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Structure-function study of deinococcal serine/threonine protein kinase implicates its kinase activity and DNA repair protein phosphorylation roles in radioresistance of *Deinococcus radiodurans*

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ABSTRACT

The DR2518 (RqkA) a eukaryotic type serine/threonine protein kinase in *Deinococcus radiodurans* was characterized for its role in bacterial response to oxidative stress and DNA damage. The K42A, S162A, T169A and S171A mutation in RqkA differentially affected its kinase activity and functional complementation for γ radiation resistance in Δ*dr2518* mutant. For example, K42A mutant was completely inactive and showed no complementation while S171A, T169A and T169A/S171A mutants were less active and complemented proportionally to different levels as compared to wild type. Amongst, different DNA binding proteins that purified RqkA could phosphorylate, PprA a DNA repair protein, phosphorylation had improved its affinity to DNA by 4 fold and could enhance its supportive role in intermolecular ligation by T4 DNA ligase. RqkA phosphorylates PprA at threonine 72 (T72), serine 112 (S112) and threonine 144 (T144) *in vitro* with the majority of it goes to T72 site. Unlike wild type PprA and single mutants of T72, S112 and T144 residues, the T72AS112A double and T72AS112AT144A triple mutant derivatives of PprA did not phosphorylate *in vivo* and also failed to complement PprA loss in *D. radiodurans*. Deletion of *rqkA* in *pprA::cat* background enhanced radiosensitivity of *pprA* mutant, which became nearly similar to Δ*rqkA* resistance to γ radiation. These results suggested that K42 of RqkA is essential for catalytic functions and the kinase activity of RqkA as well as phosphorylation of PprA have roles in γ radiation resistance of *D. radiodurans*.

1. Introduction

Deinococcus radiodurans R1 is characterized for its extraordinary tolerance to the lethal and mutagenic effects of DNA damaging agents including radiation and desiccation (Battista, 2000). An efficient DNA double strand break (DSB) repair and a strong oxidative stress tolerance (Slade and Radman, 2011) are amongst the mechanisms that could be implicated to the robustness of this organism. Recently, it has been shown that the oxidative damage of proteins also caused lethality to radiation (Daly, 2012). *D. radiodurans* has evolved extraordinarily efficient antioxidant chemical defenses mainly rich of Mn, phosphate, nucleosides and bases, and peptides that prevent protein oxidation at massive doses of ionizing radiation (Daly et al., 2010). PprA a pleiotropic protein involved in radiation resistance in *D. radiodurans* has been characterized for its roles in various molecular processes. For example, PprA role has been demonstrated in the stimulation of DNA end joining activity by T4 DNA ligase (Narumi et al., 2004) and a DNA repair ligase of *D. radiodurans* (Kota et al., 2010), and in the stimulation of *Escherichia coli* catalase activity (Kota and Misra, 2006). PprA is found to be associated with a multiprotein complex characterized from this bacterium (Kota and Misra, 2008). More recently an observation on PprA involvement in genome segregation and cell division has been reported in this bacterium (Devigne et al., 2013).

As far as DNA damage response mechanism(s) is concerned, the SOS response is a well-characterized DNA damage response mechanism in majority of the prokaryotes (Shimoni et al., 2009). *D. radiodurans* apparently lacks the classical SOS response to DNA damage (Narumi et al., 2001). Nevertheless, it adjusts its transcriptome and proteome in response to DNA damage, by both differential transcription of genes (Liu et al., 2003) and proteins turnover (Joshi et al., 2004). Recently, IrrE a γ radiation inducible protein of *D. radiodurans* was shown as a regulator of *recA* expression in response to γ radiation treatment (Earl et al., 2002). *D. radiodurans* genome encodes a large number of hypothetical proteins containing putative phosphorylation motifs nearly similar to the site of eukaryotic STPKs (eSTPKs) phosphorylation (Misra et al., 2013) and several uncharacterized response regulators, Hank family STPKs and histidine kinases, and at least one tyrosine kinase (Hanks et al., 1988; Makarova et al., 2001). Hank type kinases

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phosphorylate proteins at serine, threonine and tyrosine residues and play the key roles in regulation of signaling processes associated with the development, differentiation and DNA repair in eukaryotes (Sancar et al., 2004). In bacteria, most of the signal transduction pathways are found to be associated with two-component system (TCS) mainly comprised of histidine kinases and their cognate response regulators (Parkinson, 1993; Gao and Stock, 2009). The roles of eSTPKs have been demonstrated in the adaptation to abiotic stresses in pathogenic bacteria (Cozzone, 2005; Molle and Kremer, 2010) but very little is known in bacterial response to DNA damage. Recently, the involvement of DR2518 a eSTPK, in DNA damage tolerance of *D. radiodurans* has been demonstrated (Rajpurohit and Misra, 2010). *D. radiodurans* cells devoid of this protein become hypersensitive to γ radiation and lose the ability to repair damaged DNA. Both pyrroloquinoline quinone (PQQ) an antioxidant and linear DNA stimulated the DR2518 recombinant protein kinase activity *in solution*. Molecular mechanism underlying sensing of radiation effects by DR2518 and regulation of the activity of downstream factors regulating γ radiation resistance is not clear and would be worth investigating.

Here, we characterized DR2518 (herewith designated as RqkA, a Radiation and pyrroloquinoline quinone (PQQ) inducible protein kinase) as a eSTPK, and have identified the amino acids involved in catalytic and regulatory functions of this kinase. Mutants of these amino acids showed different levels of kinase activity and proportionally complemented the loss of γ radiation resistance in Δ*dr2518* cells of *D. radiodurans*. Further, we identified different DNA metabolic proteins including PprA in the proteome of *D. radiodurans* as substrates for RqkA. Recombinant RqkA could phosphorylate PprA at threonine 72 (T72), serine 112 (S112) and threonine 144 (T144) sites and phosphorylated PprA (P-PprA) showed improved functions as compared to unphosphorylated PprA (UP-PprA). The T72AT144A ($PprA^{T72T144}$), T72AS112A ($PprA^{T72S112}$) and S112AT144A ($PprA^{S112T144}$) double mutants and T72AS112AT144A ($PprA^{T72S112T144}$) triple mutant of PprA were studied for *in vivo* phosphorylation at Ser/Thr (S/T) and functional complementation in *pprA::cat* cells (hereafter referred as *pprA* mutant). The reduced phosphorylation of T72A single mutant by purified RqkA was correlated with reduced functional complementation in *pprA* mutant. Unlike wild type PprA ($PprA^{WT}$), the $PprA^{T72S112}$ and $PprA^{T72S112T144}$ mutants did not undergo S/T phosphorylation *in vivo* and also failed completely, in complementation of PprA loss in *D. radiodurans*. These results suggested that K42 of RqkA is catalytically important for its kinase activity, which along with the phosphorylation of DNA repair protein like PprA play important roles in radiation resistance of *D. radiodurans*.

2. Materials and methods

2.1. Bacterial strains and materials

Details of the bacterial strains and plasmids used in this study are summarized in Table S1. The wild type *D. radiodurans* R1 (ATCC13939) was a generous gift from Professor J. Ortner, Germany (Schaefer et al., 2000). 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 agar plate at 32 °C in the presence of antibiotics as required. Shuttle expression vector pRADgro and its derivatives, p11559 and its derivatives were maintained in *E. coli* strain HB101 as described earlier (Misra et al., 2006; Charaka and Misra, 2012). Molecular biology grade chemicals and enzymes were procured from Sigma Chemicals Company, USA, Roche Biochemicals, Mannheim, Germany, New England Biolabs, USA and Bangalore Genie, India.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2013.08.011.

2.2. Cloning and site directed mutagenesis

Genomic DNA of *D. radioudrans* R1 was prepared as described previously (Battista et al., 2001). Site directed mutagenesis for generating K42A, S162A, T169A and S171A single and T169A/S171A double mutants of RqkA was carried out using pET2518 (Rajpurohit and Misra, 2010) as template and site specific mutagenic primers using site directed mutagenesis kit (New England Biolabs, USA) following manufacturers protocols. All the mutations generated by *in vitro* mutagenesis, were confirmed by sequencing. For cloning in pRADgro, the coding sequences of all the mutated derivatives of RqkA were PCR amplified from respective plasmids using gene specific primer 2518F and 2518R and cloned at *Apa*I and *Xba*I sites in pRADgro. The resulting plasmids containing *rqkA* gene with S162A, K42A, T169A and S171A mutations were named as pGroS162, pGroK42, pGroT169, pGroS171 and pGroT69S71, respectively (Table S1). Plasmids were transformed into Δ*dr2518* mutant and recombinant clones were scored in the presence of chloramphenicol (5 mg/ml) and the presence of plasmid was confirmed by restriction analysis. The *pprA* mutagenesis was carried out on plasmid pETpprA (Kota and Misra, 2006) using site-specific mutagenic primers as described above. Both wild type, and T72A and T72D mutant alleles of *pprA* were PCR amplified using PprAF and PprAR primers and cloned at *Nde*I and *Xho*I sites in p11559 (Lecointe et al., 2004) and recombinant plasmids pSpecpprA, pSpk72A and pSpk72D, were obtained (Table S1). In parallel, the (His)6 tag (5′-GGAATTCCATATGCATCATCACCATCACCACGAG CTCGTC-3′) was cloned at *Nde*I and *Sac*I sites located downstream to promoter and upstream to *Xho*I in pVHS559 (Charaka and Misra, 2012) to yield pYHhis559. The wild type, T72A, S112A, T144, T72AT144, T72AS112A, S112AT144 and T72AS112AT144 mutant alleles of *pprA* were PCR amplified and cloned at *Sac*I and *Xho*I sites in pYHhis559 and recombinant plasmids pYHpprA, pYHT72, pYHS112, pYHT144, pYH7214, pYH7212, pYH1214 and pYH721214, respectively were obtained. Details of primers used in mutagenesis, sequencing and cloning on these constructs will be provided upon request. These plasmids were transformed into *D. radiodurans*, *pprA* mutant and Δ*rqkA* mutant as required and transformants were induced with 5 mM IPTG and an inducible expression of recombinant protein was confirmed by immunoblotting using PprA antibodies.

