Pnk1, a DNA Kinase/Phosphatase Required for Normal Response to DNA Damage by \(\gamma\)-Radiation or Camptothecin in *Schizosaccharomyces pombe* □

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We report the characterization of Pnk1, a 45-kDa homolog of the human polynucleotide kinase PNKP in *Schizosaccharomyces pombe*. Recombinant Pnk1 like human PNKP exhibits both 5′-DNA kinase and 3′-DNA phosphatase activities in vitro. Furthermore, we detected 3′-DNA phosphatase activity with a single-stranded substrate in extracts from wild-type yeast, but no activity was detected in *p nk1Δ* strains. We have shown that GFP-tagged Pnk1 like mammalian PNKP localizes to the nucleus. Deletion of *p nk1* does not affect cell growth under normal conditions but results in significant hypersensitivity to \(\gamma\)-radiation or camptothecin, an inhibitor of topoisomerase I, suggesting that Pnk1 plays an important role in the repair of DNA strand breaks produced by these agents. The *p nk1* deletion mutants were not hypersensitive to ethyl methanesulfonate, methyl methanesulfonate, or 4-nitroquinoline N-oxide. Expression of human PNKP in *p nk1Δ* cells restores resistance to \(\gamma\)-radiation or camptothecin, suggesting that the functions of yeast Pnk1 and human PNKP have been conserved.

DNA strand breaks arise during DNA base excision repair or can result from DNA damage by ionizing radiation and chemical agents, such as alkylating agents and topoisomerase inhibitors (1, 2). Such DNA damage often results in DNA strands with 5′-hydroxyl and 3′-phosphate termini. However, the repair of DNA damage by DNA polymerases and ligases requires 5′-phosphate and 3′-hydroxyl termini. Eucaryotic polynucleotide kinases (PNKs) were identified over 30 years ago (3, 4) and are subsequently shown to possess 3′-DNA phosphatase activity in addition to their 5′-DNA kinase activity (5, 6). The recent identification and characterization of human PNKP revealed that it too possesses both 5′-DNA kinase and 3′-DNA phosphatase activities (7, 8). Such dual DNA kinase/phosphatases could provide a mechanism for the repair of blocking lesions at the ends of DNA single strand breaks. Indeed, mammalian PNKP preferentially phosphorylates the 5′ termini associated with gaps and nicks, consistent with the idea that it is involved in the repair of DNA single strand breaks (9). More recent studies have shown that human PNKP associates with a complex containing XRCC1 (10), a protein that has been implicated in the repair of DNA single strand breaks (11–13). Furthermore, XRCC1 stimulates the DNA kinase and phosphatase activities of PNKP at damaged DNA termini. Thus, the evidence strongly suggests that PNKP is involved in the repair of DNA single strand breaks induced by DNA-damaging agents such as ionizing radiation.

A comparison of human PNKP with the DNA and protein sequence data bases revealed the existence of structurally homologous proteins among different eucaryotic organisms, including members of Animalia, Plantae, Fungi, and Protista (7, 8, 14, 15). However, several of these homologs lack either DNA kinase or phosphatase domains. For example, the budding yeast protein, Tpp1, has weak similarity to PNKP and has DNA phosphatase activity, but it lacks a DNA kinase domain and kinase activity (15). Here, we report the characterization of Pnk1, a homolog of human PNKP, in *Schizosaccharomyces pombe*. Our results demonstrate that Pnk1 has both 5′-DNA kinase and 3′-DNA phosphatase activities. Furthermore, we show that the deletion of the gene renders fission yeast hypersensitive to ionizing radiation and camptothecin, an inhibitor of topoisomerase I. Together, our results suggest that Pnk1 has a conserved role in DNA repair.

**EXPERIMENTAL PROCEDURES**

*DNA Manipulation and Analysis*—Procedures used for DNA purification, restriction-site mapping, electrophoresis, and transformation have been described previously (16).

