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Prokaryotic Expression and Characterization of Human AP DNA Endonuclease

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Abstract

The expression of major human apurinic/apyrimidinic DNA endonuclease (APEX) from its cDNA in *E. coli* (DH5α) was attempted in order to obtain a biologically active recombinant APEX. *E. coli* cells were transformed by a prokaryotic translation vector (pGEX-4T-3) harboring APEX cDNA. GST-APEX fusion protein with a molecular weight of 6.3 KDa was induced by IPTG (1.0 mM) treatment. Western blot immunodetection identified the induced protein as the GST-APEX fusion protein. The survival rate of *E. coli* cells (DH5α) transformed with pGEX-4T-3-APEX increased when the cells were treated with N-diethyl-N-nitrosamine (DENA) or 3'-methyl-4-monomethylaminoazobenzene (3'-MeMAB), indicating that APEX expression had a protective effect on the cytotoxicity of these carcinogens. The fusion protein extracted from *E. coli* cells and purified by GSH-agarose gel affinity chromatography exhibited APEX activity. Treatment of thrombin to the GST-APEX fusion protein and affinity purification followed by Sephacryl S-100 gel filtration resulted in APEX peptide with MW 36 KDa, which exhibited AP DNA repair activity (8,7000 EU/mg protein). N-ethylmaleimide (0.1 mM) or AMP (0.98 mM) inhibited APEX activity by 50% and kinetic analysis indicated that the recombinant APEX (rAPEX) had a Km value of 0.022 μM (AP sites for AP DNA) and the Ki value was 0.48 mM for AMP. These results indicated that *E. coli* cells expressing biologically active GST-APEX were resistant to the cell damage caused by chemical carcinogens and that rAPEX purified from E. coli cells transformed with APEX cDNA- inserted translation vector was similar to native APEX in some properties.

Key Words: AP DNA endonuclease, expression, *E. coli*, characterization, DNA repair

INTRODUCTION

DNA, a stable repository for genetic information, is constantly challenged by genotoxic agents.[1] The loss of bases in DNA accounts for a few thousand residues per genome per day for mammalian cells.[2,3] Apurinic or apyrimidinic (AP) DNA may be generated from spontaneous base loss or from the cleavage of the glycosidic bond between various modified bases and deoxyribose in DNA.[4] AP DNA endonuclease (APE, EC.3.1.25.2) is responsible for the repair of AP DNA by recognizing AP site and nicking the strand near the AP site of DNA, and it is known to be present in almost all organisms.[3,5,6] It has been reported that mammalian cells contain multiple forms of APE and that some of them purified from different sources of tissue have diverse properties.[7-9] Although the repair mechanisms of APE in *E. coli* are well known, the mechanisms by which APEs in eukaryotic cells repair the damaged DNA are still uncertain.[7] The general mode of action of APE in the repair of AP DNA is thought to be initiated by the excision of the phosphodiester bond near the AP site. The major human AP DNA endonuclease (APEX) has been purified from various cells[10,11] but the physicochemical nature of these preparations was reported to be controversial depending on the sources of cells. This enzyme has multifunctional activities of which the main reaction is attacking AP sites and excision of the phosphodiester bond just 5' to the AP site to generate a normal 3'-terminal 3'-hydroxyl nucleotide and a 5'-terminal deoxyribose-5-phosphate.[12] Cloning of APEX cDNA has been accomplished[13,14] and its genomic structure including the promoter region has been determined.[15,16] In the present study, APEX was expressed in *E. coli* from its cloned cDNA and purified by GSH-agarose affinity chromatography and Sephacry S-100 gel filtration, and some properties of it were characterized.

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MATERIALS AND METHODS

Construction of APEX cDNA expression vector

The entire coding region of human APEX cDNA was amplified by polymerase chain reaction (PCR) using a cloned human APEX cDNA inserted plasmid (pUAEH1) as a template and restriction sites (BamHI and XhoI)-linked appropriate oligonucleotide primers. The amplified APEX cDNA was inserted into multi-cloning sites (BamHI and XhoI) of a prokaryotic protein translation vector (pGEX-4T-3) so as to express a GST-APEX fusion protein (Fig. 1).

