently assigned
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8. AIETAMS#QIEK – site confidently assigned
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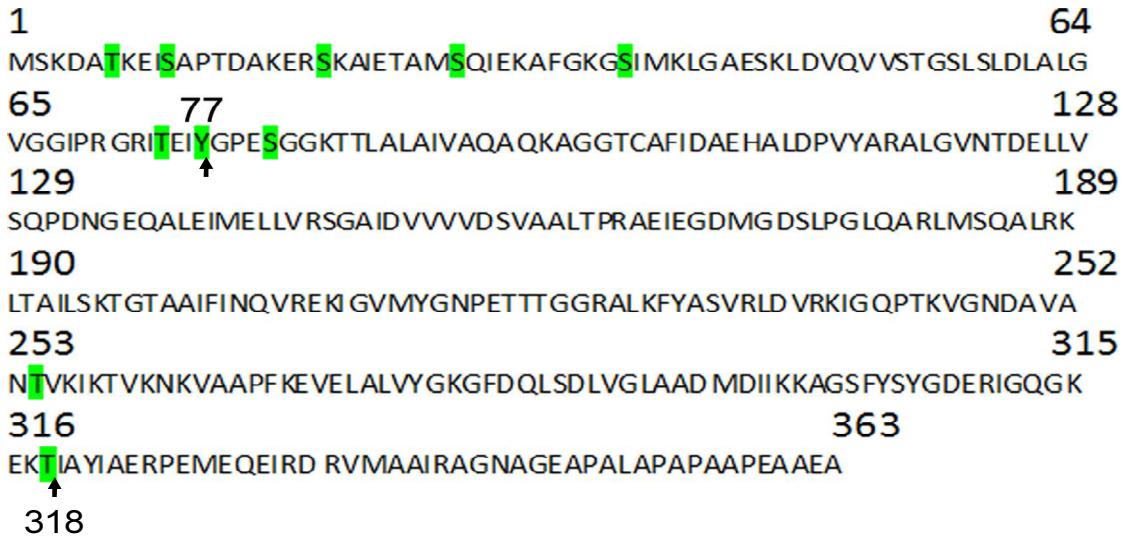

Figure S2. Mass spectrometric analysis of DrRecA incubated with RqkA in the presence of ATP and separated on SDS-PAGE. Stained band was cut and subjected to MS analysis (upper panel). Phosphosites detected were mapped on primary structure of DrRecA (lower panel).

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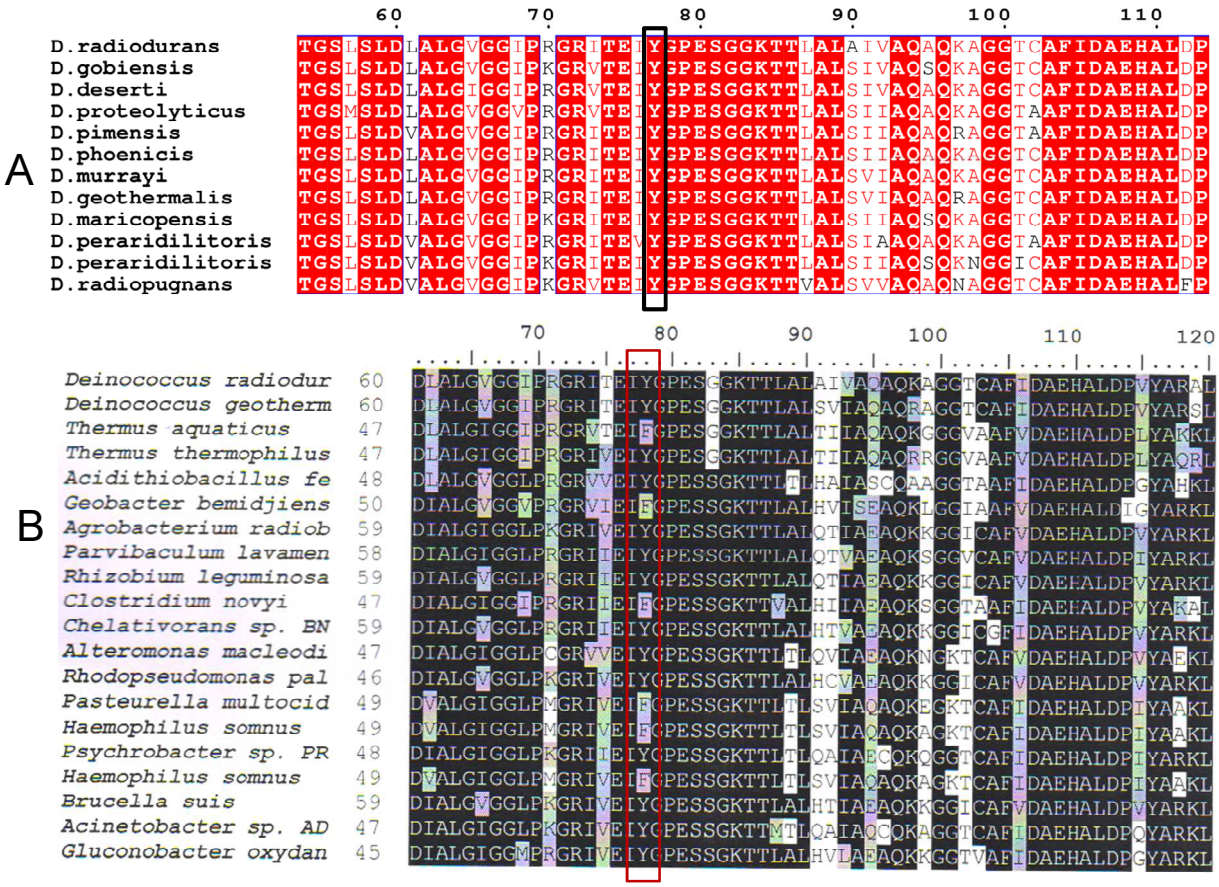

