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ORIGINAL PAPER

Identification of PprM: a modulator of the PprI-dependent DNA damage response in *Deinococcus radiodurans*

Hirofumi Ohba · Katsuya Satoh · Haïtham Sghaier · Tadashi Yanagisawa · Issay Narumi

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Abstract *Deinococcus radiodurans* possesses a DNA damage response mechanism that acts via the PprI protein to induce RecA and PprA proteins, both of which are necessary in conferring extreme radioresistance. In an effort to further delineate the nature of the DNA damage response mechanism in *D. radiodurans*, we set out to identify novel components of the PprI-dependent signal transduction pathway in response to radiation stress. Here we demonstrate the discovery of a novel regulatory protein, PprM (a modulator of the PprI-dependent DNA damage response), which is a homolog of cold shock protein (Csp). Disruption of the *pprM* gene rendered *D. radiodurans* significantly sensitive to γ-rays. PprM regulates the induction of PprA but not that of RecA. PprM belongs in a distinct clade of a subfamily together with Csp homologs from *D. geothermalis* and *Thermus thermophilus*. Purified PprM is present as a homodimer under physiological conditions, as the case with *Escherichia coli* CspD. The *pprA pprM* double-disruptant strain exhibited higher sensitivity than the *pprA* or *pprM* single disruptant strains, suggesting that PprM regulates other hitherto unknown protein(s) important for radioresistance besides PprA. This study strongly suggests that PprM is involved in the radiation response mediated by PprI in *D. radiodurans*.

Keywords *Deinococcus radiodurans* · Radiation response · PprI · PprM · DNA repair

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H. Ohba · K. Satoh · H. Sghaier · I. Narumi (✉)
Gene Resource Research Group,
Quantum Beam Science Directorate,
Japan Atomic Energy Agency,
1233 Watanuki, Takasaki 370-1292, Japan
e-mail: narumi.issei@jaea.go.jp

H. Ohba · K. Satoh · H. Sghaier · I. Narumi
DNA Repair Protein Group,
Quantum Beam Science Directorate,
Japan Atomic Energy Agency,
1233 Watanuki, Takasaki 370-1292, Japan

T. Yanagisawa
Faculty of Agriculture, Utsunomiya University,
350 Mine, Utsunomiya 321-8505, Japan

Present Address:
H. Sghaier
Centre National des Sciences et Technologies Nucléaires,
2020 Sidi Thabet, Tunis Cedex, Tunisia

Introduction

Deinococcus radiodurans belongs to a unique family of eubacteria that are characterized by an exceptional capacity to withstand the usually lethal effects of DNA-damaging conditions such as those induced by ionizing radiation, UV light and desiccation (Cox and Battista 2005; Blasius et al. 2008). The most noteworthy characteristic of *D. radiodurans* is the capacity to repair DNA double-strand breaks (DSBs) induced by ionizing radiation. This bacterium can repair over 200 genomic DSBs during post-irradiation incubation (Cox and Battista 2005), while only a few DSBs are lethal to *Escherichia coli* (Krasin and Hutchinson 1977). The highly efficient DSB repair process in *D. radiodurans* is radiation-inducible and is dependent on de novo protein synthesis following irradiation (Kitayama and Matsuyama 1968). Transcriptomic and proteomic analyses have revealed comprehensive expression profiles of radiation-inducible genes and proteins (Lipton et al. 2002; Liu et al. 2003; Tanaka et al. 2004; Zhang et al. 2005; Lu et al. 2008). It has therefore been established that *D. radiodurans*

possesses a DNA damage response mechanism. In *E. coli*, RecA and LexA proteins play central roles in the DNA damage response (known as the SOS system) (Friedberg et al. 2006). *D. radiodurans* possesses two potential DNA damage responsive repressors, LexA1 and its homolog LexA2. However, unlike the SOS system in *E. coli*, neither LexA1 nor LexA2 repressors regulate the induction of RecA following irradiation in *D. radiodurans* (Narumi et al. 2001; Bonacossa de Almeida et al. 2002; Sheng et al. 2004; Satoh et al. 2006).

Analysis of DNA-damage-sensitive strains of *D. radiodurans* identified a novel regulatory protein, PprI (also referred to as IrrE), which is involved in the induction of RecA (Earl et al. 2002; Hua et al. 2003). PprI is also involved in regulating the induction of PprA, a protein that interacts with DNA ends to protect the ends from DNA degradation and stimulate DNA ligase activity (Narumi et al. 2004), and other radiation-responsive proteins (Lu et al. 2008). PprI therefore appears to play a critical role in switching on the DNA damage response and cellular survival network following radiation damage in *D. radiodurans*. In a previous study, we identified the radiation-responsive minimal promoter region of the *pprA* gene and demonstrated that upregulation of *pprA* expression by PprI is triggered at the promoter level (Ohba et al. 2005), although we were unable to find evidence to support a direct interaction of PprI with this promoter region. This result suggests that there may be hitherto unknown components of the PprI-dependent response to radiation stress in *D. radiodurans*. In this study we describe the identification of PprM, a protein that is a novel component of the PprI-mediated signal transduction pathway.