*Plasmids*—The *p nk1* gene including flanking regions (−726 to +660 relative to the open reading frame) was amplified by PCR of *S. pombe* genomic DNA using the primers 5′-AGCTCTTAGATCTTTAAAAAGAGGCAACGC-3′ and 5′-AGCTTTGAGCTTTAGTGTCTGGATGTTTTT-3′. pBS-Pnk was constructed by cloning the PCR product into the XhoI and XbaI sites of pBluescript II SK (Stratagene). The *p nk1* open reading frame was then removed by digestion with EcoRV and BclI and replaced with a 1.8-kbp *ura4* fragment isolated from pBSUra4 by digestion with BamHI and HincII to form pBS-PnkUra4. The pREP41 and pREP41XHA expression vectors were described previously (17, 18). The *p nk1* coding sequence derived from *S. pombe* genomic DNA using the primers 5′-GATCCCTGAGATGCGTCGCTGAAAAAGG-3′ and 5′-GATCCGATTCTTATTTATATTTATTT-3′ was inserted into the pAA30 polylinker. pAA30-Pnk was constructed by the insertion of the *p nk1*-coding sequence derived from *S. pombe* genomic DNA using the primers 5′-GATCCCTGAGATGCGTCGCTGAAAAAGG-3′ and 5′-GATCCGATTCTTATTTATATTTATTT-3′.

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**FIG. 1. Comparison of yeast Pnk1 and human PNKP protein sequences.**

Protein sequences were aligned using the ClustalW alignment algorithm (37). Regions of sequence identity are shaded. Motifs conserved among PNKP-related proteins in other organisms are indicated.

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**Kinase and Phosphatase Assays**—The assays for DNA kinase activity using either micrococal nuclease-digested DNA or an oligonucleotide substrate and phosphatase activity using an oligonucleotide bearing a 3’-phosphate have been described previously (7, 9).

**Purification of Recombinant Pnk1 from Escherichia coli—**pET33-Pnk, which encodes His-tagged Pnk1, was transfected into the E. coli strain BLR(DE3) (Novagen). The bacteria were grown at 37 °C to an A_{600} of 0.6 in 100 ml of Luria-Bertani medium containing 50 μg/ml ampicillin and 12.5 μg/ml tetracycline. His6-Pnk1 expression was induced by centrifugation at 5000 × g for 5 min at 4 °C and resuspended in 1 ml of extraction buffer (50 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 4 mM 2-mercaptoethanol, and protease inhibitor mixture (Sigma)) and dialyzed overnight in the same buffer. The solution was applied on a 25-ml SP-Sepharose fast flow cation exchange column (Amersham Biosciences, Inc.). The column was washed with 80 ml of buffer A, and the enzyme was eluted with a 0.4 to 0.6 M NaCl gradient. The active fractions were eluted between 0.4 and 0.6 M NaCl. The active fractions coeluted with a 65% ammonium sulfate precipitation followed by centrifugation at 10,000 × g for 15 min. The pellet was resuspended in a minimum volume of buffer A (10 mM sodium phosphate, pH 7.3, 0.2 mM NaCl, 4 mM 2-mercaptoethanol, and protease inhibitor mixture (Sigma)) and dialyzed overnight in the same buffer. The solution was applied on a 25-ml SP-Sepharose fast flow cation exchange column (Amersham Biosciences, Inc.). The column was washed with 80 ml of buffer A, and the enzyme was eluted with a 300-ml linear gradient of buffer A containing 0.2 to 1.0 M NaCl. The active fractions were eluted between 0.4 and 0.6 M NaCl. The active fractions were pooled and concentrated by 65% ammonium sulfate. The precipitate was dissolved in buffer A and loaded onto a Superdex-75 PC 3.2/30 gel filtration column attached to a SMART micropurification system (Amersham Biosciences, Inc.). The active fractions were pooled, lyophilized, and resuspended in a minimal volume of buffer A. The purified protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the molecular mass of the protein was estimated to be 40 kDa. Mass spectral analysis of tryptic peptides confirmed that the purified protein was the Pnk1 cDNA. Eight peptides were identified by their mass, HHNVPR (786 Da), YYHNGKQ (939 Da), NSSSHFYHPKIF (1204 Da), QLPFIEAFNSFK (1294 Da), SPQFQSLSTYFEK (1513 Da), STLAE-SQVTHQ (1682 Da), DAADTWPHSPVVPK (1795 Da), and YVGDAAAPHSGFDNLSTKL (1873 Da).
Partial Purification of Polynucleotide Kinase from S. pombe—Fission yeast strains expressing either Pnk1 or PNKP were grown to a $A_{600}$ of 1.0 and were washed in $H_2O$. The cells were resuspended in 50 mM Tris-Cl, pH 8.0, 1 mM EDTA, 100 mM NaCl, and protease inhibitor mixture and disrupted by French press. To remove nucleic acid, the supernatant obtained from French press was mixed for 1.h with preswelled DE52 resin, which was then removed by low speed centrifugation. The supernatant was dialyzed against buffer A and loaded onto a 5-ml Hi-Trap SP cation exchange column (Amersham Biosciences, Inc.). The column was washed with 15 ml of buffer A, and protease inhibitor mixture and disrupted by French press. The active fractions were pooled, concentrated by 65% ammonium sulfate, dialyzed against a minimal volume of buffer A, and used for kinase and phosphatase activities.