Expression of APEX in *E. coli* cells

E. coli (DH5a) cells were transformed with the APEX cDNA-inserted plasmid (pGEX-4T-3-APEX) according to the method described by Sambrook et al.[17] and the APEX cDNA harboring colonies were identified by restriction analysis of the plasmids obtained from the transformed *E. coli* cells. A clone containing pGEX-4T-3-APEX was seed-cultured and diluted to 100 : 1 for mass culture in LB medium containing ampicilllin. As the culture reached 0.6 in OD_{600}, IPTG (1.0 mM, final concentration) was added to the culture medium and cultured for another 2 hours for the induction of GST-APEX fusion protein in *E. coli*.

Identification of recombinant APEX (rAPEX) protein

Identification of GST-APEX fusion protein induced in *E. coli* was performed by Western blot immunodetection system (ECL system kit, Amersham Life Science, Buckinghamshire, England). *E. coli* cells expressing GST-APEX were harvested by centrifugation (1,000 × g) for 10 min at 4°C and resuspended in SDS polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (50 mM Tris-Cl, pH 6.8, 5% 2-mercaptoethanol, 2% SDS, 0.1% bromopheol blue, and 10% glycerol) at a concentration of 1.0 ml of culutred cells per 100 μl of buffer. The resuspended sample was heated for 2 min at 90°C and subjected to SDS-PAGE according to the method of Laemmli.[18] Proteins separated by SDS-PAGE were electrotransferred onto a nitrocellulose membrane and GST-APEX fusion protein was identified by ECL-associated immunodetection technique described in the method.[19] Anti-APEX rabbit antiserum (kindly supplied by Dr. S. Mitra, University of Texas, Galveston, TX, USA) as a primary antibody and peroxidase-conjugated anti-rabbit IgG antibody as a secondary antibody were used.

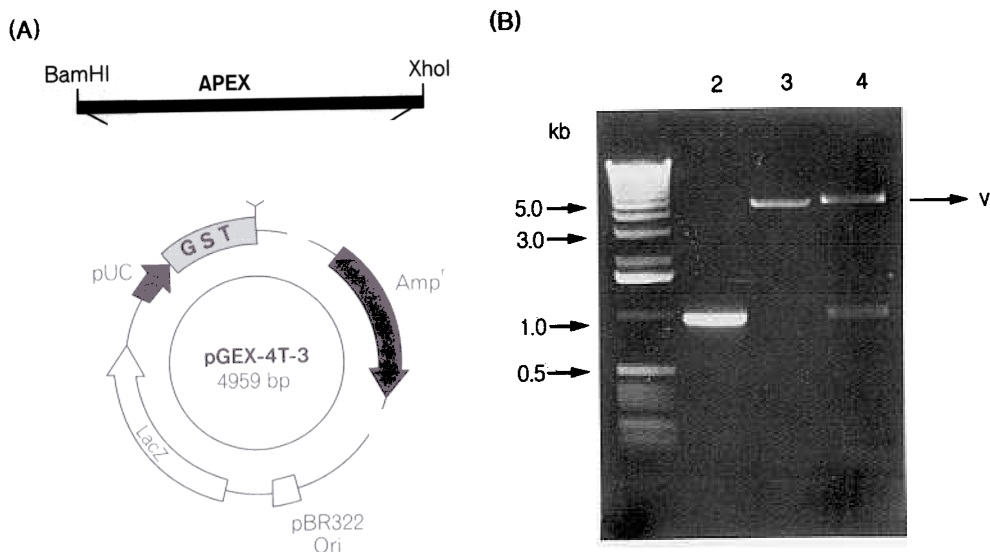

Fig. 1. Amplification of APEX cDNA and construction of APEX cDNA expressing vector. The PCR amplified APEX cDNA was inserted into BamHI and XhoI sites of pGEX-4T-3 plasmid (A) and the insertion was confirmed by restriction analysis (B) of plasmids obtained from E. coli (DH5a) transformed with the recombinant plasmid (pGEX-4T-3-APEX). Lane 1, molecular size marker; lane 2, PCR product (APEX cDNA); lane 3, vector (pGEX-4T-3); lane 4, APEX cDNA inserted recombinant vector (pGEX-4T-3-APEX) digested with BamHI and XhoI. Arrow indicates vector (V) and insert (I).