Materials and methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *D. radiodurans* strain R_1 (ATCC13939^T) was used as the parental strain. *D. radiodurans* was grown at 30°C in TGY broth (0.5% bacto tryptone, 0.1% glucose and 0.3% bacto yeast extract). *E. coli* was grown at 37°C in Luria–Bertani broth. Solid media contained 1.5% bacto agar. The following antibiotics were added when necessary: hygromycin (50 μg per ml) and chloramphenicol (3 μg per ml) for *D. radiodurans*; ampicillin (100 μg per ml), hygromycin (100 μg per ml), kanamycin (25 μg per ml) and chloramphenicol (34 μg per ml) for *E. coli*. Transformation of *D. radiodurans* was performed using PCR fragments as previously described (Kitayama et al. 1983).

Measurement of cell survival rate

D. radiodurans cells grown to early stationary phase were harvested by centrifugation, washed twice and then resuspended in 10 mM sodium phosphate buffer (pH 7.0). Cell suspensions were irradiated at room temperature with ^{60}Co γ-rays at different doses (dose rate: 2 kGy per h). Following irradiation, the cells were appropriately diluted in 10 mM sodium phosphate buffer (pH 7.0), plated onto TGY agar and incubated at 30°C for 3 days before the colonies were enumerated.

Two-dimensional PAGE analysis

D. radiodurans R_1 and JAK1 cells grown to early stationary phase were harvested by centrifugation, washed twice with 10 mM sodium phosphate buffer (pH 7.0), and then resuspended in 300 μl of lysis buffer containing 20 mM Tris–HCl (pH 8.2), 2 mM EDTA, 5 mM $MgCl_2$, 1 mM PMSF and Protease Inhibitor Cocktail Set II (Merck). Cells were then disrupted using a Micro Smash MS-100R (TOMY) with a FastPROTEIN BLUE Kit (Qbiogene). Following the removal of cell debris by centrifugation, supernatants were treated with 50 μg/ml of DNase I and 50 μg/ml of RNase A on ice for 1 h, and then subjected to two-dimensional PAGE.

Two-dimensional PAGE was performed using the immobilized pH gradient technique essentially according to the manufacturer's instructions (GE Healthcare). Five hundred micrograms of each protein extract was mixed with separating solution (9.8 M urea, 2% Triton X-100, 2.8 mg/ml DTT and bromophenol blue) to a final volume of 200 μl. Proteins were separated using an 11-cm immobiline dry strip with a pH range of 4–7 (GE Healthcare). The rehydration time was 17.5 h at 20°C, and isoelectric focusing (IEF) was performed at 500 V for 1 min by gradient, 4 kV for 90 min by gradient, and 8 kV for 90 min by a step-n-hold mode using IPGphor (GE Healthcare). Following IEF, strips were incubated in buffer 1 (50 mM Tris–HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS and 10 mg/ml DTT) for 10 min, and then incubated in buffer 2 (identical to buffer 1 except that 25 mg/ml of iodacetamide was used in lieu of DTT) for 10 min. Following incubation, SDS-PAGE was performed using an ExcelGel XL SDS, gradient 12–14% (GE Healthcare) with a Multiphor II (GE Healthcare) at 20 mA for 45 min and 40 mA for 160 min at 13°C. Gels were stained with Coomassie Brilliant Blue, and protein spots excised from the gel were subjected to MALDI-MS analysis (Relyon). The MS data were analyzed by means of peptide mass fingerprinting (PMF) using the ProFound web program (http://prowl.rockefeller.edu/).

Table 1 Strains and plasmids used in this study

Designation	Relevant description	Source or reference
D. radiodurans		
R_1	Wild-type (ATCC 13939^T)	ATCC
JAK1	R_1 but *pprI536*::*aph*	Ohba et al. 2005
XCSP1	R_1 but *pprM286*::*hph*	This study
XPA1	R_1 but *pprA428*::*cat*	This study
WPAM1	R_1 but *pprA428*::*cat pprM286*::*hph*	This study
E. coli		
JM109	Host for plasmid subclones	Takara Bio
BL21 (DE3)	Host for gene expression	Novagen
Plasmid		
pET3a	*E. coli* expression vector, Ap^r	Novagen
pET3apprIwt	pET3a with *Nde*I-*Bam*HI::PCR fragment containing *pprI*	This study
pET9a	*E. coli* expression vector, Km^r	Novagen
pET9csp1	pET3a with *Nde*I-*Bam*HI::PCR fragment containing *pprM*	This study
pLysS	*E. coli* plasmid containing T7 lysozyme gene, Cm^r	Novagen
pUC19	*E. coli* cloning vector, Ap^r	Takara Bio
pCSP1	pUC19 with 1,115-bp PCR fragment from genomic DNA, *pprM*	This study
pUPA1	pUC19 with 1,402-bp PCR fragment from genomic DNA, *pprA*	This study
pKatHPH4	Hygromycin-resistance gene cassette vector, Hyg^r (*hph*)	Satoh et al. 2006
pKatCAT5	Chloramphenicol-resistance gene cassette vector, Cm^r (*cat*)	This study
pCSPHPH	pCSP1 with 1,199-bp *Hinc*II fragment from pKatHPH4, *pprM286*::*hph*	This study
pUPACAT2	pUPA1 with 916-bp *Hinc*II fragment from pKatCAT5, *pprA428*::*cat*	This study