Polyclonal Antiserum—The antigen used to raise the antibodies was gel-purified His$_6$-Pnk1 recovered from the insoluble fraction from E. coli as described previously (24). Approximately 5–10 mg of the inclusion body material was mixed with gel loading buffer (0.1 M Tris-HCl, pH 6.8, 10% glycerol, 4% SDS, 100 mM dithiothreitol, 0.1% bromphenol blue), boiled for 5 min, and run on a 10% SDS-polyacrylamide gel. The His$_6$-Pnk1 was electroeluted by excising the 48-kDa band from the gel, transferring the gel slices to dialysis tubing containing the SDS-PAGE-running buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS) immersed in a shallow layer of the same buffer in an electrophoresis tank and electrophoresed overnight at 30 V. The protein concentration was determined by the Bradford method using a commercial protein assay system (Bio-Rad). For immunization, a New Zealand White female rabbit was given an initial intramuscular injection of 200 μg of protein followed by three boosts with 100 μg of protein injected at monthly intervals following a standard protocol (25).

RESULTS

Comparison of Fission Yeast Pnk1 and Human PNKP Sequences—A standard BLAST search of the GenBank™ data base for proteins homologous to human PNKP revealed the presence of a homologous protein in S. pombe that we have named Pnk1 (7, 8). We amplified the Pnk1 cDNA by PCR and sequenced the entire cDNA (Fig. 1). The cDNA sequence from S. pombe Pnk1 was determined by the Sanger method using a commercial kit (Perkin-Elmer). The number of nucleotides in the Pnk1 gene rather than two as reported in GenBank™, and the exons encode a predicted protein of 408 residues (Fig. 1). Human PNKP (521 residues) and fission yeast Pnk1 are structurally homologous; they share 127 identical residues (Fig. 1). An alignment of Pnk1 with human PNKP and other related PNKs revealed a strong sequence similarity within five conserved regions of the kinase and phosphatase domains (7, 8, 15). In particular, Pnk1 contains a conserved ATP-binding Walker A motif (hGXPGXGKSTh) in the kinase domain and phosphotransferase
(DXDX(T/V)) and DDDD motifs in the phosphatase domain in addition to other conserved motifs that are found in human PNKP and other related eukaryotic PNKs (Fig. 1).

Pnk1 Exhibits DNA Kinase and Phosphatase Activities—The high degree of sequence identity between the yeast Pnk1 and human PNKP suggests that they may be functionally conserved as well. Thus, we explored the possibility that Pnk1 possesses DNA kinase and phosphatase activities similar to human PNKP. First, we generated plasmids to express and purify recombinant His_{6}-tagged Pnk1 from E. coli (see “Experimental Procedures”). An analysis of purified protein fractions by polyacrylamide gel electrophoresis indicated that they contained a homogeneous protein with a molecular mass of ~48 kDa corresponding to the predicted molecular mass of His_{6}-Pnk1. We found that purified His_{6}-Pnk1 exhibited both 5'-DNA kinase and 3'-DNA phosphatase activities (Fig. 2). No activity was observed in corresponding proteins fractions from bacteria containing a control vector (data not shown).

