Human Polynucleotide Phosphorylase Protein in Response to Oxidative Stress

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ABSTRACT: 8-Oxo-7,8-dihydroguanine (8-oxoGua) is generated in nucleic acids as well as in their precursors due to the actions of oxygen radicals produced through a normal cellular metabolism. Since oxidized guanine can pair with both cytosine and adenine, it causes alterations in the phenotypic expression when it is present in RNA. To prevent such an outcome, organisms must have some mechanism for eliminating such oxidized guanine nucleotides from RNA and its precursors. In mammalian cells, MTH1 and NUDT5 proteins degrade 8-oxoGTP and 8-oxoGDP to 8-oxoGMP, which is an unusable form for RNA synthesis. In a search for proteins functioning at the RNA level, polynucleotide phosphorylase (PNP) protein has been suggested to be a good candidate for such a role. The human PNP protein has an ability to bind specifically to RNA containing 8-oxoGua. When human cells are exposed to agents that induce oxidative stress, such as hydrogen peroxide and menadion, the amounts of PNP protein decrease rapidly while amounts of other proteins in the cells do not change after such treatments. No specific decrease in the PNP protein level is observed when cells are treated with ACNU and cycloheximide at doses sufficient to provide the same degree of growth suppression. These results imply that the PNP protein might thus play a role in excluding oxidized forms of RNA from the translation mechanism.

Reactive oxygen species, such as superoxide and hydroxyl radicals, have been produced through a normal cellular metabolism, and the formation of such radicals is thus further enhanced by ionizing radiation and various chemicals. Nucleic acids exposed to oxygen radicals generate various modified bases, and more than 20 different types of oxidatively altered purines and pyrimidines have thus been detected (*1*, *2*). Among them, 8-oxo-7,8-dihydroguanine (8-oxoGua)[1] is the most abundant, and it seems to play a critical role in the maintenance and transfer of genetic information (*3*, *4*). Unlike other types of oxidatively altered bases, 8-oxoGua does not block nucleic acid synthesis but rather induces base mispairing; it can pair with cytosine and adenine with almost equal efficiencies. This mispairing is thought to significantly contribute to the levels of spontaneous mutation frequencies (*5*−*8*), and when 8-oxoGua is present in messenger RNA, it thus possibly causes alterations in gene expression (*9*).

Organisms are equipped with elaborate mechanisms for counteracting such deleterious effects of 8-oxoGua. In *Escherichia coli*, two glycosylases encoded by the *mutM* and *mutY* genes function to prevent mutations caused by 8-oxoGua in DNA. The MutM protein removes 8-oxoGua paired with cytosine, while the MutY protein excises adenine paired with 8-oxoGua (*10*−*13*). Similar mechanisms appear to function in mammalian cells. MYH has been identified as a mammalian MutY homologue, and OGG1 is a functional counterpart of *E. coli* MutM (*14*−*17*). The oxidation of guanine also proceeds in free nucleotides, and an oxidized form of dGTP, 8-oxo-dGTP, is a potent mutagenic substrate for DNA synthesis. The MutT protein of *E. coli* hydrolyzes 8-oxo-dGTP and 8-oxo-dGDP to 8-oxo-dGMP, thereby preventing the misincorporation of 8-oxoGua into DNA (*18*, *19*). Mammalian cells also possess enzymes capable of eliminating 8-oxoGua-containing nucleotides from the DNA precursor pool. These include MTH1 (NUDT1), MTH2 (NUDT15), and NUDT5, all of which are structurally similar to MutT and can replace the MutT when they are expressed in *E. coli mutT*$^-$ cells (*20*−*23*).

The persistence of 8-oxoGua in RNA may cause errors in gene expression, and some of the error avoidance mechanisms operating in DNA synthesis may thus play a role in RNA synthesis, thereby reducing the error rate (*9*, *24*, *25*). The *E. coli* MutT protein hydrolyzes 8-oxoGua-containing RNA precursor nucleotides, 8-oxoGTP and 8-oxoGDP, to 8-oxoGMP, an unusable form for RNA synthesis (*19*). Among the human MutT-related proteins, MTH1 and NUDT5 have the ability to hydrolyze 8-oxoGTP and 8-oxoGDP to the nucleoside monophosphate, respectively (*25*). In mammalian cells, these proteins appear to induce accurate protein synthesis by eliminating 8-oxoGua-containing ribonucleotides from the RNA precursor pool.

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[1] Abbreviations: 8-oxoGua, 8-oxo-7,8-dihydroguanine; 8-oxoGTP, 8-oxo-7,8-dihydroguanosine 5′-triphosphate; 8-oxoGDP, 8-oxo-7,8-dihydroguanosine 5′-diphosphate; 8-oxoGMP, 8-oxo-7,8-dihydroguanosine 5′-monophosphate; DTT, dithiothreitol; TLC, thin-layer chromatography; SDS, sodium lauryl sulfate; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl D-thiogalactopyranoside; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase; menadion, 2-methyl-1,4-naphthoquinone; ACNU, 1-(4-amino-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride; PNP, polynucleotide phosphorylase.