Gene disruption

Gene disruptions targeting *pprM* and *pprA* were performed as previously described (Funayama et al. 1999) with modifications. For the gene disruption of *pprM*, a DNA fragment containing *pprM* was amplified by PCR using *D. radiodurans* genomic DNA and the oligonucleotide primer sets DR0907DF and DR0907DR. The resulting PCR fragment was phosphorylated using T4 polynucleotide kinase and inserted into pUC19 at the *Sma*I site to yield plasmid pCSP1. The plasmid was digested with *Nsp*V, the restriction site of which is located in the *pprM* coding region, blunted using T4 DNA polymerase, and then ligated to a 1.2-kb *Hinc*II fragment (KatHPH cassette) containing the hygromycin-resistance gene (*hph*) from pKatHPH4 (Satoh et al. 2006) to yield plasmid pCSPHPH (carrying the *pprM286*::*hph* mutation). The *pprM286*::*hph* mutation was introduced into the *D. radiodurans* R_1 genome and the resulting disruptant strain was designated XCSP1. For the gene disruption of *pprA*, a DNA fragment containing *pprA* was amplified by PCR using the oligonucleotide primer sets DRA0346DF and DRA0346DR. The resulting PCR fragment was phosphorylated using T4 polynucleotide kinase and inserted into pUC19 at the *Sma*I site to yield plasmid pUPA1. The plasmid was digested with *Nsp*V, blunted and ligated to a 0.9-kb *Hinc*II fragment (KatCAT cassette) containing the chloramphenicol-resistance gene (*cat*) from pKatCAT5 to yield plasmid pUPACAT2 (carrying the *pprA428*::*cat* mutation). Plasmid pKatCAT5 is a chloramphenicol-resistance version of pKatHPH4. The *pprA428*::*cat* mutation was introduced into the R_1 and XCSP1 genomes to generate *pprA* disruptant strain XPA1 and *pprA pprM* double-disruptant strain WPAM1, respectively. Disruption was confirmed by amplifying the target allele by genomic PCR using the oligonucleotide primer sets DR0907VF/DR0907VR or DRA0346VF/DRA0346VR. Oligonucleotide primers used in this study are listed in Table 2.

Construction of *pprI* and *pprM* expression plasmids

To isolate the *pprI*- and *pprM*-coding regions, PCR was performed using *D. radiodurans* genomic DNA and oligonucleotide primer sets Nde167-F/Bam167-R and 0907Nde/0907Bam, respectively. PCR fragments were digested with *Nde*I and *Bam*HI to adapt the termini for in-frame insertion of *pprI* and *pprM* into the *Nde*I–*Bam*HI sites of the pET3a and pET9a vectors, respectively. The

Table 2 Oligonucleotide primers used in this study

Name	Sequence (5′–3′)
DR0907DF	GAACGCCGAGCAGCTGTGGGAAAC
DR0907DR	GGTACCTGGCAGGGCACTGTCACT
DRA0346DF	AACGGCGCCGGGTGCACTTTGGG
DRA0346DR	GCTGCCCCGCCAGGATGTCGCTT
DR0907VF	CCGTCAAGGCCGGCAAGAAGTACGA
DR0907VR	TTGGCTCACGCTGCTGGGAAC
DRA0346VF	GGTCACGGCCCCACTGTAGTCC
DRA0346VR	TGAAGCCTGTCCGCATGACCTCTTC
Nde167-F[a]	AATCACTGCCATATGCCCAGTGCC
Bam167-R[b]	CGGCGGATCCAGTTCACTGTGCAGCGTCC
0907Nde[a]	GAGCAAGGAGATTTCATATGGCAACTGG
0907Bam[b]	GGTCGGATCCTTACCAGCGGTCGTCGCG

[a] *Nde*I site was underlined

[b] *Bam*HI site was underlined

resultant expression plasmids were designated pET3apprIwt and pET9csp1. The DNA sequence of the expression plasmids was determined to confirm the absence of errors during plasmid construction.

