RESEARCH LETTER

Increased synthesis of signaling molecules coincides with reversible inhibition of nucleolytic activity during postirradiation recovery of *Deinococcus radiodurans*

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Abstract

Deinococcus radiodurans tolerates extensive DNA damage and exhibits differential expression of various genes associated with the growth of the organism and DNA repair. In cells treated with γ radiation, the levels of cyclic AMP (cAMP) and ATP increased rapidly by differentially regulating adenylyl cyclase (AC) and 2′3′ cAMP phosphodiesterase. The levels of cAMP, ATP, AC and protein kinases were high when phosphodiesterase activity was low. These cells exhibited *in vivo* inhibition of nucleolytic function by reversible protein phosphorylation and contained the comparatively higher levels of total phosphoproteins. We suggest that *Deinococcus*, a prokaryote, uses DNA damage-induced signaling mechanism as evidenced by γ radiation-induced synthesis of secondary messengers and signaling enzymes.

Introduction

Protein phosphorylation constitutes an important regulatory network that controls the cellular functions including cell division, cellular differentiation and signal transduction in all organisms (Pawson, 1994). At molecular levels, this regulates metabolic functions such as enzyme activity modulation, protein trafficking, protein–protein and DNA–protein interactions and recycling of proteins (Ubersax & Ferrell, 2007). By reversible protein phosphorylation, the functions of proteins can be rapidly modulated without the need for new protein synthesis or degradation. This phenomenon is regulated by the relative abundance of stress-responsive protein kinases and phosphatases in the cells (Sefton & Hunter, 1998). In eukaryotes, the significance of reversible protein phosphorylation is amply illustrated by the involvement of DNA damage-induced signal transduction and protein kinase C-mediated signaling mechanism in cell cycle regulation (Sancar *et al*., 2004; Kitagawa & Kastan, 2005). The existence of such mechanisms and their implications in DNA strand break (DSB) repair and bacterial growth would be worth investigating in *Deinococcus radiodurans*, a bacterium that confers extraordinary tolerance to DNA damage and has acquired a large number of putative sensor kinases and response regulators (White *et al*., 1999) from other organisms (Makarova *et al*., 2001).

Deinococcus radiodurans R1 is characterized by its extraordinary radioresistance, which is primarily contributed by an efficient DSB repair and a strong oxidative stress-tolerance mechanism (Battista, 2000; Makarova *et al*., 2001; Blasius *et al*., 2008). *Deinococcus radiodurans* exposed to DNA damage showed a rapid and kinetic change in gene expression profile and a rapid protein turnover (Liu *et al*., 2003; Tanaka *et al*., 2004; Zhang *et al*., 2005). *Deinococcus radiodurans* shows a biphasic DSB repair mechanism (Daly & Minton, 1996). The phase I is characterized as the reassembling of shattered genomes into larger size molecules by extended synthesis-dependent strand annealing (Zahradka *et al*., 2006) followed by RecA-dependent slow cross-over events of phase II DSB repair. During this period, the shattered genome is first protected from nucleolytic degradation by end-capping proteins such as DdrA (Harris *et al*.,

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2004) and PprA (Narumi *et al.*, 2004) and then presumably undergoes processing by a still unknown mechanism, required for further steps in DSB repair. The DSB repair kinetics monitored on pulsed field gel electrophoresis (Slade *et al.*, 2009) and using [^{3}H]thymidine labeling *in vivo* (Khairnar *et al.*, 2008) show a rapid increase in DNA degradation upon γ irradiation, which is arrested within 30 min postirradiation recovery (PIR). Although the DNA damage-induced change in gene expression and protein turnover have been reported in *D. radiodurans*, the pathways that link DNA damage response to gene expression are not known. This study reports the effect of γ radiation-induced change in levels of signaling molecules in this prokaryote and the role of radiation-inducible protein kinase function in the modulation of nucleolytic activity during PIR of *D. radiodurans*.