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Even though the misincorporation of 8-oxoGua into RNA could be prevented in such a way, there is another possible route for yielding 8-oxoGua in RNA. Guanine residues of nucleic acids may be attacked by active oxygen species, and such in situ oxidation may occur more frequently in RNA molecules, which are mostly single-stranded, than in double-stranded DNA, in which the bases are protected by their hydrogen bonding. Moreover, once the guanine bases in RNA are oxidized, they cannot be repaired. This is in contrast to the case in DNA, in which most of the oxidized bases can be excised and replaced through the work of efficient DNA repair systems. Organisms must therefore have some mechanism for eliminating RNA carrying 8-oxoGua from the translation process. Otherwise, in cells, particularly in those with long life spans, abnormal proteins would accumulate, which might thus cause a dysfunction of the cell.

In a search of the process for the quality control of RNA, we found that a 77 kDa protein of *E. coli* has the ability to bind specifically to RNA carrying 8-oxoGua (*26*). This protein was identified as polynucleotide phosphorylase (PNP), and *pnp*$^-$ mutants that lack this protein are hyperresistant to paraquat, an agent which is known to induce oxidative stress. Recent sequence analyses have revealed that the protein, which has been found in bacteria and plants, also exists in mammalian cells (*27*−*29*). These situations prompted us to investigate whether the mammalian protein possesses a capacity to scavenge oxidatively damaged RNA. We herein show that the human PNP protein interacts with 8-oxoGua-containing RNA and responds to cellular oxidative stress.

EXPERIMENTAL PROCEDURES

Chemicals. 8-OxoGTP was prepared as previously described (*9*). Poly(A)·poly(U) and poly(G)·poly(C) were prepared by heat denaturation and renaturation with poly(A) and poly(U) or poly(G) and poly(C), respectively. [^{32}P]-8-OxoGMP-labeled poly(8-oxoG-A) and [^{32}P]GMP-labeled poly(G-C) were prepared, as described previously (*9*). Briefly, the reaction was carried out with *E. coli* RNA polymerase (0.12 unit/μL) in 0.1 M Tris-HCl (pH 7.8), 2.5 mM $MnCl_2$, 0.2 mM DTT, 0.1 mM poly(dA-dT) or poly-(dI-dC), 0.2 mM ATP, and 0.05 mM [α-^{32}P]-8-oxo-rGTP or 0.2 mM CTP and 0.05 mM [α-^{32}P]GTP. After incubation at 37 °C for 60 min, RNase-free DNase I (0.5 unit/μL) was added and the mixture was incubated for a further 10 min. The reaction mixture was diluted with 5 volumes of 10 mM Tris-HCl (pH 7.5) and 0.1 M NaCl and applied to a small column containing 20 μL of AG 50W-X8 (Bio-Rad) and 100 μL of DE52 (Whatman). The column was washed with 1 mL of 10 mM Tris-HCl (pH 7.5) and 0.1 M NaCl and then with 1 mL of 10 mM Tris-HCl (pH 7.5) and 0.3 M NaCl. RNAs were eluted with 1 mL of 10 mM Tris-HCl (pH 7.5) and 0.6 M NaCl and precipitated with 2 volumes of ethanol. Poly(C-G) was partially digested with RNase A to adjust the molecular size to that of poly(8-oxoG-A). The concentrations of the RNAs were determined by using specific radioactivity and were represented as nucleotides. As competitor polymers, labeled poly(8-oxoG-A) and poly(G-C) were also prepared by using very low (1/100) radioactive trinucleotides as the substrate. The radioactivity was used to determine the concentrations of the polymers.