Protein purification

D. radiodurans pprI was expressed in *E. coli* strain BL21 (DE3) carrying pLysS and pET3apprIwt. Cells were harvested, washed and resuspended in buffer A [20 mM sodium phosphate buffer (pH 7.4) and 1 mM EDTA] containing 1 mM PMSF. All subsequent purification steps were performed at 4°C. Cell suspensions were sonicated and the debris was removed by centrifugation. Ammonium sulfate was slowly added to the supernatants to 45% saturation. Suspensions were stirred for 1 h and then centrifuged for 30 min. Protein pellets were resuspended in buffer A and then dialyzed for 18 h against the same buffer. Protein was further purified to apparent homogeneity by column chromatography on DEAE Sepharose Fast Flow, HiPrep 16/60 Sephacryl S-300, HiTrap Heparin and Mono Q HR 5/5 (GE Healthcare). Purified fractions were concentrated and then equilibrated with buffer A.

D. radiodurans pprM was expressed in *E. coli* strain BL21 (DE3) carrying pLysS and pET9csp1. Cells were harvested, washed and resuspended in buffer containing 20 mM Tris–HCl (pH 8.0), 2 mM EDTA, 1 mM PMSF and 1% Protease Inhibitor Cocktail Set II. Cell suspensions were sonicated and the debris was removed by centrifugation. Ammonium sulfate was slowly added to the supernatants to 30% saturation. Protein was purified by column chromatography on TOYOPEARL Phenyl-650S (Tosoh). Pooled fractions were further purified by an Amicon Ultra-15 30K Centrifugal Filter Device (Millipore), and the flow-through fractions were pooled. Purified fractions were concentrated and equilibrated with buffer A using an Amicon Ultra-15 5K Centrifugal Filter Device (Millipore).

Detection of intracellular PprA, RecA, PprI and GroEL levels

D. radiodurans R1, XCSP1 and JAK1 cells grown to early stationary phase were harvested by centrifugation, washed twice and resuspended in 10 mM Tris–HCl (pH 7.0). Cell suspensions were then divided into two fractions. One fraction was irradiated with 2 kGy of γ-rays, while the other fraction was not irradiated. Cells were harvested by centrifugation and incubated in a fourfold volume of fresh TGY broth for 0 or 2 h at 30°C with agitation. Cells were harvested by centrifugation, resuspended in buffer containing 50 mM Tris–HCl (pH 8.0), 5 mM EDTA, 1 mM PMSF, 1% SDS and 5% Protease Inhibitor Cocktail Set II, and then disrupted using a Micro Smash MS-100R with a FastPROTEIN BLUE Kit. Following the removal of cell debris by centrifugation, 10 μg of each protein extract was subjected to SDS-PAGE.

Electrophoresed proteins were transferred onto a PVDF membrane (Millipore) which was then incubated with *D. radiodurans* anti-PprI antibody (diluted 1:1,000), *D. radiodurans* anti-PprA antibody (diluted 1:10,000) (Narumi et al. 2004), *D. radiodurans* anti-RecA antibody (diluted 1:5,000) (Satoh et al. 2006) or *E. coli* anti-GroEL antibody (diluted 1:4,000) (StressGen Biotechnologies Co.) together with alkaline phosphatase-conjugated rabbit IgG antiserum (Applied Biosystems) or alkaline phosphatase-conjugated mouse IgG antiserum (Jackson Immuno Research). *D. radiodurans* anti-PprI antibody was raised against purified PprI at Immuno-Biological Laboratories (Gunma, Japan). Chemiluminescence signals on the PVDF membrane were visualized and the signal intensity was quantified using a Lumi-Imager Workstation (Roche Diagnostics).

Gel filtration

Purified PprM was loaded onto Sephadex 200 10/300 GL (GE Healthcare) pre-equilibrated with buffer containing 20 mM Tris–HCl (pH 7.5) and 0.3 M NaCl. Protein was eluted at a flow rate of 0.5 ml/min using the same buffer, and the elution profile was monitored by measuring the absorbance at 280 nm. The elution profiles of RNase A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa) and albumin (67 kDa) were also monitored as controls using the same column.

Results and discussion

Discovery of a novel protein involved in the PprI-dependent radiation response

In a previous study, we demonstrated that upregulation of *pprA* expression by PprI is triggered at the promoter level. However, no evidence was found to support a direct interaction of PprI with this promoter region (Ohba et al. 2005). This suggested that the effect of PprI on PprA induction is indirect, and that there may be hitherto unknown components of the PprI-dependent response to radiation stress in *D. radiodurans*. In an effort to explore this possibility, the two-dimensional protein profiles of wild-type and *pprI* disruptant strains were compared. In the course of this investigation we identified a 10-kDa protein spot during IEF analysis, the isoelectric point of which differed between wild-type and *pprI* disruptant strains (Fig. 1). The protein spot in the wild-type strain indicated higher basicity than that of the *pprI* disruptant strain, suggesting that the protein may undergo posttranslational modification via PprI. PMF analysis using MALDI-MS data identified the protein as a cold shock protein (Csp). We designated this protein as PprM (a modulator of the PprI-dependent DNA damage response) for the reason given later. Recently, Lu et al. (2008) detailed a comparative proteomic analysis of wild-type and *pprI* disruptant strains and showed that PprI affects the posttranslational modification of two proteins (serine protease and glyceraldehyde 3-phosphate dehydrogenase). However, no reference was made to PprM. PprI possesses a zinc metallopeptidase motif in the N-terminal domain (Hua et al. 2003). Recently, it has been proposed that the putative protease activity of PprI could be critical for radioresistance (Vujičić-Žagar et al. 2008). However, our two-dimensional protein profiling analysis suggests that the effect of PprI on PprM is posttranslational modification rather than proteolysis.