Materials and methods

Bacterial strains and materials

Deinococcus radiodurans (ATCC13239) was a generous gift from Dr M. Schafer, Germany (Schafer *et al.*, 2000). Wild-type bacteria 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 as required, at 32 °C. The molecular biology-grade chemicals were obtained from Roche Molecular Biochemicals (Germany) and Sigma-Aldrich Chemical Company. Restriction endonucleases and the DNA-modifying enzymes were obtained from New England Biolabs, Roche Molecular Biochemicals, GE Healthcare (Sweden) and Bangalore Genei (India).

γ Irradiation and nucleotide-binding protein purification

Deinococcus radiodurans cells were irradiated with 6.5 kGy γ radiation on ice, at 6.471 kGy h^{-1} in a Gamma chamber (GC 5000, ^{60}Co., Board of Radiation and Isotopes Technology, DAE, India) as described earlier (Khairnar *et al.*, 2008). In brief, the exponentially growing cells were harvested and suspended in 1/5 vol. of normal saline. Cells were exposed with the required dose of γ radiation and diluted 10 times in fresh TGY broth. Cells were allowed to grow and aliquots were taken at regular intervals. Cell-free extract was prepared as described earlier (Kota & Misra, 2008). In brief, the cells were sonicated in a buffer (20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA, pH 8.0, 1 mM dithiothreitol and 1 mM phenylmethanesulfonyl fluoride) containing 5 mg mL^{-1} lysozyme. Lysate was centrifuged at 20 000 ***g*** for 15 min, clear supernatant was passed through a HiTrap Heparin (GE Healthcare) column and proteins were eluted with 500 mM NaCl.

Extraction and quantitative analysis of purine nucleotides

The purine nucleotides were extracted and quantified using the modified protocols described in studies of cerebellar granule cells (Giannattasio *et al.*, 2003). In brief, the cells were treated with lysozyme (2 mg mL^{-1}) for 1 h on ice and nucleotides then extracted with ice-cold 0.5 M perchloric acid (PCA). The pH of the PCA extract was adjusted to pH 7.5 with 0.5 M KOH and incubated for 30 min on ice. The potassium perchlorate precipitate was removed by centrifugation at 20 000 ***g*** for 15 min and the supernatant was used for HPLC analysis. The individual nucleotides were identified on the basis of their retention time in C-18 column and by spiking the complex spectra with corresponding standards. The peak area of each nucleotide was obtained as arbitrary units from the spectra recorded with unirradiated control and PIR samples and was then converted into yield mg^{-1} protein.

Enzyme activity measurement

The nuclease activity was measured as described earlier (Kota & Misra, 2008). The 500-ng heparin-purified proteins were incubated with 200 ng of 1-kb PCR product from *D. radiodurans* genome, in a buffer (10 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 15 mM KCl and 2% glycerol) for 20 min at 37 °C. For ATP and calf intestinal alkaline phosphatase (AP) treatment, the proteins were preincubated with these agents for 30 min at 37 °C. For phosphatase and protein kinase inhibitor treatment, the samples were treated with 10 mM sodium fluoride and different concentrations of protein kinase inhibitors, respectively, for 20 min. The treated samples were incubated with double-stranded DNA (dsDNA) substrate for 20 min at 37 °C and reaction products were analyzed on 1% agarose gel.

Protein kinase activity was measured as described earlier (Rajpurohit *et al.*, 2008). In brief, the cell-free extract was prepared from cells treated with γ radiation and equal amounts of protein (2 μg of each sample) were incubated with 50 μCi [^{32}P] γ ATP (2500 Ci mmol^{-1}) for 1 h at 37 °C. DNAse (50 μg mL^{-1}) and RNAse (50 μg mL^{-1}) were added and further incubated for 1 h. The mixture was passed through G-25 microspin columns (GE Healthcare) to remove the unincorporated radionucleotides and smaller nonproteinaceous phospho-contaminants. Incorporation of [^{32}P] was measured by scintillation counting and the counts mg^{-1} protein were presented.