Purification of Human PNP Protein. A cDNA clone, IMAGE3919329 (BE892809), selected by using a BLAST search, was purchased from IncyteGenomics. The nucleotide sequence was determined by BigDye terminator cycle sequencing with the primers chosen by GeneWorks. To construct the GST−PNP fusion gene, a *Ssp*I fragment containing the coding sequence was ligated to *Not*I linkers. The ligation products were then cut at the *Sal*I and *Not*I sites, located in the 5′-region of the cDNA and at the 3′-linker terminus, respectively. The digest was inserted into the *Sal*I−*Not*I sites of a pGEX-4T3 cDNA expression vector in frame. When the GST−PNP protein (~110 kDa) was produced in *E. coli* SK5691 (*pnp-7*) at 37 °C (*30*), almost all of the fusion proteins were found in the inclusion body. To obtain a native form of the protein, bacteria were cultured at lower temperatures; a culture grown at 28 °C for 2 days was diluted with a 50-fold volume of medium and further incubated at 23 °C overnight. Briefly, the cells were resuspended in 25 mM Tris-HCl (pH 8.0) and 0.15 M NaCl containing protease inhibitor cocktail tablet, Complete EDTA-free (Roche), and then were disrupted by sonication. The lysate (20 mL) was mixed with 5 mL of glutathione−Sepharose 4B (Amersham Pharmacia), and then the suspension was gently rotated for 4 h at 4 °C. The mixture was placed in a column (0.56 cm^2 × 8 cm), and the column was washed extensively with the same buffer. The recombinant protein was then eluted with the same buffer containing 10 mM reduced glutathione. Although only a single band was detected on SDS−PAGE stained with Coomassie Brilliant Blue, Western blotting with anti-GST monoclonal antibody B-14 (Santa Cruz Biotechnology, Inc.) revealed that the eluate contains a small amount of truncated forms of the fusion protein. To remove the truncated forms, the eluate was concentrated with a Centriprep YM10 device (Amicon) and applied to a Superose-6 column. A nearly homogeneous preparation of GST−PNP protein was obtained, as indicated by Western blotting and silver staining.

Immunological Procedures. To obtain anti-PNP antibodies, the recombinant protein was produced in BL21-CodonPlus-RIL (Stratagene) cells at 37 °C in the presence of 1 mM IPTG. Under the conditions that were used, the recombinant protein was recovered as an inclusion body. Polyclonal antibodies were obtained by the immunization of a rabbit with the inclusion body. Anti-rabbit IgG and horseradish peroxidase-linked whole donkey antibody as the second antibody were purchased from Amersham Bioscience. Monoclonal anti-β-action clone AC-15 (A-5441) and anti-mouse IgG horseradish peroxidase-linked whole sheep antibody (NA931V) as the second antibody were purchased from Sigma and Amersham Bioscience. The cell extracts were prepared by the sonication of cells in PBS, 0.137 M NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4 (pH 7.5), containing protease inhibitor cocktail tablet, Complete EDTA-free (Roche). A Western blotting analysis was performed with a Western Lightning kit (PerkinElmer Life Sciences, Inc.), according to the manufacturer's instructions.

Immunoprecipitation was performed with anti-PNP antiserum and Protein A-Sepharose (4 Fast Flow; Amersham Pharmasia Biotech AB), according to the manufacturer's instructions. Briefly, 1 mL of whole cell extract in 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% (w/v) Triton X-100, and 10% (w/v) glycerol was mixed with 2 μL of antiserum (or preimmune serum as a control), and the mixture was kept at 4 °C for 90 min. Fifty microliters of a 50% Sepharose

suspension was added to the mixture and incubated for a further 30 min. Sepharose beads were recovered by centrifigation and washed extensively with the same buffer. This material was used for Western blotting and an enzyme assay.

Assay of Enzyme Activity. The PNP activity was determined by assessing the transfer of labeled inorganic phosphate to nucleoside diphosphates in the presence of homoribopolymers. Briefly, the reaction was carried out in a reaction mixture containing 1 mM polymer [poly(C), poly(U), poly(A), or poly(G)], 25 mM Tris-HCl (pH 9), 10 mM $MgCl_2$, 0.4 mM DTT, 0.1 M NaCl, and 0.2 μM ^{32}P-labeled sodium orthophosphate. After incubation at 37 °C, a 0.5 μL aliquot of the reaction mixture was spotted on a TLC plate (PEI-cellulose F; Merck), and the plate was developed with 0.5 M LiCl. The radioactivity was measured in a BAS2000 image analyzer, and the amounts of nucleoside diphosphates produced were determined.

Interference with siRNA. 21mers of dsRNAs corresponding to parts of the human PNP cDNA sequence were synthesized and used for the RNAi experiments. The two types of dsRNA with the following target sequences were produced: siRNA1409 (5′-AAGATTACATCTGAAGTCCT-3′) and siRNA1781 (5′-AAAACCTCGAGCATCTAGAAA-3′). As a control, nonsilencing dsRNA (catalog number 1022076) was purchased from QIAGEN Inc. Transfection was performed by using RNAiFect (QIAGEN) according to the manufacturer's instructions. Briefly, 1.5×10^5 cells were seeded in 24-well plates and incubated overnight. After the medium was exchanged with 0.3 mL of a fresh one, 0.1 mL of transfection buffer containing 1 μM siRNA was added dropwise to the culture medium. The cells were incubated at 37 °C for 1 day and then trypsinized and reseeded on 35 mm dishes. After incubation for 2 days, the analyses were performed.