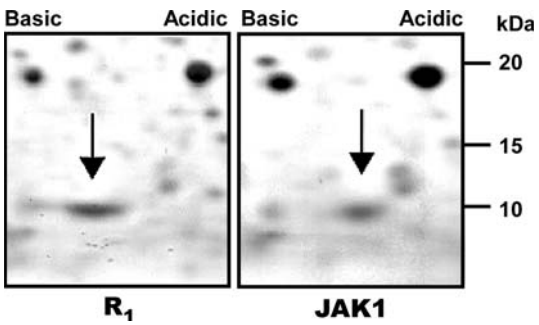

Fig. 1 Two-dimensional PAGE analysis. *D. radiodurans* R_1 (wild-type) and JAK1 (*pprI* disruptant) cells were subjected to two-dimensional PAGE analysis. A single protein spot (*arrowed*) was detected at a position corresponding to a size of approximately 10 kDa in both strains, although the protein from R_1 was located in a region that is more basic than that region occupied by the protein from JAK1. PMF analysis using MALDI-MS data identified the protein as Csp (DR0907)

In an effort to determine whether PprM is responsible for the radiation resistance of *D. radiodurans*, the *pprM* disruptant strain was generated by direct insertional mutagenesis using double-crossover recombination (Funayama et al. 1999), and the survival rate was examined. As shown in Fig. 2, the *pprM* disruptant strain exhibited markedly higher sensitivity to γ-rays than the wild-type. This result suggests that PprM plays an important role in the *pprI*-dependent radiation response in *D. radiodurans*.

PprM is involved in repressing the production of PprA

PprI has been shown to be involved in the induction of RecA and PprA, both of which play important roles in the radioresistance mechanism of *D. radiodurans* (Earl et al. 2002; Hua et al. 2003; Ohba et al. 2005). In an effort to determine whether PprM is involved in the induction of RecA and PprA, changes in the intracellular levels of RecA, PprA and PprI following irradiation were investigated. Anti-PprI antibody was generated using PprI purified from recombinant *E. coli* (Fig. 3). As shown in Fig. 4, the loss of PprI resulted in the absence of RecA and PprA induction, confirming previous results (Earl et al. 2002; Hua et al. 2003; Ohba et al. 2005). Interestingly, constitutive production of PprA at an elevated level was observed in the mock-irradiated *pprM* disruptant strain,

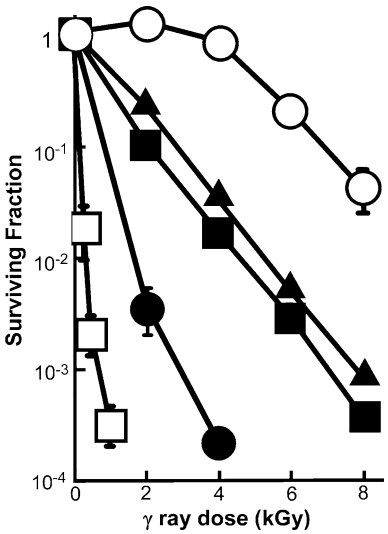

Fig. 2 Survival curves of *D. radiodurans* strains. *Open circles*, strain R_1 (wild-type); *filled squares*, strain XCSP1 (*pprM* disruptant); *filled triangles*, strain XPA1 (*pprA* disruptant); *filled circles*, strain WPAM1 (*pprA pprM* double-disruptant); *open squares*, strain JAK1 (*pprI* disruptant). Data represent the mean ± standard error of three independent experiments; $n = 9$