2.3. Protein purification

E. coli BL21 (DE3) pLysS harboring recombinant plasmid expressing different derivatives of RqkA and PprA (Table S1) were induced with 500 mM IPTG and the expression of recombinant proteins was confirmed by SDS-PAGE analysis. Recombinant proteins containing hexahistidine tag at N-terminus was purified using nickel affinity chromatography using modified kit protocol. In brief, the cells were incubated in buffer containing 50 mM Tris–HCl, pH8.0, and 300 mM NaCl containing 0.5 mg/ml lysozyme for 30 min on ice. The mixture was sonicated for 5 min on ice with 30 s pulses at 1 min interval. Pellet containing insoluble RqkA was collected by centrifugation at 12,000 × *g*. The recombinant protein was extracted from pellet with buffer containing 50 mM Tris–HCl pH 7.6, 300 mM NaCl, 10 mM β-mercaptoethanol and 0.5% Sarkosyl and incubated overnight at 4 °C. The mixture was centrifuged at 12,000 × *g* and supernatant containing recombinant protein was further treated with 1% Triton-X-100 and 10 mM CHAPS for 10 min on ice. Recombinant RqkA was further purified using nickel affinity chromatography protocols for purification under nondenaturing

conditions as described in kit above. Transgenic *E. coli* expressing PprA on pETpprA was used for purification of PprA as described in (Kota and Misra, 2006).

2.4. In vitro protein kinase activity assay

For the detection of autophosphorylation of RqkA and K42A, S162A, T169A, S171A and T169A/S171A mutants, the ~200 ng of purified recombinant proteins were incubated in 25 μL of kinase buffer (70 mM Tris–HCl pH 7, 5 mM DTT, 10 mM $MgCl_2$, 100 μM ATP, 1 μCi of [γ-^{32}P]-ATP) for 30 min at 37 °C. For transkinase activity, the ~100 ng purified recombinant RqkA kinase and its derivatives were incubated with ~200 ng of either maltose binding protein or PprA and its derivatives, and different putative phosphoprotein substrates. Deinococcal-single strand DNA binding protein (drSSB) and BSA were taken as controls. Reaction mixtures incubated at 37 °C for 30 min were separated on SDS-PAGE and protein phosphorylation was detected by autoradiography.

For large-scale phosphorylation of PprA for DNA protein interaction studies, the PprA was treated with thrombin and histidine tag was removed. The purified substrates were then incubated with His-RqkA in 1:1 molar ratio in kinase buffer (70 mM Tris–HCl pH 7, 5 mM DTT, 10 mM $MgCl_2$, 100 μM ATP) for 30 min at 37 °C. The mixture was passed through Ni-NTA matrix and PprA was recovered from flow through. Proteins were concentrated, checked for phosphorylation and used for further studies.

2.5. Cell survival studies

D. radiodurans R1, Δ*dr2518* mutant (Rajpurohit and Misra, 2010) and *pprA* mutant (Narumi et al., 2004) harboring respective vectors, and recombinant plasmids separately were generated. Cells expressing RqkA and its S162A, K42A, T169A, S171A and T169A/S171A derivatives and the PprA and its mutant derivatives were treated with different doses of γ radiations at dose rate 5.87 kGy/h Gamma cell 5000, ^{60}CO (Board of Radiation and Isotopes Technology, DAE, India) as described earlier (Misra et al., 2006). In brief, the bacteria grown in TGY medium at 32 °C were washed and suspended in sterile phosphate buffered saline (PBS) and treated with different doses of γ radiation and treated cells were spread on TGY agar plate supplemented with kanamycin (10 mg/ml) and chloramphenicol (5 mg/ml) as required, and the colony-forming units were recorded after 40 h of incubation at 32 °C.

2.6. Electrophoretic mobility shift assay

The 33 nucleotides long dsDNA oligonucleotides was made by annealing equimolar ratio of 33F (5′-AGCACATCGGCTTCGCCGGG-TGACACAACCGCT-3′) with 33C (5′-AGCGGTTGTGTCACCCGGCG-AAGCCGATGTGCT-3′). It was labeled with [^{32}P] by polynucleotide kinase, and purified by G-25 column. The 0.2 pmole of labeled probe was incubated with increasing concentration (50 nM to 3.2 mM) of both P-PprA and UP-PprA in 10 ml of reaction mixture containing 10 mM Tris–HCL, pH 7.5, 50 mM NaCl and 1 mM DTT for 20 min at 37 °C. Products were analyzed on a 12.5% native polyacrylamide gel, dried and radioactive signals were visualized by autoradiography. Intensity of DNA in free form as well as bound to protein was quantified by ImageJ software. The fraction of DNA bound to protein was plotted as a function of the protein concentration using Graphpad Prism 5. The Kd for curve fitting of individual plot was determined by the software working on the principle of least squares method applying the formula $Y = B_{max} * [X]/Kd + [X]$, where [*X*], is the protein concentration and Y is the bound fraction as described earlier (Das and Misra, 2011). Log equilibrium dissociation constant of unphosphorylated and phosphorylated PprA, was calculated by curve fitting using nonlinear regression of competition binding equation of one site Fit Ki in Graphpad PRISM software.

2.7. DNA ligation assay

The 400 ng *Bam*HI digest of pBluescript SK$^+$ plasmid DNA was incubated with increasing concentration (0.2–3.2 mM) of both P-PprA and UP-PprA in 10 mM Tris–HCL pH 7.5, 50 mM NaCl, and 1 mM DTT in 100 ml reaction volume for 30 min at 37 °C. To this, the 10 ml of 10× T4DNA ligase buffer and 2.5 units of T4 DNA ligase were added and incubated for 30 min at 16 °C. Reactions were stopped by adding 80 μL of a termination buffer containing 20 mM Tris–HCl, 20 mM EDTA, and 0.5% SDS and deproteinized with 100 μg of predigested proteinase K. Products were extracted with a phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with ethanol and analyzed on 1.0% agarose gels. DNA bands were visualized by ethidium bromide and quantified by densitometric scanning using Gene Genius tools (Syngene, UK).

2.8. Immunodetection of PprA and in vivo phosphorylation studies

The *in vivo* phosphorylation of PprA was checked by either labeling of the proteins with [^{32}P] phosphoric acid followed by immunoprecipitation (IP) or by immunobloting with phospho-Ser/Thr eipitops (P-STE) antibodies (Cat. No. 9621, Cell Signaling, USA), as described in respective figure legends. In former procedure, the cells were labeled with [^{32}P]-phosphoric acid under both γ irradiated at 6kGy dose and unirradiated conditions as described earlier (Rajpurohit and Misra, 2010). The irradiated cells were allowed to recover for 1.5 h while unirradiated cells were kept on ice for 1.5 h as control. The cells were collected and lysed by heating the cells in buffer containing 0.3% SDS, 1% β-mercaptoethanol and 50 mM Tris–HCl (pH 8) at 95 °C for 20 min. The cell free extracts obtained by centrifugation at 20,000 × *g*, were treated with DNaseI (50 mg/ml) and RNaseI (50 mg/ml) for 1 h at 37 °C. PprA protein was immunoprecipitated with PprA specific antibody; using Seize X protein-G Immunoprecipitation Kit (Pierce, Illinois) as described earlier. In brief, approximately 500 mg proteins equivalent cell free extract was incubated with PprA antibodies (Narumi et al., 2004) in binding buffer (140 mM NaCl, 8 mM sodium phosphate, 2 mM potassium phosphate and 10 mM KCL, pH 7.4). Mixture was incubated overnight at 4 °C and to this the Protein G agarose beads were added. Content was washed thrice with binding buffer and eluted with 500 mM NaCl in binding buffer. Eluted proteins were precipitated with 2.5 volume of ice-chilled acetone. Immunoprecipitate was dissolved in 2× Laemmli buffer and proteins were separated on 10% SDS-PAGE and autoradiogram was developed. For detection of phosphorylation by immunobloting with P-STE antibodies, the wild type and PprA mutant derivatives expressing in *D. radiodurans* cells harboring recombinant plasmids like pYHpprA, pYHT72, pYHS112, pYHT144, pYH7214, pYH7212, pYH1214 and pYH721214 under IPTG inducible promoter, were purified using metal affinity chromatography. Nearly equal amount of these proteins were separated on SDS-PAGE, and blotted with P-STE antibodies using protocols as described elsewhere in this manuscript. The intensities of immunosignals were quantified using Image J software and normalized by dividing with the intensity of coomossie blue stained protein bands of respective samples.