Treatment of Cells with Chemical Agents. One million cells were placed in 35 mm dishes and incubated at 37 °C for 1 day. The medium was replaced with a fresh one containing various chemicals at the following concentrations: 2 mM hydrogen peroxide, 0.5 mM menadion, 2.5 mM ACNU, and 1 μM cycloheximide. These concentrations correspond to 10 times those used to produce a 50% growth suppression. At the indicated times, whole cell extracts were prepared by sonication, and the enzyme assay and Western blotting were performed.

Other Procedures. The protein concentrations were determined using Bio-Rad protein assay kits with bovine serum albumin as a standard (*31*). All other procedures have been described previously (*24*).

RESULTS

Specific Binding of Human PNP Protein to 8-OxoGua-Containing RNA. A GST-fused form of human PNP protein (GST−PNP) was produced in *E. coli pnp*$^-$ cells and purified. Figure 1A shows Western blotting with the mouse anti-GST monoclonal antibody and silver staining of the protein. The bands obtained with the two analyses closely corresponded to one another, and no other band was detected on the gels. As shown in Figure 1B, the purified recombinant protein possesses an activity for catalyzing the conversion of polynucleotide to nucleoside diphosphate upon consumption of inorganic phosphate. Under the conditions that were used, formation of UDP from poly(U) occurs most rapidly, which

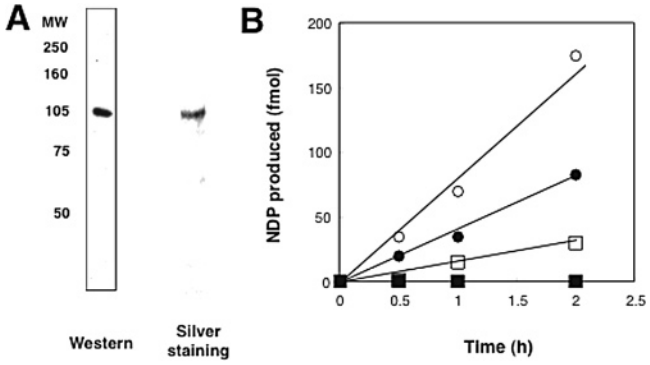

FIGURE 1: Purification of the PNP protein. (A) SDS−PAGE of a purified preparation of recombinant protein. Thirty microliters of a Superose-6 peak fraction was subjected to SDS−8% PAGE followed by Western blotting using monoclonal antibody and silver staining. (B) Phosphorylase activities of the recombinant protein toward various polyribonucleotides. One picomole of [^{32}P]orthophosphate and 5 nmol of polynucleotides were incubated with 2 μL of the Superose-6 peak fraction in a reaction mixture (5 μL). After incubation at 37 °C for the indicated times, 0.5 μL aliquots of the reaction mixture were spotted on a TLC plate and developed in 0.5 M LiCl. Total amounts of nucleoside diphosphates produced were determined by measuring the radioactivity: (●) CDP, (○) UDP, (□) ADP, and (■) GDP.

is followed by the formation of CDP and ADP from the corresponding polynucleotides. Only a small amount of GDP was produced when poly(G) was used as a substrate.

The *E. coli* PNP protein binds specifically to 8-oxoGua-containing RNA to form stable RNA−protein complexes (*26*). To clarify whether the human PNP protein possesses a similar ability, an RNase protection assay was performed. When poly(8-oxoG-A), in which the 8-oxoGMP moieties of the polymer were labeled with ^{32}P, was incubated with RNase A, all the radioactive materials were converted to nucleotides (Figure 2A, right panel). However, when the polymer was preincubated with the purified GST−PNP protein and then was subjected to RNase digestion, significant amounts of radioactive materials remained at a position for the polymer, even after prolonged treatment. When the polymer was preincubated with the same amounts of GST protein (without the PNP sequence), no protection from the RNase digestion existed. Moreover, poly(U-A), in which the UMP moieties were labeled with ^{32}P, was completely digested even in the presence of GST−PNP (Figure 2A, left panel). The protection of 8-oxoGua-containing RNA from nuclease attack would thus mean that the PNP protein binds to poly(8-oxoG-A).

To confirm the findings described above, a gel shift assay was performed. In this experiment, the results of which are shown in Figure 2B, poly(8-oxoG-A) was incubated with either the preparation of GST−PNP or GST protein, and then the mixtures were subjected to native gel electrophoresis. When the polymer was incubated with GST−PNP, three distinct bands were detected (lane 2), and one of the bands (the upper one) remained intact even after digestion by RNase A (lane 5). No such band was detected with the GST protein alone (lane 3). To clarify whether the binding is poly(8-oxoG-A) specific, labeled poly(G-C) was also incubated with GST−PNP. As shown in the lane 8, no band was detected. To confirm the specificity, various polymers were added to the reaction mixture as competitors, and then their effects on complex formation were examined (Figure 2C). When a