To further investigate the function of Pnk1, we generated fission yeast strains lacking pnk1 by gene replacement with the ura4-selectable gene (see “Experimental Procedures”). Deletion of pnk1 did not have any apparent effect on cell growth, mating, or germination under normal conditions (data not shown). We generated an anti-Pnk1 polyclonal antibody, and we examined wild-type and pnk1Δ yeast cell extracts by Western blot analysis to verify the presence or absence of Pnk1 expression. We detected a 45-kDa protein corresponding to the predicted size of Pnk1 in wild-type yeast, however, we did not detect a similar protein in pnk1Δ cells (Fig. 3A). We also detected a similar band in pnk1Δ cells overexpressing HA-Pnk1, but the antibody did not detect human PNKP in cells expressing this protein. Expression of human PNKP in S. pombe was verified by Western blotting with an antibody to PNKP (24).

Next, we examined DNA phosphatase activities in partially purified extracts from pnk1Δ and wild-type cells. To avoid any potential contribution to 3'-phosphatase activity arising from an apurinic/apyrimidinic endonuclease in the cell extract, we used a single-stranded oligonucleotide substrate, because all known apyrimidinic endonucleases appear to be double strand-specific (1, 26). Our results show that we detected significant 3'-DNA phosphatase activity in extracts from wild-type cells, but no detectable activity was observed in pnk1Δ cell extracts (Fig. 3B). The phosphatase activity in the cell extracts coeluted from a cation-exchange column with recombinant Pnk1. We also detected 3'-DNA phosphatase activities in pnk1Δ cells expressing either yeast Pnk1 or human PNKP (Fig. 3B). Attempts to recover 5'-DNA kinase activity from cell extract were unsuccessful. Our experience with the recombinant Pnk1 indicated that the kinase activity is less robust than that of human PNKP.

Pnk1 Is Localized in the Nucleus—Human PNKP has been previously shown to localize in the nucleus of human A549 cells (7). To examine the localization of Pnk1 in fission yeast, we expressed GFP-tagged Pnk1 in wild-type cells. An examination of cells expressing GFP-Pnk1 by fluorescence microscopy revealed that it colocalized with 4',6-diamidine-2'-phenylindole dihydrochloride staining, indicating that it was predominantly localized in the nucleus, whereas, GFP alone was distributed throughout the cell (Fig. 4). This observation is consistent with

**FIG. 4. Pnk1 localizes to the nucleus.** Normal (AS7) fission yeast cells expressing GFP, GFP-Pnk1, or GFP-Pnk1*K4E,R6D were briefly fixed before being mounted in medium containing 4',6-diamidine-2'-phenylindole dihydrochloride as described previously (38). Cells were visualized by differential interference contrast (DIC) microscopy. DNA (DAPI) and GFP localization were visualized using fluorescence microscopy. Expression plasmids used were pAALNGFPHA, pAALNGFPHA-Pnk1, and pAALNGFPHA-Pnk1*K4E,R6D.

**FIG. 5. Deletion of pnk1 results in hypersensitivity to γ-radiation and camptothecin, and expression of human PNKP rescues pnk1 null phenotypes.** A, to assay for radiosensitivity, overnight cultures of normal (WT) and pnk1Δ strains were serially diluted, plated onto 60-mm minimal medium plates, and irradiated with 1000 Gy using a Gammapac 1000 137Cs x-ray source (Nordion) at 2.5 Gy/min. Colony number was counted after 3 days of incubation at 30 °C. The graph indicates the percentage survival (y axis) of WT or pnk1Δ cells harboring a control vector or pnk1Δ cells expressing either Pnk1 or human PNKP. B, to test for sensitivity to camptothecin, cells were grown at 30 °C for 4 days on PMA plates containing 0.1% Me_{2}SO, 25 mm HEPES, pH 7.2, in the presence or absence of 5 μg/ml camptothecin (Sigma). Expression plasmids used were pREP4IHX (control vector), pREP4IHX-Pnk, and pREP41-HPnk.
conserved nuclear roles for human PNKP and fission yeast Pnk1. We also expressed mutant GFP-Pnk1[K448,R456] in which residues in the putative N-terminal nuclear localization signal have been replaced. As predicted, this mutant protein failed to localize to the nucleus (Fig. 4).