The acid and alkaline phosphatases were assayed in 100 mM acetate buffer (pH 5.0) and 50 mM Tris-HCl buffer (pH 9.0), respectively, using disodium salt of *p*-nitrophenyl phosphate (pNPP) as described earlier (Bolton & Dean, 1972). In brief, 2 μg of total protein was incubated with the respective substrate in a corresponding buffer for 20 min. The levels of *p*-nitrophenol produced from pNPP substrate

were measured at 440 nm against substrate blank. The phosphatase activity was expressed as nmol *p*-nitrophenol formed min^{-1} mg^{-1} protein. Similarly, the total phosphodiesterase activity was measured in a reaction mixture containing 2 μg total protein and 1 mM bis-pNPP substrate in 50 mM sodium acetate, pH 5.0, at 30 °C as described earlier (McLoughlin *et al.*, 2004). The 2′,3′ cyclic AMP (cAMP) phosphodiesterase activity was measured using the procedure essentially as described by Kier *et al.* (1977). In brief, the assay mixture contained 40 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM 2′,3′ cAMP, 4 μg total protein and 10 units of AP (New England Biolabs). The reaction was initiated by the addition of substrate and assayed at 37 °C for 30 min. The release of free inorganic phosphate was determined using the method of Bencini *et al.* (1983).

The adenylyl cyclase (AC) activity was measured using a modified protocol described earlier (Post *et al.*, 2000). In brief, the α[^{32}P]-ATP was replaced with 500 μM ATP and cAMP was omitted from the standard reaction mixture. The other modifications were the use of caffeine in place of isobutylmethylxanthine and estimation of the levels of cAMP by HPLC, as described above.

All the experiments were repeated at least three times and results were reproducible. Data presented without statistical analysis are from a typical experiment.

Results and discussion

Levels of cAMP and ATP increased in response to γ irradiation

The purine nucleotide profile of exponentially growing unirradiated cells of *D. radiodurans* R1 was determined using ion-pair reverse-phase HPLC as described in Materials and methods. The peaks corresponding to ATP, cAMP, ADP, NAD^+ and GTP nucleotides were assigned on the basis of the retention time of respective standard in hydrophobic column and by spiking the spectra with known compound (Fig. 1). The levels of purine nucleotides were measured in the exponentially growing cells irradiated with 6.5 kGy γ radiation and the aliquots were taken at different time during PIR. The results of γ-irradiated cells were compared with unirradiated controls. The levels of ATP, GTP, NAD^+ and cAMP nucleotides showed significant changes during PIR (Fig. 2). The ATP and cAMP levels increased rapidly within 30 min, peaking at 1 h PIR. The levels of GTP and NAD^+ increased slowly and reached a maximum at 3 and 4 h PIR, respectively, and subsequently returned to unirradiated control levels. The levels of ADP did not change significantly during PIR. This indicated that the γ radiation-induced DNA damage affects the nucleotide metabolism in *Deinococcus*. The higher levels of these nucleotides could be accounted for by either increased synthesis and/or reduced degradation of individual species. However, the possibility that other nucleotides such as CTP, TTP and their derivatives also change during PIR cannot be ruled out. Earlier, it has been shown that the differential levels of AC and 2′,3′ cyclic phosphodiesterase activities determine the cellular levels of cAMP (Anderson *et al.*, 1973). Therefore, the possible regulation of these stress-responsive signaling enzymes in response to DNA damage was hypothesized.

