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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