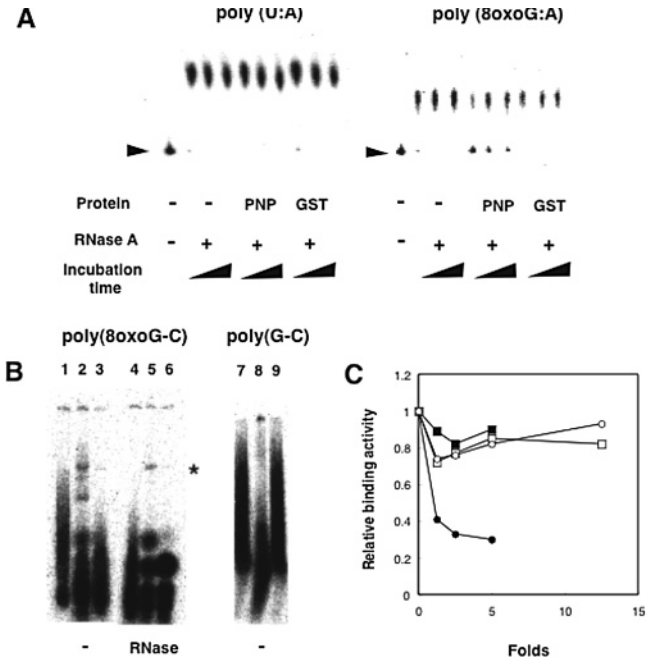

FIGURE 2: Capacity of binding of human PNP protein to 8-oxoGua-containing polyribonucleotide. (A) RNase protection assay. Ten picomoles of [^{32}P]UMP-labeled poly(U-A) and [^{32}P]-8-oxoGMP-labeled poly(oxoG-A) were preincubated with or without 5 pmol of GST−PNP or GST protein in a reaction mixture (5 μL) containing 0.1 M Tris-HCl (pH 7.4), 5 mM $MgCl_2$, and 0.4 mM DTT for 10 min on ice and then incubated in the presence or absence of RNase A (0.08 μg) at 30 °C. At the indicated times, 0.5 μL aliquots of the reaction mixture were spotted on a TLC plate and run in 1 M LiCl. Radioactive spots were monitored using a BAS2000 image analyzer. The arrowheads show the polynucleotides which remain at the origin. The triangles show the duration of the incubation times: 10, 20, and 30 min. (B) Gel shift assay. For the reaction, 0.5 pmol of ^{32}P-labeled poly(8oxG-A) or poly(G-C) was incubated with or without 36 pmol of GST−PNP or GST protein in 10 μL of a reaction mixture containing 0.1 M Tris-HCl (pH 7.4), 5 mM $MgCl_2$, and 0.4 mM DTT for 10 min on ice, and then it was incubated with or without RNase A (0.08 μg) at 37 °C for 10 min. The reaction mixtures were analyzed in native 6% PAGE. Electrophoresis was performed at 10 V/cm for 2.5 h in 90 mM Tris-borate (pH 8.0)/2 mM EDTA buffer. The gels were dried, and radioactive bands were monitored. Samples 1−3 and 7−9 were incubated without RNase A, while samples 4−6 were incubated with RNase A. Samples 1, 4, and 7 were preincubated without protein; samples 2, 5, and 8 were preincubated with GST−PNP, and samples 3, 6, and 9 were preincubated with GST protein. The asterisk indicates a complex remaining intact after digestion with RNase A. (C) Competition for binding to 8-oxoguanine-containing polynucleotide. Labeled poly(8-oxoG-A) was incubated with purified GST−PNP in a reaction mixture containing various amounts of polymers, including poly(8-oxoG-A), poly(G-C), poly(A)·poly(U), and poly(G)·poly(C). The levels of radioactivity of the complexes that formed were monitored. The relative binding activities were determined by dividing the levels of radioactivity in the presence of competitor polymers by those without the competitor: (●) poly(8-oxoG-A), (■) poly(G-C), (□) poly(A)·poly(U), and (○) poly(G)·poly(C).

5-fold excess of poly(8-oxoG-A) was added to the reaction mixture, complex formation was significantly abolished. On the other hand, the same amounts of poly(G-C), poly(A)·poly(U), and poly(G)·poly(C) had no appreciable effect on complex formation (Figure 2C).

Immunological Quantification of PNP Protein. It is difficult to assay the PNP activity in crude extracts of human cells, since the extracts contain various interfering enzyme activities and substances to inhibit the PNP activity. To overcome such difficulty, we developed an immunological assay procedure. Antibodies against human PNP were prepared by using a purified preparation of recombinant protein and then were used for Western blotting analyses. As shown in Figure 3A, a major band corresponding to the human 80 kDa PNP protein was detected in the extracts prepared from human cell lines Jurkart, HeLa, and HepG2. Other human cell lines, including PL14, OUR10, TCO-1, XP2YO(SV), KPK13, and KB, contain almost the same amount of PNP protein (data not shown).