2.9. Generation of double mutant of D. radiodurans

The Δ*rqkApprA:cat* knockout mutant of *D. radiodurans* was generated using procedure as described earlier (Khairnar et al., 2008). In brief, pNOK2518 plasmid was constructed by cloning the 1 kb upstream and 1 kb downstream sequences to the dr2518 coding

region on restriction sites flanking to kanamycin selection marker (*nptII*) cassette as described earlier (Rajpurohit and Misra, 2010). The pNOK2518 was linearized and transformed into *pprA:cat* cells and transformants were scored on TGY agar plate supplemented with Kan (8 μg/ml) and chloramphenicol (5 μg/ml) and grown several generation under selection pressures of both the antibiotics. Homozygous replacement of *dr2518* (*rqkA*) by *nptII* was confirmed by PCR amplification using both internal as well as diagnostic pairs of primers in chromosome I and *nptII* specific primer. Details on the primers will be provided on request. The clone showing complete replacement of *dr2518* with *nptII* in *pprA* mutant was named as Δ*rqkApprA:cat* mutant.

Data presented without standard deviations are illustrative of a typical experiment and represent the average of three replicates wherein the variation among replicates was less than 15%. All experiments were repeated at least three times.

3. Results

3.1. RqkA has a typical eSTPK type structural organization

DR2518 (RqkA) a radiation inducible quinoprotein kinase is characterized for its role in radiation resistance and in the reassembly of shattered genome during post irradiation recovery of *D. radiodurans* (Rajpurohit and Misra, 2010). The N-terminus of RqkA contains a kinase domain similar to eukaryotic type Ser/Thr protein kinases (Hanks et al., 1988), while C-terminus has a putative PQQ interacting domain made of 7 tandem β propeller repeats. The kinase domain of RqkA shows 40% identity with PknB of *M. tuberculosis* (Ortiz-Lombardia et al., 2003). The lysine 42 (K42) located in N-terminal lobe of kinase domain of RqkA was structurally identical to the lysine 40 of PknB. The S162, T169 and S171 amino acids were present in the activation loop and hypothesized as the possible phosphoacceptor sites (Fig. 1). The RqkA had all the conserved motifs like P-loop, Helix-C, DFG motif, and catalytic loop, as known in other eSTPKs. These enzymes share well-conserved catalytic scaffold and active site architecture. The activation loop, to which phosphate is added during autokinase activity, is diverse and has multiple sites for phosphorylation in different members of this sub-family. The SMART server (http://smart.embl-heidelberg.de/) predicted the presence of seven-tandem β-propeller repeats at C-terminal domain of RqkA. Multiple sequence alignment of the C-terminal domain of RqkA with the conserved amino acid sequences of each class of β-propeller family proteins was carried out using Clustal X program analyses. It showed that the C-terminal sensory domain of RqkA had seven WD repeats of approximately 40 amino acids starting with glycine (G) and end with tryptophan (W) (Fig. S1). Topology analysis predicted RqkA as an N-in, C-out membrane protein with three transmembrane domains hypothetically placing the catalytic domain in cytoplasm and the sensory C-terminal domain possibly in periplasmic space (http://www.ch.embnet.org/software/TMPRED_form.html). The structural similarities of RqkA with eSTPKs and WD family proteins and γ radiation induced synthesis and activity (Rajpurohit and Misra, 2010); together suggested a strong possibility of RqkA as a sensor kinase, which might constitute a part of the hypothetical alternate DNA damage response mechanism in *D. radiodurans*.

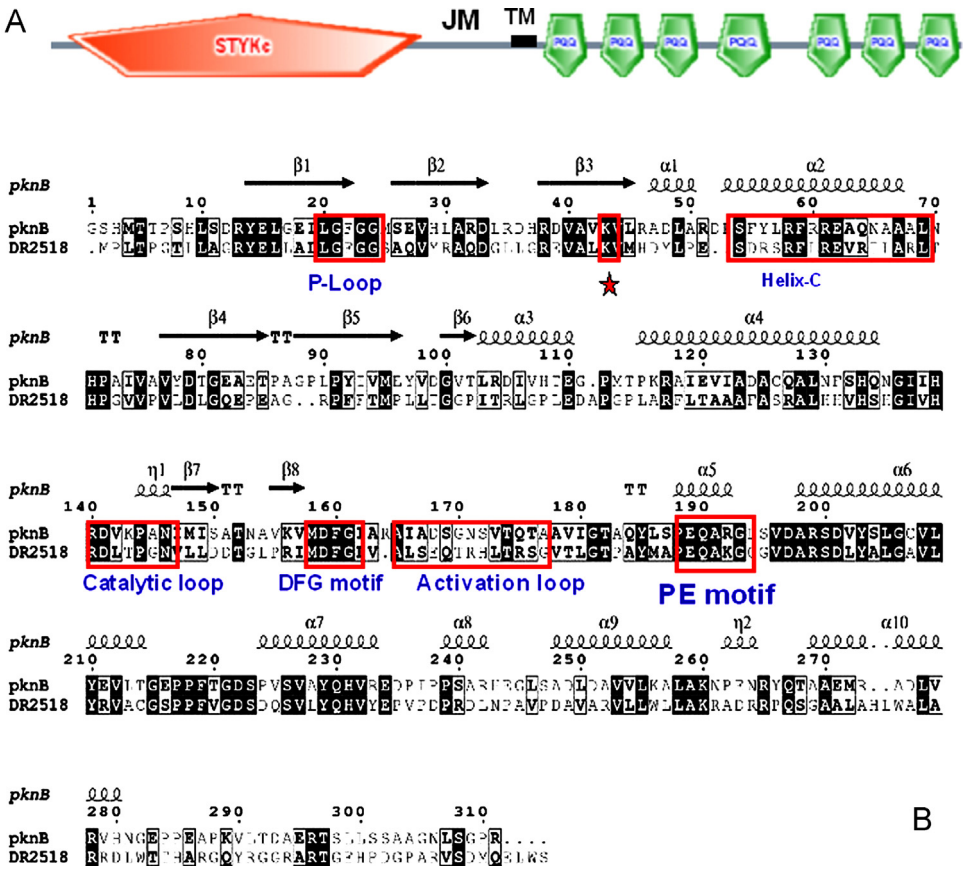

Fig. 1. Functional domain analysis in DR2518 (RqkA) kinase of *D. radiodurans*. Different regions of RqkA kinase representing various functions like Ser/Thr/Tyr/Kinase (STYKc), juxta-membrane region (JM), transmembrane region (TM) and PQQ binding motifs (PQQ) are shown in schematic representation of this protein (**A**). Primary sequence alignment of catalytic domain of RqkA kinase (DR2518) with PknB of *Mycobacterium* showing the functionally important conserved residues (marked in box) like lysine 42 in catalytic cleft, S162, T169 and S171 located in activation loop region of RqkA and their similarities with PknB (B).

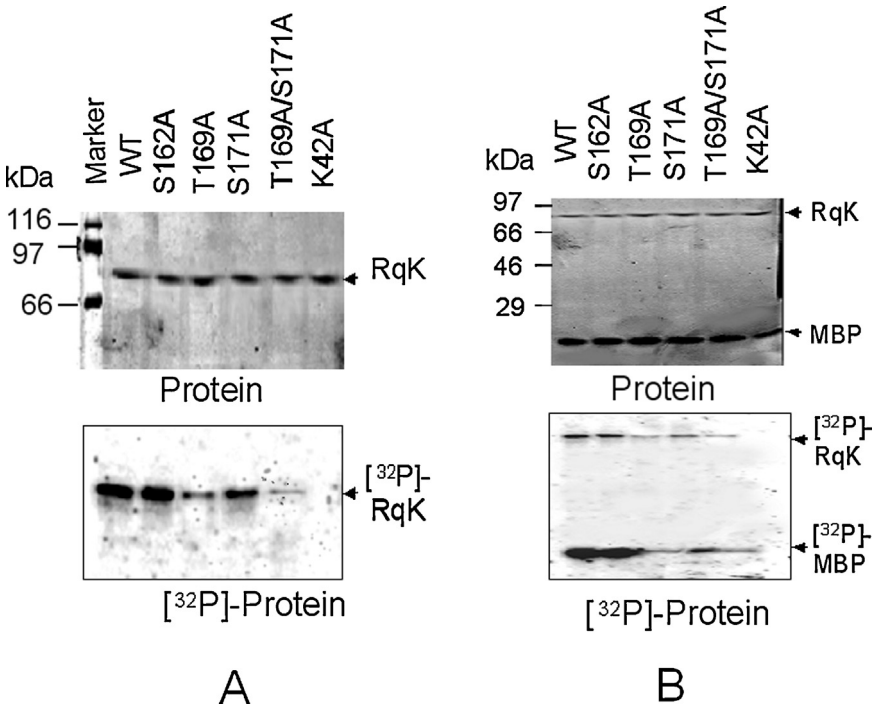

Fig. 2. Protein kinase activity characterization of different mutant derivatives of RqkA *in vitro*. Recombinant RqkA (WT) and its mutants like K42A, S162A, T169A and S171A and T169A/S171A were purified to near homogeneity and incubated with [^{32}P] γATP in kinase buffer (A). These proteins were also incubated with maltose binding protein (MBP) in kinase buffer supplemented with [^{32}P] γATP (B). Products were analyzed on SDS-PAGE, stained with coomassie blue (Protein), dried and [^{32}P]-phosphosignal was detected by autoradiography ([^{32}P]-protein).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2013.08.011.