Purification of rAPEX

GST-APEX fusion protein was induced in *E. coli* (DH5α) cells transformed by a recombinant plasmid (pGEX-4T-3-APEX). The cells cultured in LB medium containing IPTG (1.0 mM) for 3 hours were harvested by centrifugation (1,000×g) for 10 min and washed with 0.1 M sodium phosphate buffer (pH 7.0) containing 6 mM 2-mercaptoethanol and 0.2 mM PMSF (buffer A) and stored at −70°C until use. After thawing the frozen cells, lysozyme (4 mg/ml) was added and incubated for 20 min at 4°C for the lysis of cells. The digested cells were centrifuged at 10,000×g for 30 min at 4°C in order to remove cell debris. Soluble cell extract was applied to a glutathione agarose gel column (1.2×5.0 cm) equilibrated with buffer A. The column was washed with 3 volumes of buffer A and GST-APEX fusion protein bound to the gel was eluted with buffer A containing 10 mM reduced glutathione (GSH), pH 6.0. Fractions containing GST-APEX were pooled and treated with thrombin (10 NIH unit/ml) for 4 hours at 37°C to cleave APEX from GST-APEX fusion protein. For the separation of APEX from GST in the thrombin-treated contents, the digested mixture was applied onto a GSH-agarose gel column equilibrated with buffer A and fractions passed through the column (unbound to the gel) were collected. The collected fractions were pooled and concentrated to 3 ml in volume and applied to a Sephacryl S-100 gel filtration column (1.2×78.0 cm) equilibrated with buffer A. The column was eluted with buffer A and fractions containing APEX activity were pooled and saved for the characterization of its nature.

Enzyme assay

APEX activity was measured by the method of Thibodeau et al.[20] To the [^{32}P]AP DNA dissolved in 100 μl of 0.1 M Tris-Cl buffer (pH 8.0) 100 μl of enzyme solution was added and reacted for 10 min at 37°C. The reaction was stopped by transfer of reaction mixture to ice-cold water, and then 100 μl of 200 mg% of bovine serum albumin (BSA) solution and 60 ul of 30% perchloric acid were added and mixed. After standing for 15 min at 0°C, the mixture was centrifuged for 15 min at 10,000×g, and radioactivities in the supernatant were counted in a liquid scintillation counter (Beckman, LS6500). The radioactivities in the supernatant after the enzyme reaction with a sufficient amount of APEX for a prolonged time (30 min) were assumed to be a representation of 100% of AP sites in the substrate excised, and the relative radioactivities were used for the calculation of enzyme unit (EU) which is defined as pmole of substrate (AP site) excised per min. [^{32}P]AP DNA was prepared by PCR amplification of a specific sequence of human blood coagulation factor VIII light chain cDNA (1.0 kbp). PCR was performed by the method of Saiki et al.[21] and ^{32}P-labeled dCTP (1.0 μCi/50 μl) was added to the PCR reaction mixture. The PCR product was subjected to agarose gel (1%) electrophoresis and the amplified DNA (1.0 kbp) was confirmed under UV illumination. The amplified DNA was extracted with phenol-chloroform-isoamylalcohol (25 : 24 : 1) and precipitated with 2.5 volumes of cold ethanol. AP sites in the DNA were generated by incubation of the amplified DNA in 1.0 M acetate buffer (pH 4.0) at 70°C for 2 hours. [^{32}P]AP DNA was precipitated by the addition of 2.5 volumes of cold ethanol to the incubation mixture and left to stand at −20°C for 4 hours. The precipitated [^{32}P]AP DNA was resuspended in the appropriate amount of TE buffer (pH 8.0) and the number of AP sites per molecule of AP DNA was determined by the method described by Kim and Oh.[22]

Kinetic analysis of rAPEX

Km value of recombinant APEX for the AP DNA was determined from a Lineweaver-Burk double reciprocal plot[23] on the concentration of substrate vs enzyme activity. The inhibitiory mode of AMP on APEX was determined from a double reciprocal plot, and the Ki value for AMP was determined from a Dixon plot on the inhibitor concentration vs reaction velocity.