Deletion of pnk1 Results in Hypersensitivity to DNA-damaging Agents—To further investigate the roles of Pnk1, we examined pnk1Δ cells for sensitivity to DNA damage by ionizing radiation. We found that pnk1Δ cells were hypersensitive to γ-radiation compared with wild-type cells (Fig. 5A), suggesting that Pnk1 is important for the normal cellular response to DNA damage, and may be directly involved in DNA repair. We also found that pnk1Δ cells were hypersensitive to camptothecin (5 μg/ml), an inhibitor of topoisomerase I (Fig. 5B). However, we did not observe a significant difference in the sensitivity to other DNA-damaging agents including ethyl methanesulfonate, methyl methanesulfonate, and 4-nitroquinoline N-oxide. Expression of Pnk1 restored normal resistance to γ-radiation or camptothecin in pnk1Δ cells. Furthermore, we found that the expression of human PNKP in pnk1Δ cells restored normal resistance to either γ-radiation or camptothecin, suggesting that the functions of these proteins have been conserved (Fig. 5).

DISCUSSION

DNA damage can lead to serious cellular consequences including mutation, gene rearrangement, cell cycle arrest, and cell death. Thus, mechanisms to repair DNA lesions are important for the selective fitness and survival of all species. Consequently, several DNA repair mechanisms have evolved and are highly conserved among eucaryotes. A role for polynucleotide kinases in DNA repair has been previously suggested, and recent evidence has implicated mammalian PNKP in single strand break repair, although the details remain to be determined. The high degree of conservation of PNKP with related proteins in other eucaryotic organisms suggests that a role for polynucleotide kinase/phosphatase-related proteins in DNA repair.

It has been shown previously that the expression of human PNKP into HeLa cells or the XRCC1 mutant Chinese hamster ovary cell line, EM9, affords a degree of resistance to the alkylating agent ethyl methanesulfonate (10). Here, we provide clear evidence for the involvement of Pnk1 in the normal response of S. pombe to ionizing radiation and the antineoplastic agent, camptothecin. It has long been known that ionizing radiation can generate strand breaks with 3′-phosphate and 5′-hydroxyl termini (27, 28). Thus, there is an important role for a polynucleotide 5′-kinase/3′-phosphatase in the restoration of these termini to a configuration suitable for strand rejoining by a DNA polymerase and ligase. In many organisms, 3′-phosphate groups can be removed by an apyrimidinic endonuclease (29). However, little progress has been made in identifying an active apyrimidinic endonuclease in S. pombe extracts (30, 31), and it remains to be seen whether Pnk1 is the major 3′-DNA phosphatase in this organism.

Camptothecin inhibits topoisomerase I after the enzyme has incised the DNA, thus trapping the enzyme while it is covalently attached to the DNA terminus via a 3′-phosphate linkage (32). The 5′ terminus of the DNA at such a break bears a hydroxyl group. Several investigators have proposed that the repair of such strand breaks would require a polynucleotide kinase to phosphorylate the 5′ terminus and remove the 3′-phosphate if such a group remains at the 3′ terminus after hydrolysis of the DNA-topoisomerase linkage (33–35). Support for this supposition was enhanced with the discovery and cloning of a gene in S. cerevisiae encoding a tyrosine-DNA phosphodiesterase that can hydrolyze the topoisomerase I-DNA complex (36). Our data showing that the deletion mutants of Pnk1 are hypersensitive to camptothecin provide substantial support for a DNA repair mechanism involving a 5′-kinase/3′-phosphatase. That human PNKP can efficiently substitute for the fission yeast enzyme suggests that a similar mechanism for repair of camptothecin-blocked topoisomerase-DNA complexes may be operative in human cells, and this could have implications for the resistance of tumor cells to camptothecin and similar topoisomerase I inhibitors.

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