3.2. Lysine 42 of RqkA is essential for its protein kinase functions

For determining the involvement of lysine 42 (K42) and serine 162 (S162), threonine 169 (T169) and serine 171 (S171) amino acids in the functions of RqkA kinase, the K42A, S162A, T169A, S171A single and T169A/S171A double mutants were generated and both autokinase and transkinase activities of purified recombinant proteins were checked on maltose binding protein (MBP) a ubiquitous phospho-substrate of STPKs. The K42A mutant showed neither autophosphorylation nor phosphorylation of MBP (Fig. 2). The T169A, S171A and T169A/S171A mutants of RqkA showed ~5%, ~10% and <1% of wild type protein kinase activity, respectively (Fig. 2). The S162A mutant showed nearly similar levels of kinase activity as that of wild type protein *in vitro* (Fig. 2). The activation loop is an important regulatory domain for kinase function, and is known for conformational and functional plasticity in other characterized eSTPKs (Johnson et al., 1996). Such structural and functional plasticity of the activation loop might account for the residual activity in T169A/S171A double mutant of RqkA. Loss of autokinase activity for a catalytically important conserved lysine and reduced autokinase activity for activation loop mutants have also been demonstrated for many other Hank family Ser/Thr kinases in bacteria including Pkn kinases of mycobacteria (Duran et al., 2005). Thus, these results suggested that RqkA is an eSTPK in this prokaryote, and the K42 of RqkA is catalytically essential for its activity while T169 and S171 are regulatory phosphorylation sites in its activation loop.

3.3. Protein kinase activity of RqkA is required for radiation resistance

RqkA mutants having different levels of kinase activities were expressed in Δ*dr2518* (Δ*rqkA*) mutant on the plasmids and the γ radiation survival of these recombinant cells was monitored. K42A an inactive mutant of RqkA, failed to recover the loss of γ radiation resistance in Δ*rqkA* cells, while T169A and S171A proteins could recover partially, which decreased further in double mutant T169A/S171A. Level of complementation was nearly 3–5 fold less by T169A and S171A derivatives and more than 20 folds less by T169A/S171A double mutant as compared to wild type RqkA at 10kGy γ radiation dose (Fig. 3) indicating the regulatory roles of T169 and S171 amino acids in kinase activity regulation of RqkA. Interestingly, the S162A derivative, which was as active as wild type

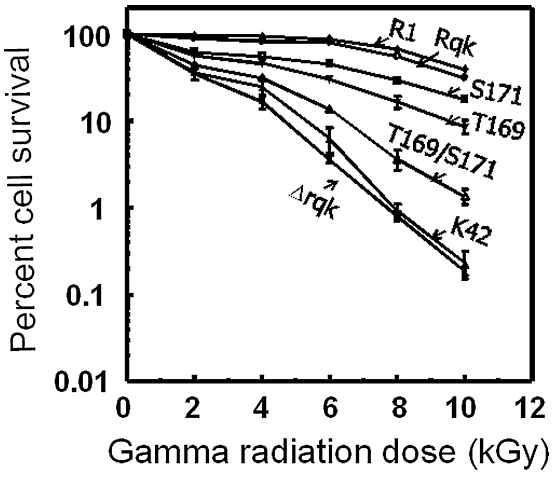

Fig. 3. Effect of kinase activity of RqkA (DR2518) on functional complementation in Δ*dr2518* mutant. The *Δdr2518* cells expressing wild type RqkA (Rqk) and K42A (K42), T169A (T169), S171A (S171) and T169A/S171A (T169/S171) proteins on pGro2518, pGroK42A, pGroT169A, pGroS171A, and pGroT69S71 plasmids respectively, were treated with different doses of γ-radiation and cell survival was compared with wild type *D. radiodurans* (R1) and *Δdr2518* mutant (ΔRqk) cells. The pGro162A showed a curve almost similar to wild type and therefore, omitted for clarity

Table 1
Selected proteins of *D. radiodurans* showing putative phosphorylation motif (-S/T-Q-X-Hydrophobic-Hydrophobic-) where T is phosphoacceptor and X can be any amino acid residues except the positively charged (Kang et al., 2005).

Protein ID (ORFs)	Protein name (annotated)	Putative phosphomotifs	No. motif present	Molecular weight (kDa)
DR_0002	DnaA	M**SQEIW**AD	1	52
DR_0012	ParB1	RA**SQLAG**L	1	31.8
DR_0400	FtsK-like protein	MM**SQVGA**K	1	107
DR_0911	DNA-directed RNA polymerase subunit β	LM**SQGAP**D	1	171.8
DR_1696	MutL	TV**SQLF**AR	1	57.9
DR_1984	Thymidine kinase	ATR**TQRLI**GG	1	22.3
DR_2417	β CASP family nuclease	FA**SQVYRI** PA**SQAHP**D	2	68
Dr_A0282	Ku 80 type DNA binding protein	PY**SQVAF**AG		54.8
DR_A0346	PprA	GL**SQWAA**LGEG VD **SQIAA**LA AAL**TQSL**QEA	3	32.2
DR_B0002	ParB type from megaplasmid (M1B)	IQ**SQGIL**QP	1	32

Bold letters indicate the phosphomotifs in different proteins.

also showed functional complementation very similar to wild type protein in Δ*rqkA* mutant (data not shown). These results supported that K42, T169 and S171 residues are essential for protein kinase activity of RqkA and the protein kinase activity of RqkA in turn, is required for its role in γ radiation resistance of *D. radiodurans*.

3.4. Deinococcal proteins have putative phosphorylation motifs of STPKs

The eSTPKs phosphorylate only that serine/threonine residues in the target protein, which are present in the characteristic motifs and such sequences are nearly conserved across the protein substrates for STPKs. Since, the functional and structural domains of RqkA are similar to eSTPKs, the proteome of *D. radiodurans* R1 was scanned using www.scansite.mit.edu motif search engine for the presence of phosphomotifs either like "X-X-S/T-Q-X-Hydrophobic-Hydrophobic-" (PMotif1) where T is phosphoacceptor and X can be any amino acid except with positive charge (Kang et al., 2005) or like "XαααααTX(X/V)$(P/R)I" (PMotif2), where T is phosphoacceptor site, $ a large hydrophobic residue and X any amino acid (Prisic et al., 2010). We observed that there are many proteins of *D. radiodurans* genome that have conserved phosphomotifs similar to that observed in STPKs phosphosubstrates (Tables 1 and 2). This includes the DNA metabolic proteins either known from their characterized functions or hypothetical but have domains relate the functions associated with DNA metabolism, and cell division proteins. This indicated that *D. radiodurans* genome encodes proteins with putative phosphomotifs belonging to different functional categories including DNA metabolism.

3.5. Purified RqkA kinase could phosphorylate proteins involved in DNA metabolism

Some of the deinococcal proteins having eSTPK specific phosphomotifs (Tables 1 and 2) were purified from recombinant *E. coli* and checked for phosphorylation with purified RqkA kinase. These include PprA, ParB1 (ParB homologue in chromosome I), ParB3 (ParB homologue in megaplasmid), DRA0282 and DR2417, and the single stranded DNA binding protein (drSSB) of *D. radiodurans* as control. Among these, the PprA showed relatively higher levels of phosphorylation with RqkA, as compared to ParB1, ParB3, DRA0282 (a Ku homologue) and DR2417 while drSSB phosphorylation was not detected (Fig. 4A). The specificity of PprA phosphorylation by RqkA kinase was also checked with different mutant derivatives of RqkA. Results showed the K42A was unable to phosphorylate PprA while T169A, S171A and T169A/S171 proteins phosphorylated PprA at different levels (Fig. 4B). Amongst these PprA (Narumi et al., 2004) and DRA0282 (Das and Misra, 2011) have been shown

Table 2
Selected proteins of *D. radiodurans* showing putative phosphorylation motif "X-X-T-Q/D/E-X/V-$-X-$", where T is phosphoacceptor site and α is an acidic residues, $ a large hydrophobic residue and X any amino acid (Prisic et al., 2010).