Survival test

Sensitivities of *E. coli* cells transformed with pGEX-4T-3-APEX plasmid to N-nitrosodiethylamine (DENA) or 3'-methyl-4-monomethylaminoazobenzene (3'-MeMAB) were measured essentially as described.[14,24] Saturated overnight cultures were diluted into 25 volumes of LB medium, and cultured for 5 hours at 37°C in the presence of IPTG (1.0 mM). Ten-fold serial dilutions of this culture were made, and 0.1 ml of each diluted culture was added to 2.0 ml of 0.7% molten top agar (at 46°C) containing 2 μM of DENA or 3'-MeMAB. The mixture was poured on top of a LB agar plate and incubated overnight. As a control, *E. coli* cells transformed with pGEX-4T-3 plasmid were used. Colonies grown in each plate were counted and DNA repair activity was

evaluated from the number of surviving *E. coli* under the conditions employed.

RESULTS

Expression of GST-APEX fusion protein in *E. coli* cells

Amplification and insertion of APEX cDNA into pGEX-4T-3 plasmid vector were identified by agarose gel electrophoresis of the PCR product and restriction fragments of the plasmids obtained from *E. coli* transformed with pGEX-4T-3-APEX (Fig. 1). The right orientation of inserted APEX cDNA sequence was confirmed by DNA sequencing. GST-APEX fusion protein induced by IPTG (1.0 mM) in *E. coli* cells was identified by SDS-PAGE and Western blot immunodetection system (Fig. 2). A strong protein band with 63 KDa appeared when the cell extracts of *E. coli* induced by IPTG (1.0 mM) were subjected to SDS-PAGE, and ECL-associated Western blot immunodetection revealed that the induced protein with 63 KDa protein was a GST-APEX protein. The fusion protein induced in *E. coli* cells was partially soluble and a part of it was present as insoluble inclusion bodies in cell extracts.

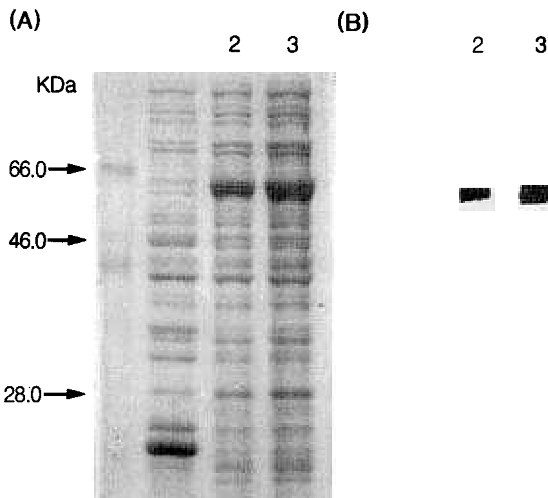

Fig. 2. Detection of GST-APEX induced in E. coli transformed with pGEX-4T-3-APEX plasmid. (A) SDS polyacrylamide gel electrophoresis of extracts of E. coli transformed with pGEX-4T-3 or pGEX-4T-3-APEX and subjected to IPTG (1.0 mM) induction. (B) Western blot immunodetection (ECL kit) of APEX using anti-APEX primary antibody (rabbit antiserum) and; peroxidase-conjugated anti-rabbit IgG antibody as secondary antibody. Lane 1, E. coli transformed with pGEX-4T-3 (10 μg protein); lane 2, E. coli transformed with pGEX-4T-3-APEX (5 μg protein); lane 3, E. coli transformed with pGEX-4T-3-APEX (10 μg protein).

Purification of GST-APEX fusion protein and preparation of rAPEX

The GST-APEX fusion protein was induced in pGEX-4T-3-APEX transformed *E. coli* by IPTG (1.0