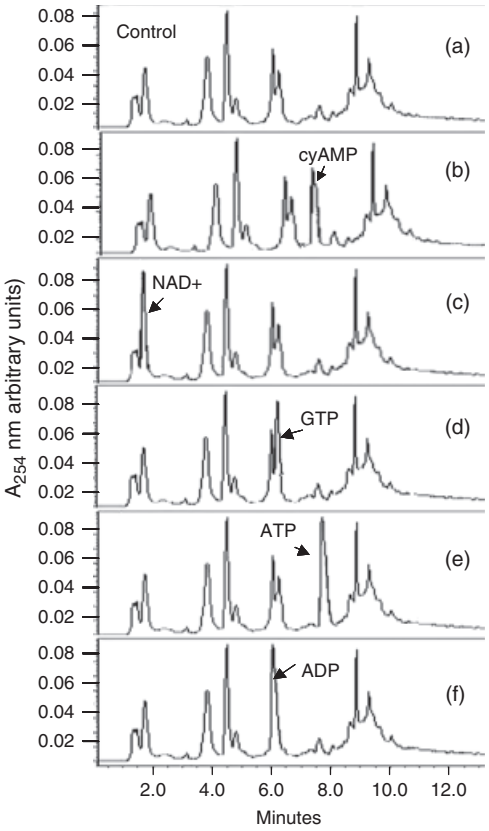

Fig. 1. The nucleotide profile of exponentially growing *Deinococcus* cells. The mid-log phase cells of *Deinococcus* were collected and total purine nucleotides were extracted with PCA and separated on HPLC as described in Materials and methods. The nucleotide profiles obtained from unirradiated cells were used as control (a), for comparing the spiked spectra with known nucleotides. The peaks corresponding to cAMP (b), NAD^+ (c), GTP (d), ATP (e) and ADP (f) were assigned by monitoring the spiking of that peak in the complex spectra on addition of one known nucleotide at a time.

AC and phosphodiesterases were differentially regulated in response to γ irradiation

The activity of the soluble protein kinases, 2′3′ cAMP phosphodiesterase (cyclic phosphodiesterase), total phosphodiesterases, AC and the phosphatases was measured in cells recovering from γ radiation effects (Fig. 3). The AC activity increased rapidly following γ irradiation and reached a maximum in 0.5 h PIR (Fig. 3a), during which

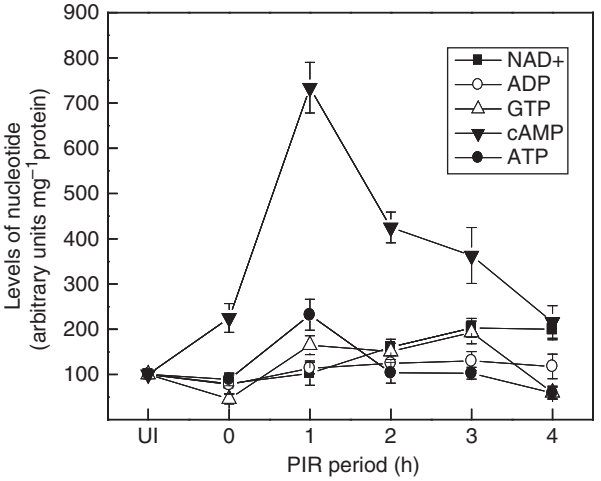

Fig. 2. Change in the levels of purine nucleotides during PIR of *Deinococcus radiodurans*. Total nucleotides were extracted with PCAs from unirradiated and γ-irradiated cells collected at 0, 1, 2, 3 and 4 h of PIR and their levels were determined as described in Materials and methods.

the activity of phosphodiesterases and phosphatases was low. Whereas the AP did not change significantly during PIR, the acid phosphatase increased nearly 1.5-fold from 1 h PIR (5.146 μmol min^{-1} mg^{-1} protein) to 4 h PIR (8.243 μmol min^{-1} mg^{-1} protein) (Fig. 3b). The levels of cyclic phosphodiesterase decreased rapidly in 1 h PIR followed by an increase of nearly threefold in 4 h PIR (Fig. 3c). These results might support the argument that the net increase in the cAMP levels was due to differential regulation of AC and cyclic phosphodiesterase activities in response to DNA damage. Although, *D. radiodurans* R1 genome does not annotate the classical bacterial AC and 2′3′ cAMP phosphodiesterase, it encodes for protein with a phosphodiesterase-type functional domain with nearly 30% genome without annotated functions, leaving the strong possibility that unknown proteins are responsible for these activities. The amino acid sequence of AC from *Escherichia coli* was subjected to multiple sequence alignment, which showed different levels of amino acid similarities with some of the deinococcal ORFs. Among them, DR_1433 showed close to 75% match with *E. coli* protein in PSIBLAST analysis. The