Fig S3. Alignment of tyrosine 77 in the amino acid sequences of DrRecA with its bacterial homologues. Multiple sequence alignment of amino acids from 54 to 113 in DrRecA were aligned with corresponding regions of RecA from other members of Deinococcaceae family (A) and 60-120 amino acids of DrRecA with corresponding regions of RecA from other bacteria (B).

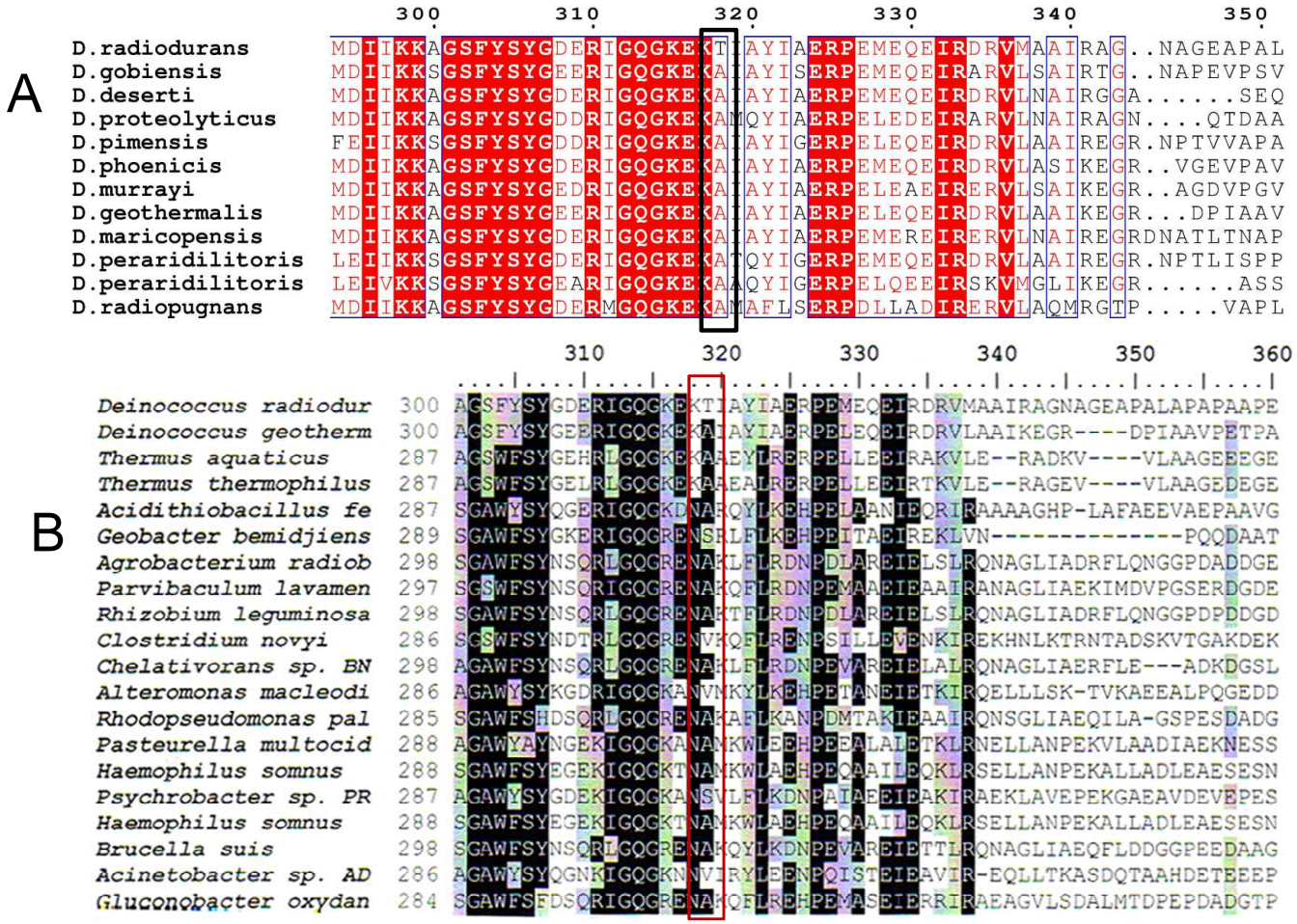

Fig S4. Alignment of threonine 318 in the amino acid sequences of DrRecA with its bacterial homologues. Multiple sequence alignment of amino acids from 294-351 in DrRecA with corresponding regions of RecA from other members of Deinococcaceae family (A) and 300-360 amino acids of DrRecA with corresponding regions of RecA from other bacteria (B) were carried out.

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