Protein ID	Protein name	Putative phosphomotif	Occurrence (No. of time)	Size kD
DR_A0065	HU protein	VAK**T**QLVEMV	1	12.289
DR_1424	DnaJ	VE**T**QQVCPTC	1	40.23
DR_2069	DNA ligase	LD**T**DDFTFTG AE**T**EAAPAES LV**T**QLLHEG	3	75.5
DR_0507	DNA polymerase III subunit alpha	LAM**T**DHGNM	1	149.2
DR_2263	Hypothetical	DAR**T**QVADLV	1	23.03
DR_0493	Fpg (MutM)	RN**T**ERAHGRQ	1	30.8
DR_1984	Thymidine kinase	TR**T**QRLIGGQ	1	22.2
DR_A0344	LexA	QV**T**DRARAA	1	22.3
DR_A0346	PprA	AL**T**QSLQEA	1	32.2
DR_2340	Protein RecA	VN**T**DELLV	1	38.14
DR_1089	RecF	GE**T**EAYVRA LG**T**EIMLFRR	2	39.14
DR_0198	RecR	LEY**T**DEVTLG	1	23.7
DR_0939	Rex	LQ**T**QDLHLPE	1	25.09
DR_0912	RpoB	VVLQ**T**QDLHLPEA GDI**T**EVIPLP	2	128.7
R_0911	RpoC	KPK**T**QAVVAD RSL**T**DLLGGK	2	171.3
DR_0440	RuvC	LT**T**ESAWLMP	1	19.6
DR_2509	Hypothetical	RFT**T**QRARALGA	1	14.86
DR_1922	SbcC	DIE**T**QAAEAGR	1	100
DR_0689	UDG	EL**T**EDIPGFVA	1	27.7
DR_1771	UvrA	SEV**T**DRLLAG	1	112.1
DR_1354	UvrC	GDK**T**DLIEMAQ	1	68.9
DR_0012	ParB1	TG**T**QVQTL	1	31.8
DR_B0002	ParB of Megaplasmid	GL**T**EVPVIV	1	32
DR_A0282	Human Ku 80 type	GE**T**QILSNLQG	1	54.8

Bold letters in different proteins indicate the putative site of phosphorylation.

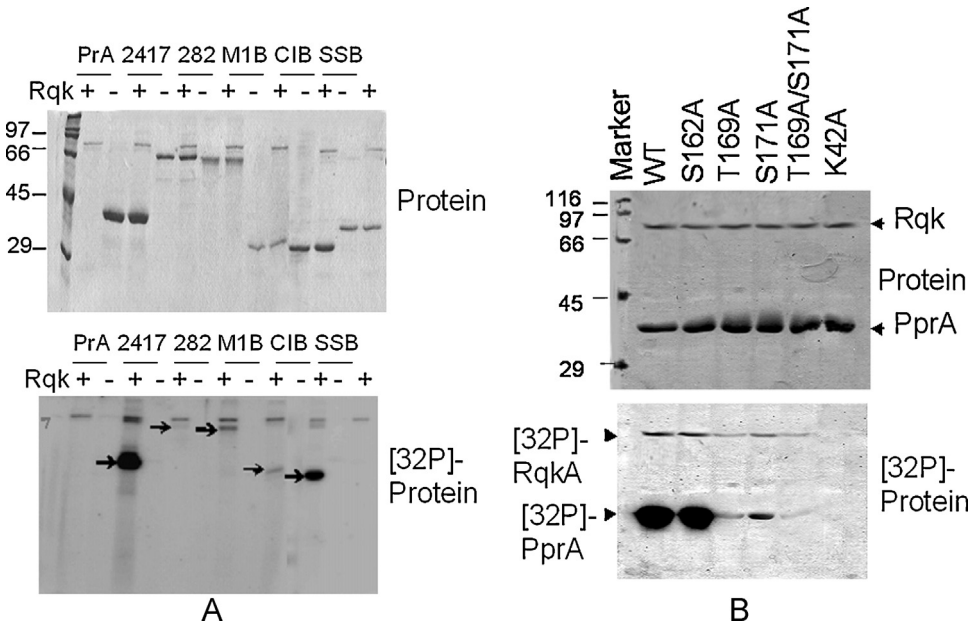

Fig. 4. Phosphorylation studies on identified protein substrates by RqkA kinase. Purified recombinant proteins PprA (PrA), ParB of chromosome 1, ParB1 (CIB), ParB type protein of megaplasmid (M1B), DR2417 (2417), DRA0282 (282) and single stranded DNA binding protein (SSB) of *Deinococcus radiodurans* were incubated with [32P]-γ-ATP in presence, and absence of RqkA (Rqk) kinase (A). Similarly, different mutants of RqkA kinase (Rqk) were incubated with purified PprA in the presence of [32P]-γ-ATP in kinase buffer (B). Products were separated on SDS-PAGE, stained with coomassie blue (protein) and autoradiography was done for imaging protein phosphorylation ([^{32}P]-protein).

for their roles in radiation resistance and ParB1 is characterized as a centromere binding protein involved in genome segregation (Charaka and Misra, 2012). Recently, DR2417 has been characterized as a novel member of β-CASP family nuclease (Das and Misra, 2012). These results suggested that RqkA kinase could phosphorylate several DNA metabolic proteins including PprA, of *D. radiodurans in vitro*. Since, the levels of PprA phosphorylation was highest as compared to other protein, and the mechanisms underlying PprA roles in radiation resistance and in the regulation of cell division and genome segregation (Devigne et al., 2013) are better understood, the effects of phosphorylation on some of the known functions of PprA and in radiation resistance, were evaluated.

3.6. PprA phosphorylation improves its activity in vitro

Since, PprA is a DNA binding protein (Narumi et al., 2004) and has been shown to stimulate DNA ligase activity *in vitro*, the effect of phosphorylation on these characteristics of PprA was checked. Results showed that the P-PprA binds 33mer dsDNA with nearly 4 fold higher affinity as compared to UP-PprA (Fig. 5A). The Kd values of phosphorylated and unphosphorylated PprAs were 0.348 ± 0.081 mM and 1.766 ± 0.70 mM, respectively (Fig. 5B). These results were further supported by competition binding assay with both unphosphorylated and phosphorylated PprA (Fig. 5C and D). The log equilibrium dissociation constants (Ki) of both forms of PprA with dsDNA, was calculated by curve fitting using nonlinear regression of competition binding equation of one site Fit Ki, in Graphpad PRISM software. The log Ki for P-PprA (63.82 ± 0.612) was nearly 4 fold higher than UP-PprA (16.3 ± 0.672) (Fig. 5E). These results suggested that phosphorylation of PprA increases its DNA binding affinity for dsDNA.

Since, PprA could stimulate DNA ends joining activity of DNA ligase *in vitro* (Narumi et al., 2004, Kota et al., 2010), the effect of PprA phosphorylation on stimulation of DNA ligase activity was examined. Results showed that both phosphorylated and unphosphorylated PprA could improve the total DNA end joining activity of T4 DNA ligase *in vitro* (Fig. 6A). However, the levels of intermolecular ligation by T4 DNA ligase increased several folds in the presence of P-PprA as compared to UP-PprA at its 0.4 mM concentration (Fig. 6B). This might suggest that the P-PprA could bring intermolecular ends closer for higher T4 DNA ligase activity. Interestingly, it was observed that the T4 DNA ligase activity stimulation by UP-PprA increased gradually as a function of PprA concentration, while this increase was very rapid with P-PprA and reached to the maximum at a much lower protein concentration as compared to UP-PprA control. At higher concentration of P-PprA, the total ligase activity was less than same concentration of UP-PprA. This could be accounted to higher DNA binding activity upon phosphorylation. Such types of T4 DNA ligase activity at inhibition at higher concentration of PprA has been observed earlier (Narumi et al., 2004) where it was argued that this effect was due to excessive binding of PprA with DNA molecules. Nevertheless, these results suggest that PprA undergoes phosphorylation by RqkA *in vitro* and the PprA phosphorylation improves its DNA binding activity and enhances it supports to intermolecular DNA end joining activity of T4 DNA ligase.

3.7. RqkA phosphorylates PprA at threonine 72, serine 112 and Threonine 144 sites in vitro

The phosphorylation sites of PprA were analyzed bioinformatically and observed that there are multiple putative sites to which PprA could be phosphorylated by STPKs. These include serine 28, threonine 72, serine 86, serine 90, serine 112, threonine 144, threonine 147, serine 153, threonine 269/270 and serine 274, which were present either in the consensus phosphosite or in close vicinity to this motif. Therefore, all these sites were mutagenized with alanine, recombinant proteins were purified and their phosphorylation by RqkA was examined *in vitro*. Amongst these mutants, T72A showed maximum loss of phosphorylation by RqkA kinase (Fig. 7A). It may however, be noted that there are three other sites like S112, T144 and T269/270 showing reduced phosphorylation by RqkA kinase as compared to control. The levels of RqkA autophosphorylation when incubated with different PprA variants did not change (Fig. 7B). These results therefore, suggested