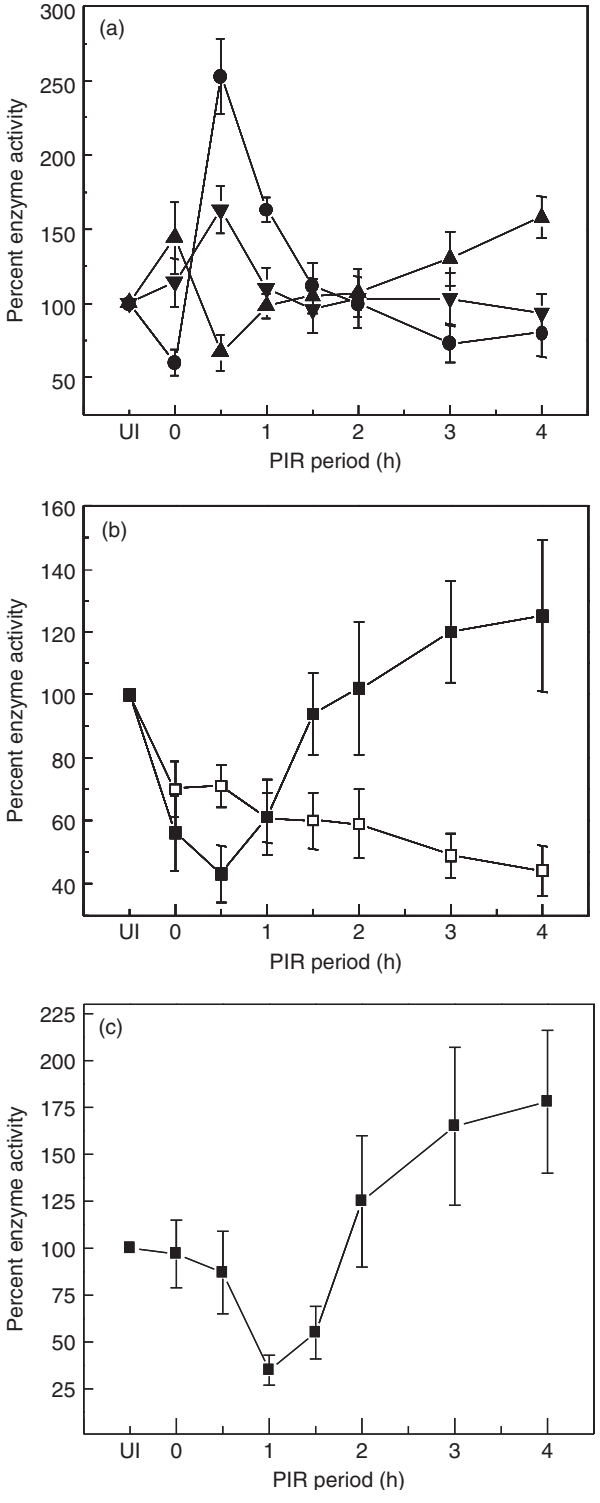

Fig. 3. Change in the levels of stress-responsive enzymes during PIR of *Deinococcus radiodurans*. The cell-free extracts of unirradiated and γ-irradiated cells collected at 0, 0.5, 1, 1.5, 2, 3 and 4 h of PIR were used for monitoring the total protein kinase (-▼-), AC (-●-) and phosphodiesterase (-▲-) (a). The acid phosphatase (-■-) and AP (-□-) activities were also measured using these samples (b). Phosphodiesterase activity was also measured using 2′,3′ cAMP as a substrate (c) and release of phosphorous was measured against substrate blank. The activity of all the enzymes was measured as the amount of products formed per unit time per milligram total proteins.

presence of AC and cyclic phosphodiesterase activities in cell-free extracts of this bacterium suggested the strong possibility of AC and cyclic phosphodiesterase activities containing uncharacterized proteins in bacterial genome and it will be interesting to investigate these activities separately.