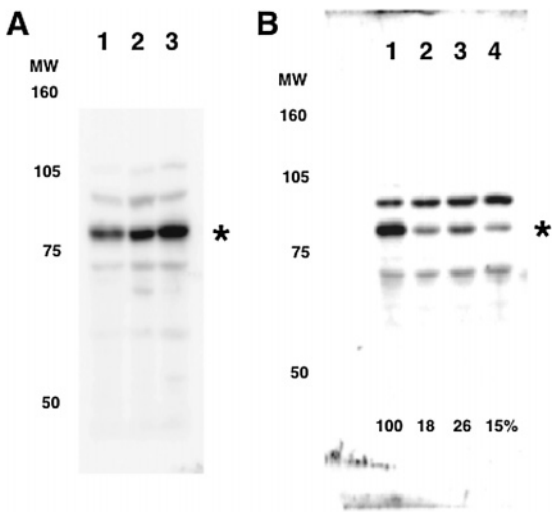

FIGURE 3: Detection of PNP protein in crude extracts of human cells. (A) Western blotting of the whole cell extracts. Fifty micrograms of the cell extracts from various cell lines was subjected to SDS−8% PAGE, and then antiserum against PNP protein was applied: lane 1, Jurkat; lane 2, HeLa; and lane 3, HepG2. (B) Effects of siRNAs. Twenty-five micrograms of whole cell extracts, prepared from HeLa cells transfected with various siRNAs, was subjected to SDS−8% PAGE followed by Western blotting: lane 1, 100 pmol of nonsilencing control dsRNA; lane 2, 100 pmol of dsRNA1409; lane 3, 100 pmol of dsRNA1781; and lane 4, 50 pmol each of dsRNA1409 and dsRNA1781. Asterisks indicate the positions where human PNP protein migrates. The numbers (%) indicate the relative amounts of PNP protein in the extracts.

To confirm that the 80 kDa protein is the PNP, an RNAi experiment was performed. HeLa cells were transfected with siRNAs carrying antisense sequences for PNP, and amounts of the protein were measured by a quantitative Western blotting analysis (Figure 3B). A 15−26% reduction in the levels of the 80 kDa protein was achieved by the application of the siRNAs, while no significant reductions were observed in other bands. To show that the 80 kDa protein possesses an activity for catalyzing the PNP reaction, the protein was partially purified by concomitant treatment of the whole cell extract with antiserum and Protein A-Sepharose. Only the 80 kDa protein recovered from the immunoprecipitate and then was applied to the enzyme assay. As shown in Figure 4, it carried a PNP activity. On the basis of the Western blotting analysis shown in Figure 4B, the specific activity of cellular PNP protein was estimated to be 10 fmol of UDP produced h^{-1} (pmol of protein)$^{-1}$, which almost corresponds to the value obtained with a purified preparation of recombinant protein [18 fmol of UDP produced h^{-1} (pmol of protein)$^{-1}$].

Response of PNP Protein to Oxidative Stress. With an appropriate assay procedure in hand, it is possible to explore

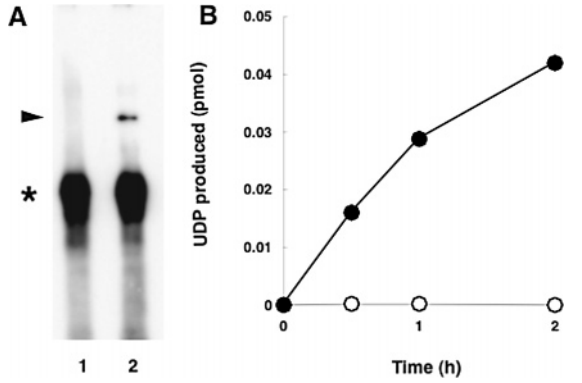

FIGURE 4: PNP activity of cellular extracts. (A) Western blotting of immunoprecipitates. One milliliter of whole cell extract was mixed with 2 μL of antiserum or preimmune serum. After immunoprecipitation, the total precipitate was subjected to SDS−PAGE followed by Western blotting: lane 1, preimmune serum; and lane 2, antiserum against PNP protein. The arrowhead shows PNP protein, and the asterisk indicates rabbit IgG protein. (B) Production of UDP from poly(U) and [^{32}P]orthophosphate by the reaction with the immunoprecipitate. The immunoprecipitated complexes were suspended in 5 μL of the reaction mixture for assay of PNP activity and incubated at 37 °C with occasional shaking. At the indicated times, amounts of UDP produced were determined by measuring radioactivity: (●) antiserum against recombinant protein and (○) preimmune serum.