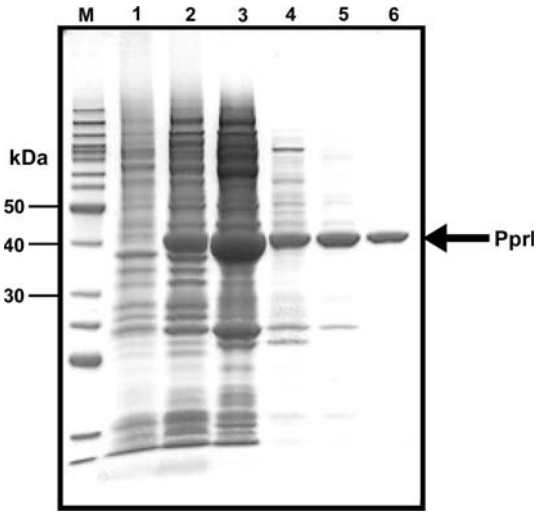

Fig. 3 Purification of *D. radiodurans* PprI protein. Samples were subjected to SDS-12.5% PAGE and then stained using Coomassie Brilliant Blue. *Lane M* BenchMark Protein Ladder (Invitrogen), *lane 1* total cellular protein from *E. coli* BL21(DE3)/pLysS/pET3a, *lane 2* resuspended 45% ammonium sulfate-precipitated total cellular protein from *E. coli* BL21(DE3)/pLysS/pET3pprIwt induced by IPTG, *lane 3* pooled PprI fractions following DEAE sepharose fast flow chromatography, *lane 4* pooled PprI fractions following HiPrep 16/60 Sephacryl S-300 High Resolution chromatography, *lane 5* pooled PprI fractions following HiTrap Heparin chromatography, *lane 6* pooled PprI fractions following MonoQ HR 5/5 chromatography. The position of the 40-kDa PprI band is indicated on the right. The purified protein was used to generate anti-PprI antibody

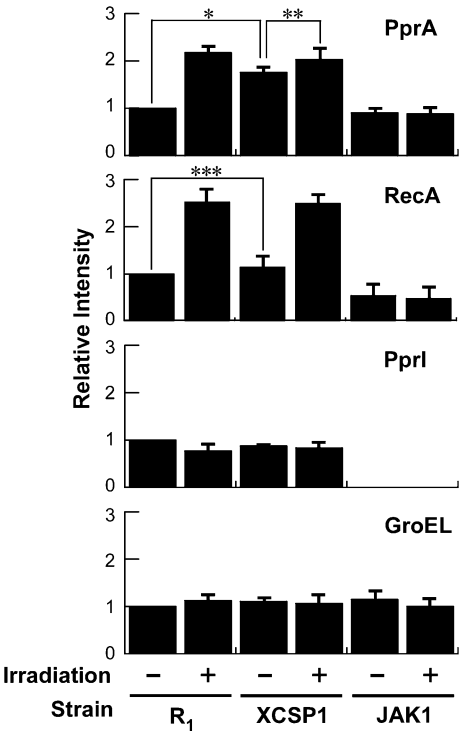

Fig. 4 Changes in intracellular PprA, RecA and PprI levels following irradiation in *D. radiodurans* strains R_1 (wild-type), XCSP1 (*pprM* disruptant) and JAK1 (*pprI* disruptant). Cells were irradiated (+) or mock-irradiated (–) with 2 kGy of γ-rays, incubated in fresh TGY for 2 h, and then subjected to Western blot analysis using anti-PprA, anti-RecA or anti-PprI antibodies. Anti-GroEL antibody was used to gauge the sample loading control. Values represent relative intensity of Western blot signals normalized to that of mock-irradiated wild-type for each antibody as 1 (the mean ± standard error of four or five independent experiments). *Asterisks* indicate statistical difference determined by the Student's *t* test. *$P < 0.01$; **not significant ($P = 0.51$); ***not significant ($P = 0.61$)

where the level of PprA was comparable to that observed in irradiated cells of wild-type and *pprM* disruptant strains. On the other hand, the induction of RecA was not affected by *pprM* disruption. Additionally, the level of PprI in the wild-type and *pprM* disruptant strains remained unchanged irrespective of irradiation (Fig. 4). These results suggest that PprM is involved in repressing the production of PprA, but not that of RecA. As already mentioned, PprM from the wild-type strain showed a high isoelectric point (Fig. 1). Taken together, we propose that only the basic form of PprM can be involved in derepressing PprA production following irradiation in *D. radiodurans*.

PprM is a homolog of Csp

Figure 5a shows the phylogenetic relationship between PprM and the *E. coli* Csp family of proteins together with Csp homologs from *D. geothermalis*, another member of the genus *Deinococcus* (Makarova et al. 2007), and *Thermus thermophilus*, an extreme thermophile phylogenetically related to *Deinococcus* (Henne et al. 2004). PprM belongs in a distinct clade of a subfamily together with Csp homologs from *D. geothermalis* and *T. thermophilus*. Whereas *D. geothermalis* and *T. thermophilus* have two Csp homologs, *D. radiodurans* only has a single Csp homolog (White et al. 1999). The PprM protein shows high sequence identity to bacterial Csp homologs, all of which possess two RNA-binding motifs (Fig. 5b). Furthermore, these proteins possess a high content of aromatic residues, an essential feature utilized by Csp homologs in the binding to RNA (Yamanaka et al. 1998). To date, nine Csp homologs have been identified in *E. coli*, designated CspA–CspI, and these have been implicated to act in a variety of cellular processes including adaptation to low temperature, cellular growth, nutrient stress and the stationary phase (Graumann and Marahiel 1998; Trun and Johnson 2003). In this study, we demonstrated that PprM modulates the induction of PprA in *D. radiodurans*. This is the first report to show that a Csp homolog is implicated in the signal transduction pathway response to radiation stress.