Nucleolytic function was modulated by γ-induced reversible protein phosphorylation during PIR

Aliquots of γ-irradiated cells were collected during PIR and nucleotide-binding proteins were purified by heparin-sepharose affinity chromatography. Fractions were tested for nucleolytic activity on dsDNA substrate. Results showed the presence of nucleolytic activity in unirradiated and zero PIR-irradiated samples. This activity was completely absent in 1- and 2-h PIR samples (Fig. 4a) but reappeared in 3- and 4-h PIR samples. This indicated that the bacterium has an as yet unidentified mechanism to regulate the nuclease activity during different stages of PIR. It may be speculated that during early PIR, i.e. before 2 h PIR, the bacterium needs to protect its shattered genome and very low nuclease activity might be required for DSB end-joining, whereas at a later stage, i.e. after 2 h PIR, high recombinase functions are needed, which requires the high nuclease activity observed at 3 and 4 h PIR. Except for the unirradiated control, all the samples, including 1 and 2 h PIR, showed inhibition of nucleolytic function with 2 mM ATP (Fig. 4b), whereas this function was induced upon dephosphorylation of 1 and 2 h PIR samples with AP (Fig. 4c). These results suggested the involvement of protein phosphorylation in ATP attenuation of nucleolytic function. When AP-treated samples were incubated with ATP in the presence of 10 mM sodium fluoride, a phosphatase inhibitor (Mishra & Parnaik, 1995), the nucleolytic activity was inhibited in all PIR fractions, but not in unirradiated control (Fig. 5a). The 1-h PIR sample was dephosphorylated with AP and subsequently treated with sodium fluoride to inactivate phosphatase enzyme. When such samples were incubated with ATP in the presence or absence of protein kinase A (PKA) inhibitor (H89) and protein kinase C inhibitor (staurosporine) separately, the ATP attenuation of nucleolytic degradation was observed only in the absence of protein kinase inhibitors

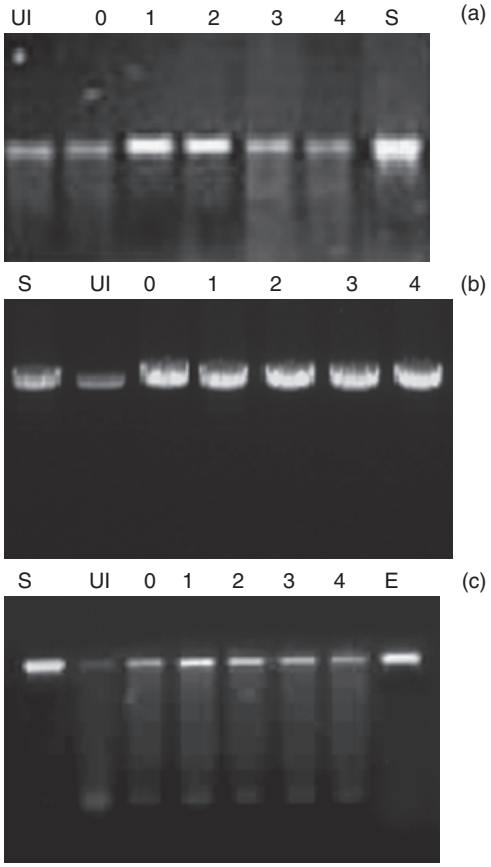

Fig. 4. Regulation of nucleolytic functions during PIR of *Deinococcus radiodurans*. Heparin-binding proteins were purified from cells collected at different time intervals (0, 1, 2, 3 and 4 h) of PIR and unirradiated control. These proteins were incubated with PCR-amplified DNA (S) for 30 min. The nucleolytic activity (a) was monitored in the presence of ATP (b) and upon dephosphorylation with AP (c), which alone does not confer nuclease activity (E) in solution. The alkaline reaction products were analyzed on 1% agarose gel.