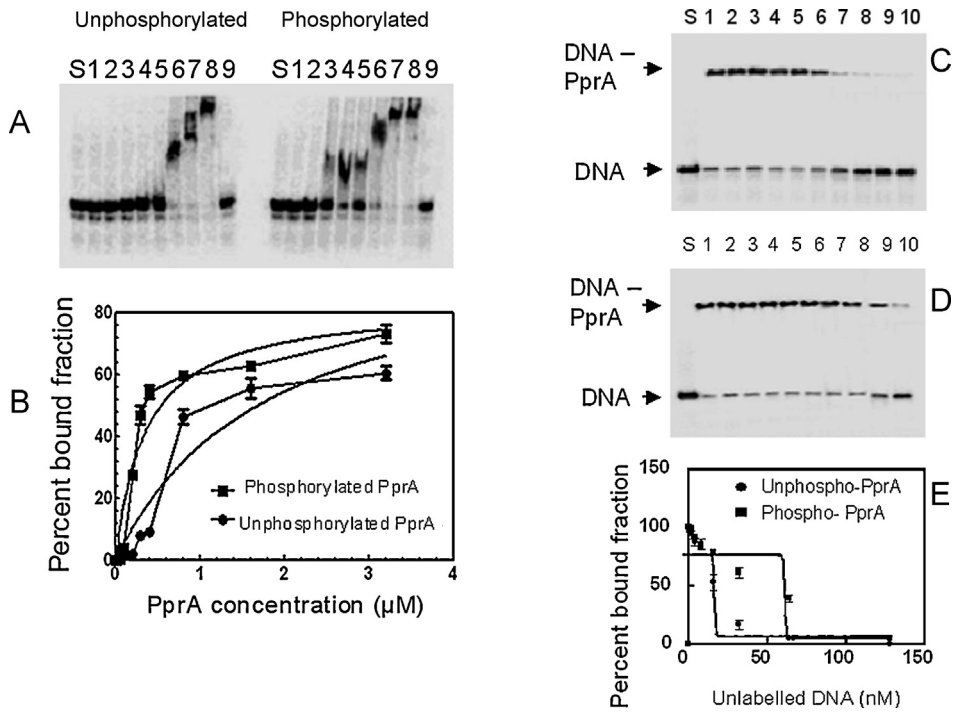

Fig. 5. Effect of phosphorylation on DNA binding activity of PprA. The 0.2 pmol of [^{32}P] labeled 33 mer dsDNA (S) was incubated with 0.05 (1), 0.1 (2), 0.2 (3), 0.4 (4), 0.8 (5), 1.6 (6) and 3.2 mM (7) of unphosphorylated and phosphorylated PprA, separately. The BSA in unphosphorylated (9) and RqkA (9) in phosphorylated panels were negative and protein kinase controls, respectively. Products were analyzed on native PAGE and autoradiogram was developed (A). Intensity of free DNA and DNA complexes with protein was determined by densitometry scanning. Percent fraction of DNA bound to proteins was calculated and plotted as a function of PprA concentration using Graphpad Prism 5 (B). Similarly, the 150 nM unphosphorylated (C) and phosphorylated (D) PprAs were incubated with 0.2 pmol of [^{32}P] labeled 33mer dsDNA (S) in 10 ml reaction mixture and chased with 0.25 (1), 0.5 (2), 1.0 (3), 2.0 (4), 4.0 (5), 8.0 (6), 16.0 (7), 32.0 (8), 64.0 (9) and 128 nM (10) cold DNA and products were analyzed on 12.5% native PAGE. Autoradiograms were developed and the intensity of individual bands was determined by densitometry scanning. The percent bound fraction of DNA to protein was calculated and plotted as a function of protein concentration (E). Both Kd and Ki values were determined for curve fitting of individual plot using nonlinear regression equation using Graphpad PRISM software, as described in methods.

that RqkA kinase phosphorylates PprA largely at T72 but to a little extent also at S112 and T144 amino acids.

3.8. *PprA is phosphoprotein in vivo and T72, S112 and T144 are sites for phosphorylation*

The possibility of PprA undergoing *in vivo* phosphorylation was monitored in the cell free extract of radiolabeled wild type, Δ*rqkA*, and *pprA* mutant cells by immunoprecipitation using antibodies against PprA. A [^{32}P] labeled phosphoprotein was detected in immunoprecipitate of wild type and Δ*rqkA* mutant cells extract, while cell free extract of *pprA* mutant did not cross-react with PprA antibodies (Fig. 7C). Absence of PprA-antibody reaction with any phosphoproteins in *pprA* mutant while its doing so in wild type ascertained the specificity of antigen-antibody reactions. On the other hand the cross reactivity of PprA antibodies with phosphoprotein in Δ*rqkA* cells and enhanced PprA phosphorylation in unirradiated Δ*rqkA* mutant cells are intriguing observations. Although the molecular basis of PprA phosphorylation in RqkA mutant is not clear, it might be possible that some other kinases are phosphorylating PprA in absence of RqkA. It may be noted that *D. radiodurans* genome encodes 11 Pkn2-type kinases (Makarova et al., 2001). The possibility of RqkA specific phosphorylation negatively regulating PprA phosphorylation on other sites/other kinases may be speculated. Effect of phosphorylation affecting kinase substrate interaction by changing the conformation of protein has been demonstrated. Therefore, in order to get deeper insights on the regulation of PprA phosphorylation by STPKs in *D. radiodurans* and the sites undergoing STPK mediated phosphorylation *in vivo*, both double and triple mutants of PprA were generated for T72, S112 and T144 residues. The recombinant proteins were expressed in *D. radiodurans*, purified and levels of phosphorylation was monitored in γ treated and untreated cells. Results showed that the levels of phosphorylation in PprA^{T72AS112A} and PprA^{T72AS112AT144A} proteins were nearly absent in cells grown under both unirradiated and irradiated conditions (Fig. 8A). Further we observed that the levels of *in vivo* phosphorylation in T72A (PprA^{T72A}) and PprA^{T72AT144A} derivatives of PprA were significantly higher in untreated cells than respective γ radiation treated controls (Fig. 8B). This agrees with the pattern of *in vivo* phosphorylation of PprA detected in Δ*rqkA* cells under unirradiated conditions (Fig. 7C), supporting although indirectly, that T72 is the specific site of PprA phosphorylation by RqkA. Furthermore, the comparison of the levels and trends of S/T phosphorylation in PprA^{T72A}, PprA^{T72AT144A} and PprA^{T72AS112A} together indicated that S112 and T144 are the other sites in PprA for STPK phosphorylation, and T72 phosphorylation seems to negatively regulates the PprA phosphorylation on these sites. These results suggested that PprA undergoes *in vivo* phosphorylation in *D. radiodurans* and T72, S112 and T144 are the potential sites of PprA phosphorylation by STPKs in *D. radiodurans.*

3.9. *Ser/Thr phosphorylation of PprA supports its role in radioresistance of D. radiodurans*

Results presented so far suggested that RqkA kinase primarily phosphorylates PprA at T72 residue and T72 phosphorylation negatively regulate S/T phosphorylation on the other sites in response to γ radiation. Therefore, the PprA^{T72A} and PprA^{T72D} derivatives were generated and the complementation of these derivatives to PprA loss in *D. radiodurans* was checked. Purified PprA^{T72A} phosphorylation by RqkA kinase was reduced significantly (Fig. 8C). The expression of recombinant PprA^{WT}, and both PprA^{T72A} and

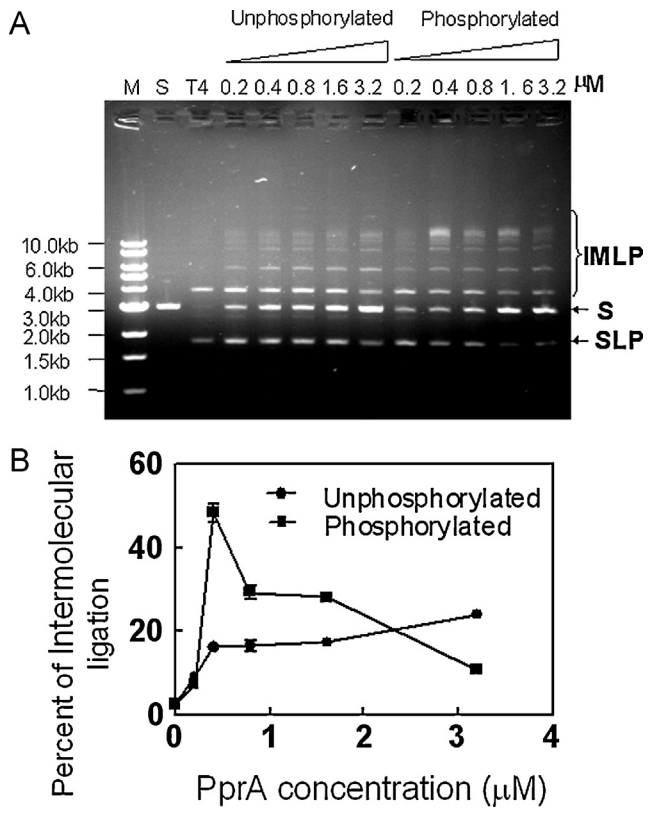

Fig. 6. Effect of PprA phosphorylation on T4DNA ligase activity stimulation. The 400 ng *Bam*HI digested pBluescriptSK+ (S) was ligated with T4 DNA ligase (T4) in the presence of increasing concentration (0.2–3.2 mM) of unphosphorylated and phosphorylated PprAs in ligation buffer at 16 °C. Products were analyzed on agarose gel (A). The intensity of both self ligated product (SLP) and intermolecular ligated products (IMLP) were determined by densitometry scanning and plotted as a function of protein concentration (B) using Graphpad PRISM 5.