To date, only a few reports have detailed the physiological properties of PprM. Airo et al. (2004) showed in their proteomic analysis that PprM was not a cold shock-induced protein, but a heat shock-induced protein. In

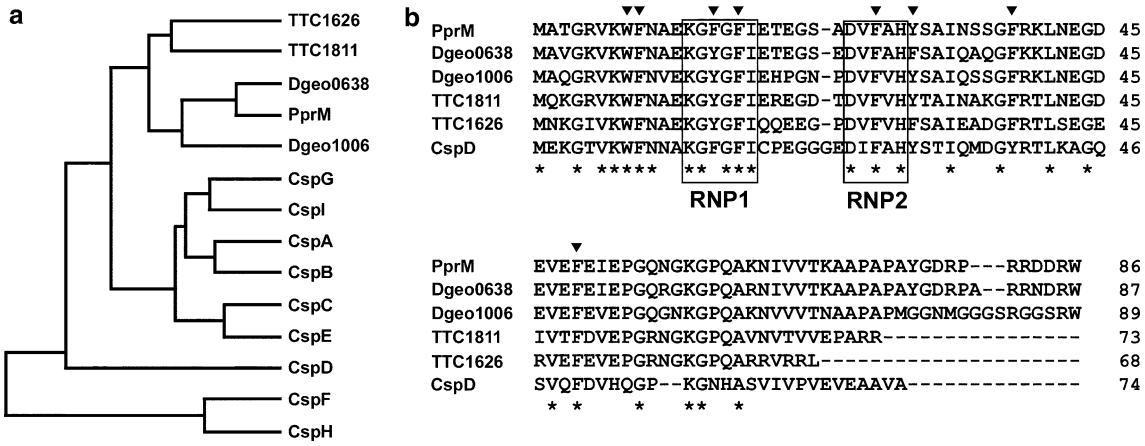

Fig. 5 Amino acid sequence comparison of PprM. **a** Phylogenetic relationship of Csp homologs among *E. coli* (CspA to CspI), *D. radiodurans*, *D. geothermalis* and *T. thermophilus*. Multiple alignment was employed using the CLUSTAL W program (Thompson et al. 1994). A phylogenetic tree was constructed based on the multiple alignment using the unweighted pair group method with arithmetic mean (Sokal and Michener 1958). **b** Multiple amino acid sequence alignment of PprM, *D. geothermalis* DSM 11300 Csp homologs (Dgeo0638 and Dgeo1006) (Makarova et al. 2007), *T. thermophilus* HB27 Csp homologs (TTC1811 and TTC1626) (Henne et al. 2004) and *E. coli* CspD. Sequences were obtained from GenomeNet (http://www.genome.jp/en/). *Numbers* on the *right* are the coordinates of each protein. *Asterisks* indicate identical residues, and *dashes* indicate gaps in the alignment. RNA-binding motifs, RNP1 and RNP2 are *boxed*. *Triangles* indicate aromatic residues that are implicated to be involved in the binding of Csp homologs to RNA (Yamanaka et al. 1998)

contrast to this, Schmid et al. (2005) were unable to confirm in their proteomic analyses that PprM was induced by heat shock, and showed by means of microarray analyses that *pprM* gene transcription was repressed by heat shock. Further investigations are required to delineate the physiological role of PprM in *D. radiodurans*.

PprM forms a dimer

It has been shown that *E. coli* CspD adopts a homodimeric form in solution (Yamanaka et al. 2001), whereas *E. coli* CspA adopts a monomeric form in solution (Newkirk et al. 1994; Schindelin et al. 1994). With a view to investigating the quaternary structure of PprM, recombinant PprM protein was purified from *E. coli* (Fig. 6a). Consistent with the results of the two-dimensional PAGE analysis (Fig. 1), purified PprM was found to be approximately 10 kDa in size following SDS-PAGE analysis. Gel filtration analysis revealed that purified PprM had a molecular size of approximately 20 kDa (Fig. 6b, c), suggesting that PprM is present as a homodimer under physiological conditions. The stable dimeric form of *E. coli* CspD has been suggested to possess an amino-terminal end that is shorter than *E. coli* CspA by three residues, which facilitates intermonomer antiparallel β-strand interactions between the β4 strand from each CspD monomer (Yamanaka et al. 2001). Given that a shorter amino-terminal end represents a common feature in *E. coli* CspD and PprM (Fig. 5b), PprM dimer formation may be facilitated by the same mechanism employed for *E. coli* CspD.