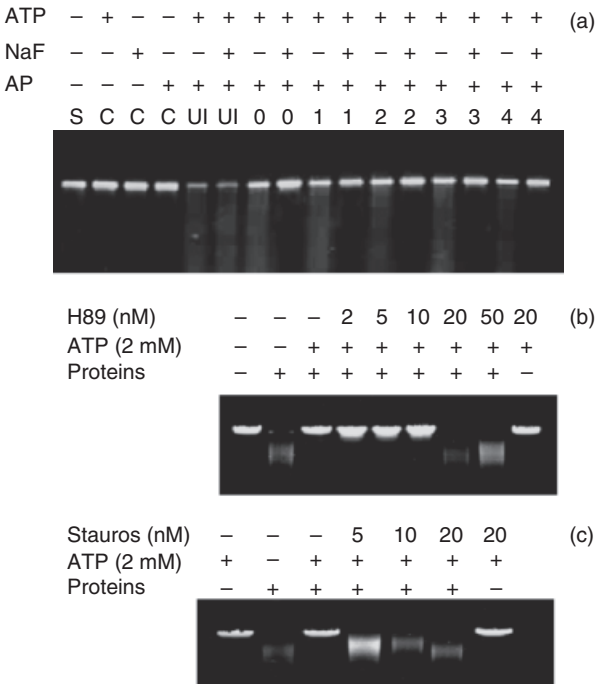

Fig. 5. Contribution of protein kinases and reversible protein phosphorylation in regulation of nucleolytic functions during PIR. Heparin-purified proteins from unirradiated and different time intervals of postirradiation (0, 1, 2, 3 and 4 h PIR) were dephosphorylated with AP and incubated with 1-kb PCR-amplified DNA substrate (S) and with 2 mM ATP in the presence and absence of sodium fluoride (NaF), an inhibitor of phosphatase activity. The nuclease activity was compared against DNA incubated with each addition (C) and products were analyzed on agarose gel (a). The dephosphorylated 1-h PIR sample was incubated with 2 mM ATP with increasing concentrations of H89 (b) and staurosporine (stauros) (c), and the effect on nuclease activity was assayed.

(Fig. 5b and c). As both, H89 and high concentrations of staurosporine inhibit PKA activity, it appears that ATP attenuation of nucleolytic function might be regulated through PKA-type kinase(s). These results indicated that the DNA damage induces an ATP-responsive function and the nucleolytic activity was modulated by reversible protein phosphorylation.

DNA damage showed stimulation of protein kinase functions in *D. radiodurans*

The effect of γ radiation on phosphoproteins and protein kinase activity was measured both *in vivo* and *in vitro*. *In vivo* phosphoprotein profiles were monitored on cells labeled with [^{32}P]phosphoric acid and changes in phosphoprotein profile were detected by autoradiography. The protein kinase activity in γ-irradiated cell-free extract monitored *in vitro* was highest in 0.5-h PIR cells, which subsequently decreased to the levels of unirradiated control in 3 h PIR (Fig. 3). Similarly, 1-h PIR cells showed very high levels of protein phosphorylation as compared with unirradiated sample and the samples beyond 1 h PIR (Fig. 6). These results suggest that γ radiation induces protein kinase activity, possibly leading to the enhanced protein phosphorylation, which are considered strong indicators of signal transduction mechanisms in any organism.

Recently, the involvement of protein phosphorylation in bacterial radiation resistance and DSB repair has gained significant importance. It has been demonstrated that (1) *D. radiodurans* showing relatively low protein kinase activity also exhibits DSB repair impairment and γ radiation sensitivity (Rajpurohit *et al*., 2008); (2) a multiprotein complex isolated from *D. radiodurans* contains DNA repair proteins along with protein kinase and phosphoproteins (Kota & Misra, 2008) and (3) the deletion of deinococcal-response regulator DR_2418 from bacterial genome leads to repression of catalase, *recA* and *pprA* gene expression and decreased γ radiation resistance (Wang *et al*., 2008). A periplasmic protein kinase activity required for radiation resistance and DSB repair in *E. coli* (Khairnar *et al*., 2007), as well changes in the DNA substrate-binding preference of *Bacillus subtilis* single-stranded DNA-binding protein by phosphorylation (Mijakovic *et al*., 2006) have also been reported in other bacteria.