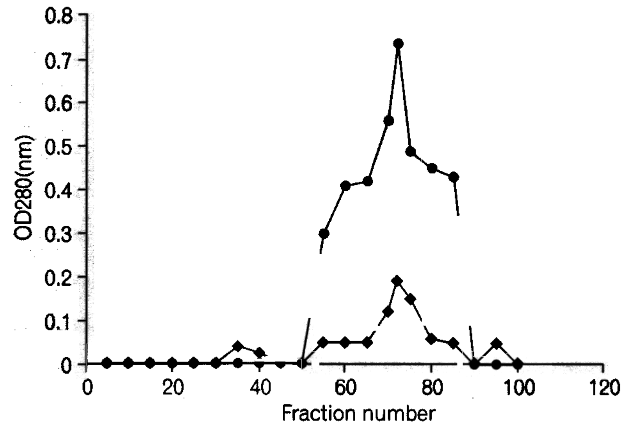

Fig. 3. Sephacryl S-100 gel filtration of recombinant APEX expressed in E. coli cells. GST-APEX expressed in E. coli was affinity purified by GSH-agarose gel chromatography and the purified GST-APEX fusion protein was digested with thrombin (10 NIH unit/ml culture extract) for 26 hours at room temperature followed by gel filtration through a Sephacry S-100 column (1.2 × 78 cm). rAPEX was eluted with 0.1 M Tris-Cl buffer (pH 7.0) at a flow rate of 20 ml/hr. -◆- (protein, A_{280}), -●- (enzyme activity, cpm $^{32}P \times 10^{-3}$).

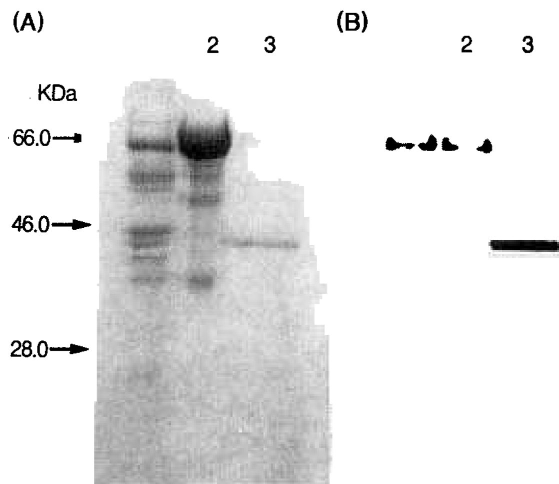

Fig. 4. Western blot immunodetection of purified rAPEX by GSH-agarose gel affinity chromatography and Sephacry S-100 gel filtration. (A) SDS-PAGE of protein fractions obtained from each purification step. (B) Western blot immunodetection (ECL kit) of Panel A using anti-APEX antiserum (rabbit) as primary antibody and anti-rabbit IgG antibody as secondary antibody. 1, Soluble extracts of E. coli transformed with pGEX-4T-3-APEX and treated with IPTG (1.0 mM); 2, samples obtained after GSH-agarose gel affinity chromatography; 3, samples obtained after Sephacry S-100 gel filtration (final step).

Table 1. Some Properties of Recombinant Major Human Apurinic/Apyrimidinic DNA Endonuclease (rAPEX) Expressed in *E. coli*

Preparation	Specific activity (EU*/mg protein)	Molecular weight (Daltons)	Km (μM AP site)	Ki (for AMP, mM)
GST-APEX †	3,300	63,000	0.030	0.54
rAPEX ‡	8,700	36,000	0.022	0.48

*Enzyme activity was assayed using ^{32}P-labeled AP DNA (5.8 AP sites/Kbp DNA) and enzyme unit (EU) is defined as pmole AP site hydrolyzed per min.
†GST-APEX fusion protein was isolated by GSH-agarose gel chromatography from the extract of *E. coli* transformed with pGEX-4T-3-APEX and treated with IPTG (1.0 mM).
‡APEX was prepared from GST-APEX fusion protein by Sephacryl S-100 chromatography after digestion with thrombin.

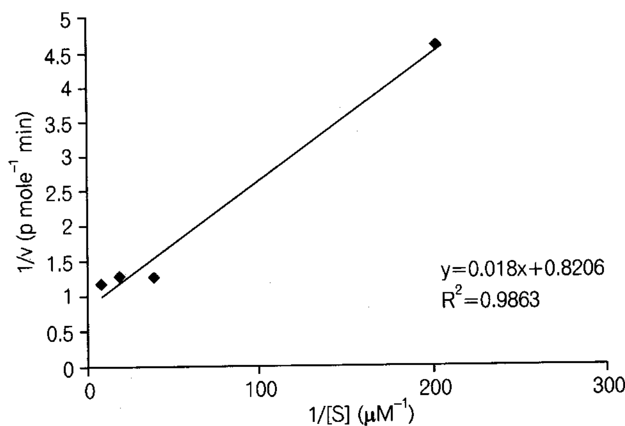

Fig. 5. Lineweaver-Burk reciprocal plot for APEX: 1/v vs 1/ [S]. −1/Km = −44.6, Km = 0.022 μM AP site.