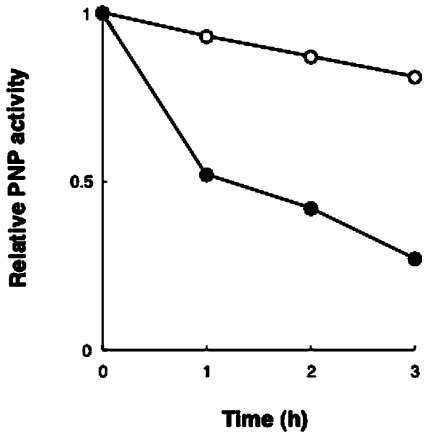

FIGURE 5: PNP activity in cells treated with hydrogen peroxide. HeLa cells were cultured in 6 cm (diameter) dishes overnight. The cells were then placed in a fresh medium containing 2 mM hydrogen peroxide and incubated at 37 °C. At the indicated times, the cells were collected and extracts were prepared by sonication. The PNP activity was determined using 2 μg of cellular protein: (●) cells treated with 2 mM hydrogen peroxide and (○) cells without treatment.

how the human PNP protein behaves against oxidative stress. HeLa cells were incubated at 37 °C in the growth medium containing 2 mM hydrogen peroxide and, at various times of treatment, were withdrawn for an enzyme assay. As shown in Figure 5, the PNP activity decreased rapidly during incubation with hydrogen peroxide whereas only a small decrease was observed with a control culture.

To see if this decrease in the enzyme activity reflects the actual loss of the PNP protein from the cells, Western blotting analyses using specific antibodies were performed. As shown in Figure 6, the amounts of the PNP protein decrease rapidly when the cells are treated with two types of oxidative agents, hydrogen peroxide and menadion. On the other hand, no significant change was observed in the cells treated with ACNU and cyclohexamide, which block cellular DNA replication and protein synthesis, respectively. To rule out any direct oxidative degradation of the PNP protein by these oxidants, cellular extracts were incubated in the presence of 2 mM hydrogen peroxide or 0.5 mM menadion for 3 h. The amounts of PNP protein were not significantly changed by these treatments (data not shown). It seems that the PNP protein thus specifically degrades when cells experience oxidative stress.

DISCUSSION

Polynucleotide phosphorylase is the first enzyme found to form a polynucleotide from constituent nucleotides (*32*). This enzyme catalyzes the conversion of polynucleotide to ribonucleoside diphosphate upon consumption of inorganic phosphate, and with the use of high initial nucleoside diphosphate concentrations, it can produce polyribonucleotides with random base compositions. Subsequently, this enzyme has been defined as a component of a multiprotein complex called the exosome, which plays an important role in the mRNA turnover in *E. coli* cells (*33*). Despite increasing interest in this unique enzyme, its existence was thought to be limited to bacteria and plants. Recent genome sequence analyses as well as cDNA isolation, however, revealed that this enzyme is present in humans and other animals (*27*−*29*). In this study, we cloned a cDNA encoding the human polynucleotide phosphorylase protein, and the human enzyme was expressed in *E. coli* as a GST-fused recombinant protein. The recombinant protein is able to degrade RNA polymers to corresponding nucleoside diphosphates. Under the conditions that were used, the formation of UDP from poly(U) occurs most rapidly, which is followed by the formation of CDP and ADP from the corresponding polynucleotides. Only a small amount of GDP was produced when poly(G) was used as a substrate. The *E. coli* enzyme also possesses an ability to degrade preferentially polyadenylated and polyuridinylated RNAs, while it hardly degraded polyguanylated RNA (*34*). The human enzyme resembles the bacterial enzyme in this respect.

Although it is difficult to detect the enzyme activity in crude extracts of human cells, because of the existence of interfering enzyme activities and inhibitors, we were able to determine the amounts of PNP protein using specific antibodies. The immunologically isolated protein indeed carried the PNP enzyme activity. Taking advantage of this assay procedure, we have shown in this study that the amounts of PNP protein in the cell change profoundly in response to oxidative stress. Together with the finding that the human PNP protein binds specifically to 8-oxoGua-containing RNA, we speculate that the PNP might thus play an important role in maintaining the high fidelity of translation by sequestering the oxidatively damaged RNA molecules. Human YB-1 protein, namely, Y box-binding protein 1 with multiple regulatory activities (*35*−*38*), has been shown to have a potential to bind to 8-oxoGua-containing RNA (*39*). There is a possibility that both YB-1 and PNP function cooperatively to carry out such a role.