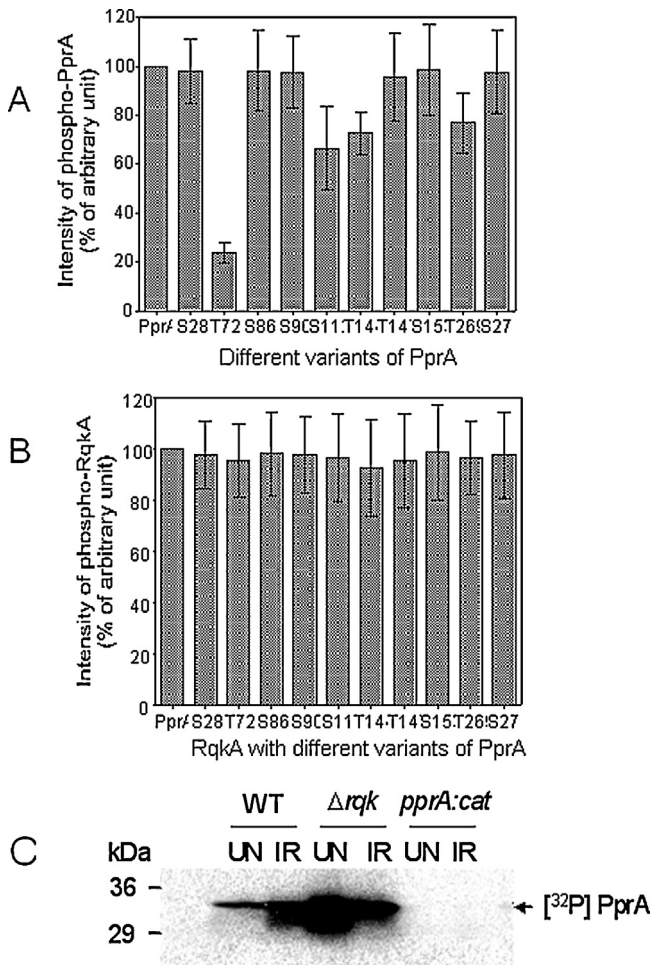

Fig. 7. *In vivo* phosphorylation of PprA and mapping of its phosphosites. The S28A (S28), T72A (T72), S86A (S86), S90A (S90), S112A (S112), T144A (T144), T147A (T147), S153A (S153), T269A (T269) and T274A (T274) mutants of PprA were generated through site directed mutagenesis and purified proteins were incubated with and without recombinant DR2518 (RqkA) in the presence of [^{32}P] γ ATP, and the levels of phosphorylation of PprA derivatives in the presence of RqkA (A) and autophosphorylation of RqkA itself (B) was determined densitometrically. For *in vivo* phosphorylation, the [^{32}P] labeled phosphoproteins of *D. radiodurans* R1 (WT) and its RqkA deletion mutant (Δ*rqkA*) and PprA mutant (*pprA:cat*) grown under normal conditions (UN) and treated with 6kGy γ radiation (IR), were precipitated with antibodies against PprA. Immunoprecipitates were analyzed on SDS-PAGE and the presence of [^{32}P] PprA was detected by autoradiography (C).

PprA^{T72D} derivatives was ascertained by immunoblotting (Fig. 8D). The γ radiation survival of the cells expressing PprA^{WT} showed nearly complete recovery of γ radiation resistance in *pprA* mutant but not when PprA^{T72A} and PprA^{T72D} proteins were expressed *in trans* (Fig. 9A). The PprA^{T72A} and PprA^{T72D} proteins expressing cells showed ~10 and ~50 fold higher resistance as compared to *pprA* mutant, while nearly 2- and 1.3-log cycle higher sensitivity as compared to wild type cells respectively, at 12kGy dose of γ radiation (Fig. 9A).

Since, PprA^{T72A} protein continued to get phosphorylated though low levels, both *in vitro* and *in vivo*, the possibility to this residual phosphorylation keeping PprA partially active in spite of mutation in T72 site and thus showing partial complementation in *pprA* mutant was hypothesized. In order to understand the significance of S/T phosphorylation in the role of PprA in radioresistance, the different mutants of PprA showing different levels of *in vivo* phosphorylation (Fig. 8B) were expressed in *pprA* mutant and these cells were monitored for cell survival in response to γ radiation. Interestingly, we observed that levels of functional complementation were mostly proportional to the levels of *in vivo* phosphorylation (Fig. 9B). Two proteins namely PprA^{T72AS112A} and PprA^{T72AS112AT144A}, which were nearly phosphonegative *in vivo* did not complement to the loss of γ radiation resistance in *pprA* mutant (Fig. 9B) indicating that PprA phosphorylation by STPK(s) contributes to its role in radioresistance of *D. radiodurans*. Further, the genetic interaction of *pprA* with *rqkA* in radioresistance of *D. radiodurans* was monitored in *pprA::cat*Δ*rqkA* double mutant. The deletion of *rqkA* in *pprA* minus background resulted to an increased radiosensitivity in *pprA* mutant, which became nearly similar to Δ*rqkA* mutant (Fig. 9C), indicating that *rqkA* plays a bigger role in radioresistance of this bacterium perhaps beyond its interaction with *pprA*. These results suggested that (i) PprA phosphorylation at T72, S112 and T144 sites contributes to radiation resistance of *D. radiodurans* and (ii) RqkA although phosphorylates PprA, it seems to have roles in radioresistance beyond PprA, possibly through phosphorylation of other DNA metabolic proteins in this bacterium.

4. Discussion

The involvement of RqkA in *D. radiodurans* response to DNA damage and radiation resistance has been demonstrated recently (Rajpurohit and Misra, 2010). This kinase is found to be a phosphoprotein *in vivo* and its transcription as well as phosphorylation is induced in response to γ radiation. This suggests the involvement of RqkA in radiation response of this bacterium. Molecular basis of this STPK role in radiation resistance was not known. Bioinformatic analysis showed that RqkA is a putative membrane kinase having kinase domain at N-terminal and PQQ binding motifs with WD

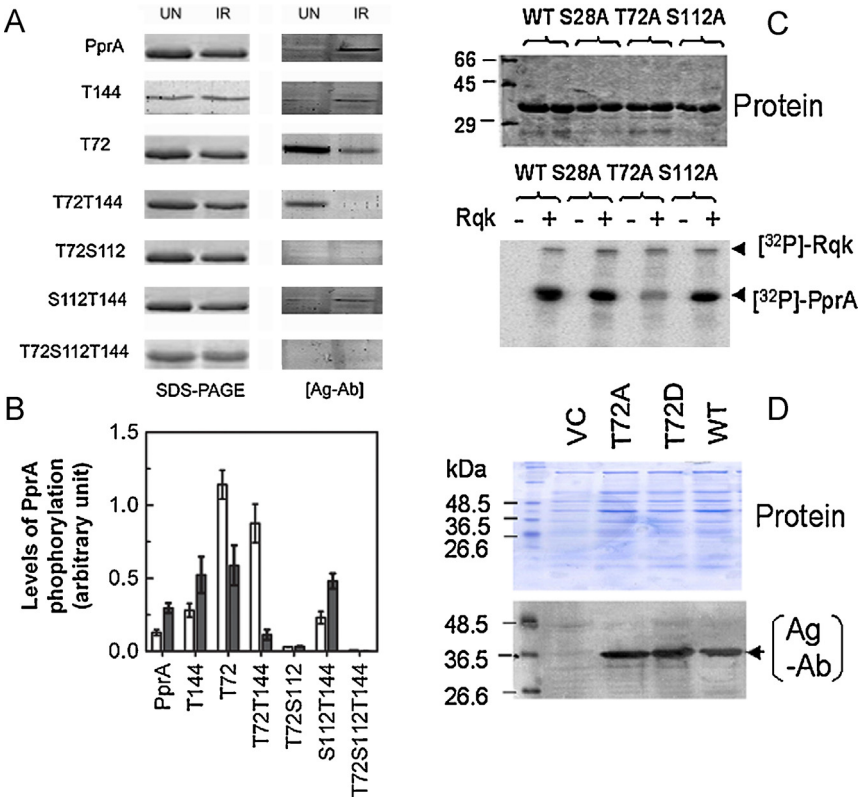

Fig. 8. *In trans* expression and checking of phosphorylation status in different mutant derivatives of PprA. (A) The wild type (PprA), T144A (T144), T72A (T72), T72AT144A (T72T144), T72AS112A (T72S112), S112AT144A (S112T144) and T72AS112AT144A (T72S112T114) derivatives of PprA were expressed *in trans* into *D. radiodurans*. These cells expressing these proteins under IPTG inducible promoter were grown under normal (UN) as well as post γ radiation exposure (IR). The recombinant proteins were purified and nearly equal amount (Protein) of these were probed with phospho-Ser/Thr epitops antibodies (Ag-Ab). (B) Both phosphobands and coomassie stained proteins (CBB) bands were scanned densitometrically and the ratio of phosphobands intensity to respective CBB bands intensity was plotted. (C) The serine 28 (S28A), threonine 72 (T72A) and serine 112 (S112A) sites of PprA (WT) were replaced with alanine. Purified recombinant proteins (Upper panel) were incubated with RqkA kinase (Rqk) in the presence of [^{32}P]-γ-ATP and phosphorylation was detected on autoradiogram (lower panel). (D) The T72A, T72D and wild type alleles were cloned in *Deinococcus* expression vector (VC) and transformed into *pprA* mutant. Recombinant proteins were separated on SDS-PAGE (Protein) and blotted with PprA antibodies (Ag-Ab).