PprM regulates other protein that is important for radioresistance besides PprA

Loss of PprA renders *D. radiodurans* highly sensitive to γ-rays (Fig. 2) (Narumi et al. 2004; Tanaka et al. 2004). On the other hand, the *pprM* disruptant strain, which produced high amounts of PprA under unirradiated conditions (Fig. 4), also exhibited high sensitivity to γ-rays (Fig. 2). Two possibilities could account for the radiosensitivity of the *pprM* disruptant strain; (1) a defect in the precisely timed induction of PprA following irradiation, and/or (2) a defect in the regulation of hitherto unknown protein(s) necessary for radioresistance besides PprA.

The former possibility is supported by our previous experiments demonstrating that the *lexA2* disruptant strain, in which enhancement of *pprA* promoter activation was observed following irradiation, exhibited much higher resistance to γ-rays than wild-type (Satoh et al. 2006). Zahradka et al. (2006) proposed a model referred to as extended synthesis-dependent strand annealing (ESDSA) that utilizes DNA polymerase I (PolA) at an early stage of the DNA repair process in *D. radiodurans*. Since PprA preferentially binds to double-strand DNA carrying strand breaks (Narumi et al. 2004), excessive amounts of PprA at an early DNA repair stage may inhibit the ESDSA process.

In order to confirm the possibility that PprM regulates other protein(s) intricately related to the radioresistance of *D. radiodurans*, a *pprA pprM* double-disruptant strain was constructed and the survival rate was examined. It was

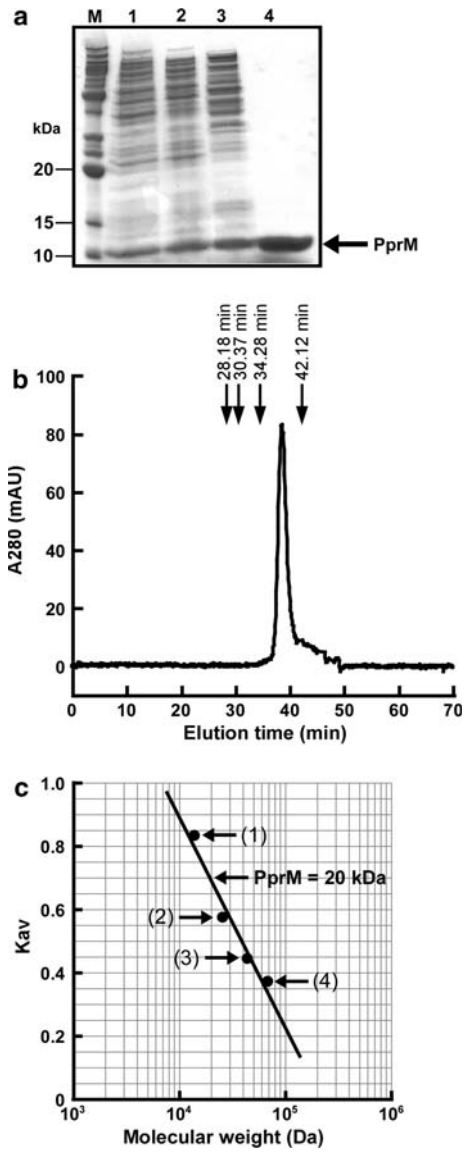

Fig. 6 Properties of the PprM protein. **a** Purification of *D. radiodurans* PprM protein. Samples were subjected to SDS-15% PAGE and then stained with Coomassie Brilliant Blue. *Lane M* BenchMark Protein Ladder (Invitrogen), *lane 1* total cellular protein from *E. coli* BL21 (DE3)/pLysS/pET9csp1 induced by IPTG, *lane 2* resuspended 30% ammonium sulfate-precipitated protein, *lane 3* pooled PprM fractions following TOYOPEARL Phenyl-650S chromatography, *lane 4* pooled PprM fraction following ultrafiltration on an Amicon Ultra-15 5K Centrifugal Filter Device. The position of the PprM band (10 kDa) is indicated on the right. **b** Gel filtration analysis. *Vertical* and *horizontal axes* indicate the absorbance (280 nm) intensity and elution time (min), respectively. PprM was eluted at 38.60 min. *Arrows* (*left* to *right*) indicate the elution times of albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and RNase A (13.7 kDa). **c** Molecular weight of PprM protein estimated by gel filtration. K_{av} was calculated using $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e, V_t and V_0 represent the elution, bed and void volumes (elution volume of Blue Dextran 2000), respectively. (*1*) RNase A, (*2*) chymotrypsinogen A, (*3*) ovalbumin and (*4*) albumin

found that the *pprA pprM* double-disruptant strain exhibited much higher sensitivity to γ-rays than *pprA* or *pprM* single disruptant strains (Fig. 2). Our study strongly suggests that PprM is involved in the unique radiation response mediated by PprI, and plays a crucial role in the induction of PprA together with other protein(s) that are important for the radioresistance of *D. radiodurans*. Further investigation is required to delineate the radiation response mechanism in *D. radiodurans*. Our efforts are currently being directed towards investigating the nature of the modification PprM is subjected to *via* PprI and identifying the protein(s) controlled by PprM.

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