8-OxoGua can be formed in RNA by the direct oxidation of the constituent guanine base and also by the incorporation of oxidized guanine nucleotide into RNA. Regardless of the cause, once 8-oxoGua is formed in RNA, it cannot be eliminated. This is in contrast to the case of DNA, in which

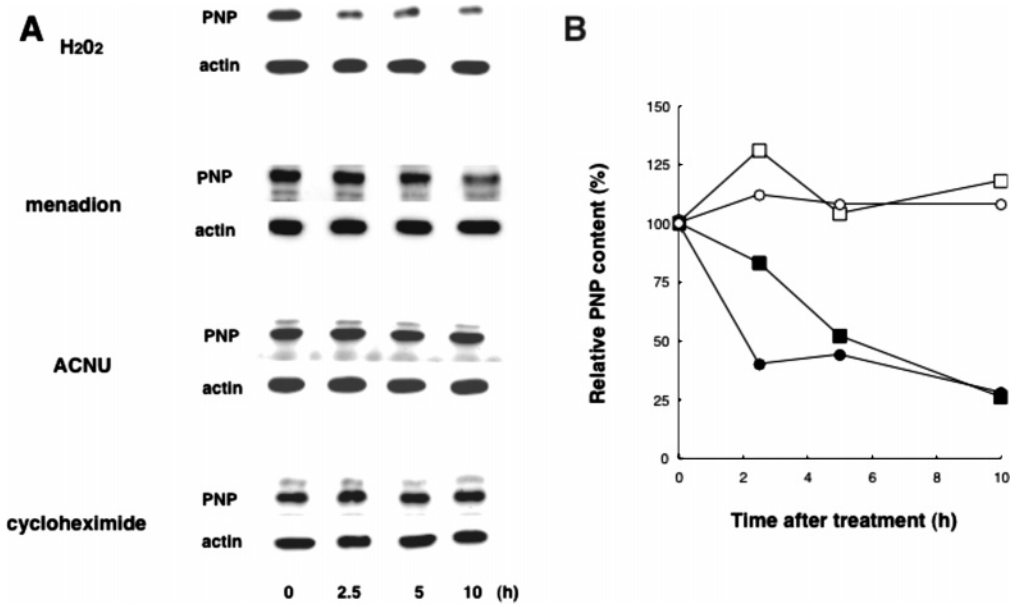

FIGURE 6: Amounts of PNP protein in cells under oxidative stress. The cells were treated with various agents: 2 mM hydrogen peroxide, 0.5 mM menadion, 2.5 mM ACNU, and 1 μM cycloheximide. At the indicated times, whole cell extracts were prepared and a Western blotting analysis was performed. In panels A and B, the actual Western blotting data and the values were normalized by the amounts of β-actin protein as shown, respectively: (●) hydrogen peroxide, (■) menadion, (○) ACNU, and (□) cycloheximide.

the oxidized bases are excised by specific glycosylases and repaired. As a result, organisms must be equipped with other means of maintaining the high quality of RNA against oxidative stress. Proteins that specifically bind to 8-oxoGua-containing RNA have been implicated in a mechanism for scavenging damaged RNA molecules, and a search for such proteins in *E. coli* showed the PNP protein to possess such characteristics (*26*). Mutants with defective PNP protein are hyperresistant to paraquat, a drug that induces oxidative stress in cells. This can be taken as proof that, even within cell, the PNP protein is capable of discriminating 8-oxoGua-containing RNA from normal RNA. The binding of PNP protein to 8-oxoGua-containing RNA would inhibit cell growth, probably due to the withdrawal of most of mRNA from the translational mechanism. In the PNP-deficient mutant cells, partially oxidized RNA can enter into the translation process, which thus allows certain low levels of protein synthesis for cell survival, still yielding some error-containing proteins.

In this study, we demonstrated that the human PNP protein possesses an ability to bind specifically to 8-oxoGua-containing RNA. To ascertain whether the human protein functions in the response to oxidative stress, we applied siRNA to reduce the cellular PNP level, but no significant change in the sensitivity to hydrogen peroxide and menadion was observed (unpublished result). Since siRNA-transfected cells still contain considerable amounts of PNP protein (15−26% of the normal level), it was difficult to make any definite conclusions, based on the findings of these experiments. The construction of PNP-defective mice by gene targeting and the establishment of cell lines from such mice may help to solve this problem, and studies along this line are now in progress.

Oxidative damage to RNA may be a prominent feature of vulnerable neurons in mammals. According to an in situ immunostaining analysis, a large amount of 8-oxoGua was found in the cytoplasm of neurons of patients with Alzheimer's disease but not in normal subjects. The oxidized base appears to be predominantly associated with RNA since immunoreactions were inhibited by preincubation with RNase but not with DNase (*40*). Similar results have also been obtained in cases of Down's syndrome and Parkinson's disease; in the case of Parkinson's disease, both RNA and DNA were oxidatively damaged (*41*−*43*). It will be interesting to see whether such proteins that are capable of binding to 8-oxoGua-containing RNA play a role in the nervous system.

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