repeats at C terminal. Similar types of C-terminal signatures having β-Propeller repeats have been found in β-subunit of G protein a classic WD40 family signaling protein; in low-density lipoprotein receptor belonging to YWTD class, and in the NHL domain of BraT protein (Edwards et al., 2003). The WD-repeat proteins perform wide range of functions, which include regulatory functions in signal transductions, RNA synthesis and processing, chromatin assembly, vesicular trafficking, cytoskeletal assembly, cell cycle control and apoptosis in eukaryotes (Li and Roberts, 2001). Although, the presence of Glycine-Histidine (GH) and Trypthophan-Glutamate (WD) dipeptides are the characteristics of WD repeats subfamily proteins, neither of these are conserved (Smith et al., 1999) across the members of this family. Interestingly, RqkA kinase has structure and function similarities with other eSTPKs. We found that K42 in the catalytic domain and T169 and S171 in the activation loop of RqkA kinase are required for its catalytic functions, and the kinase activity of RqkA, is required for its role in γ radiation resistance of *D. radiodurans*. Furthermore, we have also shown that RqkA kinase activity is stimulated by PQQ (Rajpurohit and Misra, 2010). PQQ also works as an antioxidant (He et al., 2003; Misra et al., 2004) and as an inducer of STPKs involved in cellular differentiation in mammals (Rucker et al., 2009). The level of expression of *pqqE*, a gene responsible for PQQ synthesis in *D. radiodurans* (Rajpurohit et al., 2008), also gets induced several folds in response to γ radiation. These findings might argue that the antioxidant nature of PQQ is probably acting as a sensor to oxidative stress effect of γ radiation, which collectively stimulate RqkA kinase activity leading to the phosphorylation of deinococcal proteins, and thereby regulating the functions of these proteins roles in radiation resistance. Thus, a strong possibility of RqkA kinase acting as a signaling enzyme in response to γ radiation could be suggested.

Amongst the number of DNA metabolic proteins identified as substrates for this kinase, the PprA is an important DNA repair protein unique to the members of Deinococacceae family. It has been shown that PprA is a phosphoprotein *in vivo* and the phosphorylation of PprA improves its dsDNA binding activity and also its support to intermolecular ligation functions of T4 DNA ligase *in vitro*. The protein phosphorylation mediated increase in DNA binding affinity has been reported earlier for single stranded DNA binding protein (SSB) from *Bacillus subtilis* (Mijakovic et al., 2006). They showed that phosphorylation of SSB increased its affinity for ssDNA by almost 200-fold in *vitro*. Mapping of RqkA phosphorylation sites on PprA showed that RqkA phosphorylates PprA largely at T72 and to a little extent at S112 and T144 residues *in vitro*. However, enhanced phosphorylation of PprA^{T72A} in wild type on one hand and PprA^{WT} in Δ*rqkA* cells on the other hand in cells growing under normal condition (Figs. 7C and 8A) might support that T72 is a preferred site for RqkA phosphorylation in PprA. Therefore, the effect of T72 replacement with alanine and aspartate on functional complementation in *pprA* mutant was checked. Both PprA^{T72A} a phospho-ablative mutant and PprA^{T72D} a phospho-mimetic mutant showed reduced yet some functional complementation in *pprA* mutant when compared with *pprA* mutant and these cells expressing PprA^{WT}. The molecular basis to this result is not clear. However, it may be hypothesized that

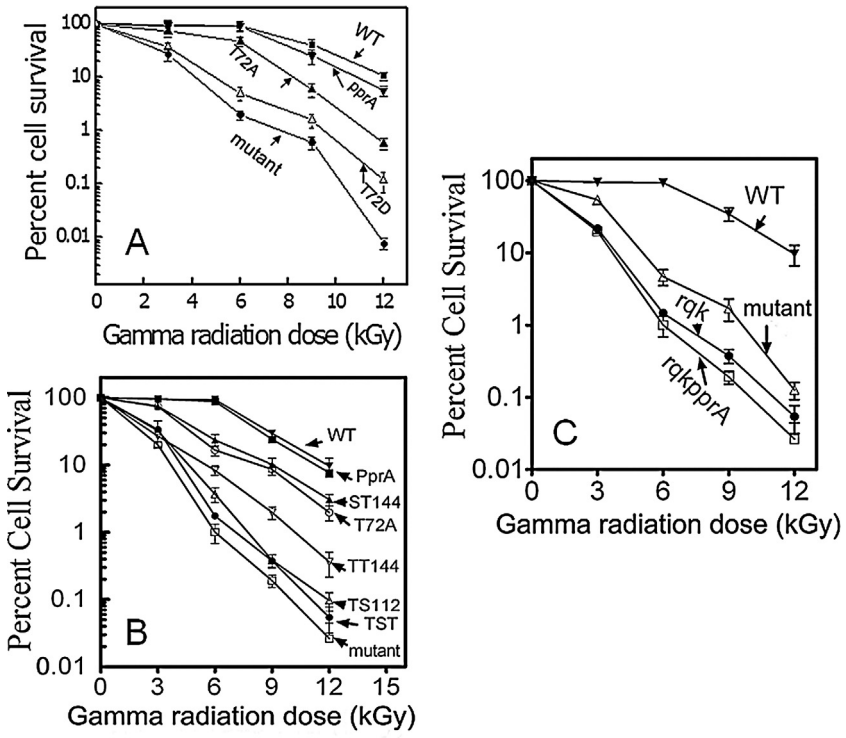

Fig. 9. Involvement of PprA phosphorylation in radiation resistance of *D. radiodurans*. (A) *D. radiodurans* R1 (WT), *pprA* mutant (mutant) and *pprA* mutant expressing wild type allele of *pprA* (pprA), T72A (T72A) and T72D (T72D) were exposed to different doses of γ radiation and cell survival was monitored. (B) Similarly, *pprA* mutant (mutant) expressing wild type (PprA), T72A (T72A), T72AS112A (TS112), T72AT144A (TT144), S112AT144A (ST) and T72AS112AT144A (TST) derivatives of PprA were exposed to different doses of γ radiation and cell survival was compared with *D. radiodurans* R1 (WT). (C) The *rqkA* deletion was introduced into *pprA::cat* background and *pprA::cat*Δ*rqkA* (rqkApprA) double mutant was obtained. The γ radiation survival of this double mutant was compared with *D. radiodurans* R1 (WT), *pprA* mutant (mutant), Δ*rqkA* (rqk) cells.

both PprA^{T72A} and PprA^{T72D} proteins are either inactive or partially active and the partial functional complementation by these mutant derivatives could be due to the phosphorylation of PprA at other S/T sites in absence of T72. The results obtained on phosphorylation of PprA^{T72AS112A} and PprA^{T72AS112AT144A} proteins (Fig. 8A) and their inability to complement the PprA loss in this bacterium (Fig. 9B) supported the hypothesis. Results reported earlier in case of cytosolic aconitase (Pitula et al., 2004) and Human Pregnane X Receptor (Pondugula et al., 2009) also showed the differential effects of phosphor-ablative and phosphor-mimetic mutant derivatives. Protein phosphorylation/dephosphorylation are well-characterized mechanism that living cells use for communicating the surrounding changes to cellular and genetic levels (Kennelly and Potts, 1996). PprA has been shown to exist with other DNA repair proteins, protein kinases and phosphoproteins in a multi-protein DNA processing complex identified from *D. radiodurans* (Kota and Misra, 2008). A TCS role in γ radiation resistance of *D. radiodurans* (Desai et al., 2011) is the other most recent findings corroborating protein phopshorylation roles in bacterial response to γ radiation. Stimulation of intermolecular ligation by phosphorylated PprA might suggest the regulatory roles of this protein during cohesin-like functions *in vivo*. Recently the intermolecular ends joining of linear DNA molecules resulting in multimer formation has been shown in case of native RecN, which has been therefore, named as bacterial cohesin-like protein (Reyes et al., 2010). These results indicated that PprA activity could be modulated through phosphorylation by STPKs including RqkA kinase. Thus, the results shown here collectively suggest that (i) the lysine 42 of RqkA is essential for its kinase activity, which is required for RqkA role in radiation resistance of *D. radiodurans*, (ii) there are DNA metabolic proteins in proteome of this bacterium, which could be substrates for RqkA, (iii) the phosphorylation of PprA at T72 seems to negatively regulating PprA phosphorylation at other sites, and (iv) phosphorylation of PprA improves its functions and eventually contribute to the PprA's role in radioresistance of *D. radiodurans*.

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