The signal transduction mechanisms in response to nutritional stress and other abiotic stresses besides DNA damage have been shown in bacteria (Parkinson, 1993). In this study, we highlight, for the first time, the presence of a γ radiation-induced signaling mechanism in a prokaryote, *D. radiodurans*. We demonstrate that the DNA damage-induced synthesis of cAMP and ATP was possibly manifested by upregulation of AC and downregulation of 2′,3′ cAMP phosphodiesterase activities during PIR. The presence of different ACs and their involvement in bacterial signal transduction are well established (Linder & Schultz, 2003; Shenoy & Visweswariah, 2006). Although, the mechanism by which cAMP regulates DNA damage response is not clear; it can presumably act as an inducer of protein kinase activity and a signaling molecule in bacteria, as is known in eukaryotes (De Gunzburg, 1985). Similarly, the effects of DNA damage and oxidative stress on AC and 2′,3′cyclic phosphodiesterase enzymes have not been studied, but the regulation of cyclic phosphodiesterase and AC activities by a membrane receptor relaxin-mediated tyrosine phosphorylation has been demonstrated in mammalian cells (Bartsch *et al*., 2001). As cAMP is a known activator of mitogen-activated protein kinases and other soluble as well as membrane-bound protein kinases (Stork & Schmitt, 2002; Sanz, 2008) in eukaryotes, it is likely that the higher levels of cAMP and AC activity in 1- and 0.5-h PIR samples, respectively, regulate protein phosphorylation in this bacterium by similar mechanisms. Our results show that (1) the levels of cAMP and ATP change in response to DNA damage, possibly manifested by differential regulation of

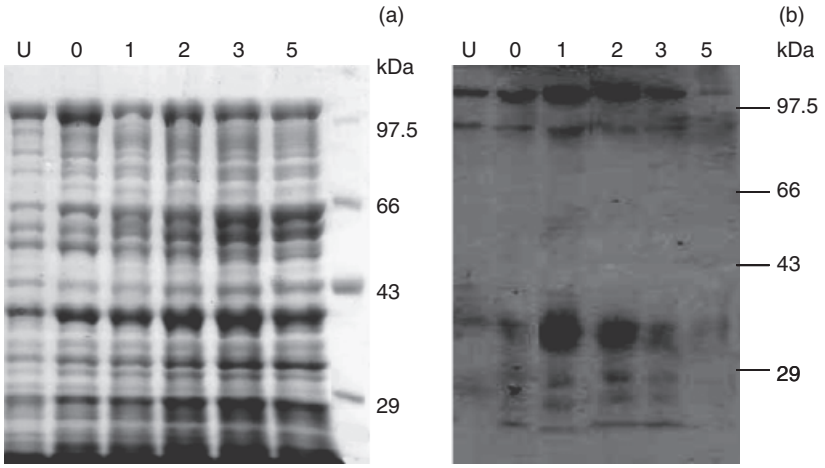

Fig. 6. Effect of γ radiation on total phosphoprotein profile change during PIR of *Deinococcus radiodurans*. *Deinococcus* cells were grown in the presence of [^{32}P]phosphoric acid to mid-logarithmic phase. These cells were collected, washed with sterile normal saline and irradiated with 6.5 kGy γ radiation. Aliquots were collected at 0, 1, 2, 3 and 5 h PIR and proteins were separated on 5–14% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis. Protein bands were visualized with Coomassie brilliant blue (a) staining and [^{32}P]-labeled phosphoproteins were detected by autoradiography (b).

AC and cyclic phosphodiesterase enzymes and (2) DNA damage-inducible protein kinase-mediated ATP attenuation of nucleolytic activity is involved during PIR. This is consistent with the activation of protein kinase by DNA damage in eukaryotes (Kitagawa & Kastan, 2005). Thus, there exists a DSB-induced signaling mechanism in this extremophile, which is known to have acquired the genetic elements from higher organisms through horizontal gene transfer (Makarova *et al.*, 2001; Blasius *et al.*, 2008). The possibility that this superbug has acquired the DNA damage-induced signaling pathway from other organisms during evolution cannot be ruled out and would be worth investigating.

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