mM) and purified from the cell extracts by GSH-agarose affinity gel chromatography. After washing out the proteins unbound to the gel, GST-APEX fusion protein was eluted from the column with buffer A containing 10 mM GSH. GST-APEX fusion protein with MW of 63 KDa exhibited APEX activity and fractions containing enzyme activity were pooled. Treatment of the pooled sample with thrombin (10 NIH unit/ml) resulted in cleavage of the fusion protein into GST and APEX to which extra two amino acids (Ser-Gly) added to the N-terminal of it. GST peptide was removed from the thrombin-treated mixture by passing through a GSH-agarose gel column and rAPEX protein was separated from other molecules including thrombin by Sephacryl S-100 gel filtration (Fig. 3). The purified rAPEX was almost homogeneous and had APEX activity (Fig. 4). The purification steps and specific activities of the enzyme in each step are shown in Table 1.

Some properties of the rAPEX

The purified GST-APEX had a specific activity of 63,000 EU/mg and the thrombin treatment did not abolish the APEX activity. The rAPEX required Mg^{++} for the catalytic activity and N- ethylmaleimide (0.1 mM), a sulfhydryl group inhibitor, as well as AMP (0.98 mM) inhibited 50% of the enzyme activity. Km value of the rAPEX for AP DNA was estimated as 0.22 μM AP sites and Ki value of AMP for the enzyme was 0.48 mM (Fig. 5, Table 1).

Resistance to carcinogens in *E. coli* expressing rAPEX

The survival rate of *E. coli* harboring pGEX-4T-3-APEX was higher than the control *E. coli* cells harboring pGEX-4T-3 plasmid when these cells were treated with DENA (Fig. 6) or 3'-MeMAB (Fig. 7). These results indicate that the resistance to an alkylating agent (DENA) or an ultimate adduct-forming agent (3'-MeMAB) was increased by the expression of rAPEX in *E. coli* cells. The survival rate of *E. coli* transformed with pGEX-4T-3-APEX was also higher than the control cells transformed with pGEX-4T-3 plasmid without the treatment of carcinogens.

DISCUSSION

APEX, a major human AP DNA endonuclease belongs to class II AP endonuclease (5' AP endonucleases) which catalyzes incision of the 5' side of AP sites of DNA to produce 3'-hydroxylnucleotide and 5'-deoxyribose-phosphate termini.[3,25,26] *E. coli* has two types of 5' AP endonuclease, exonuclease III and

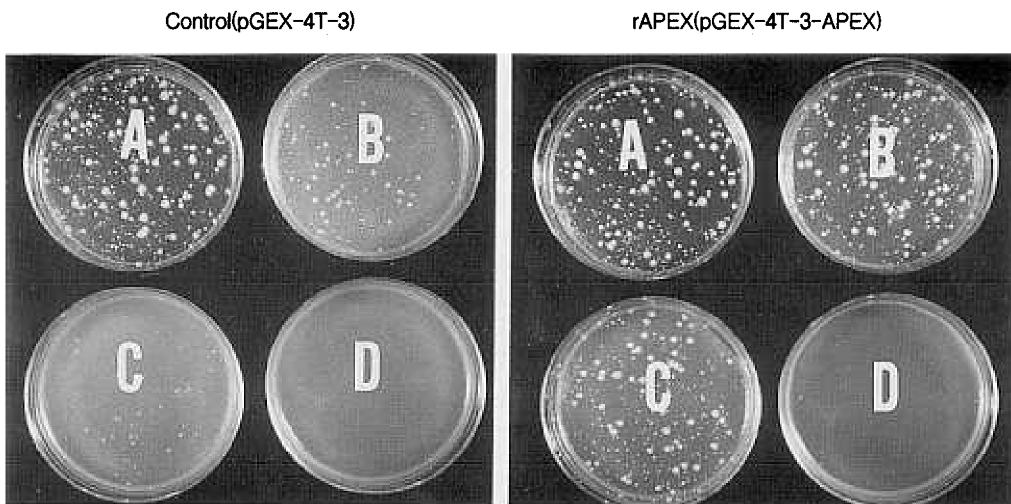

Fig. 6. Survival of E. coli transformed with pGEX-4T-3-APEX under exposure to chemical carcinogens. The resistance of E. coli cells expressing GST-APEX to carcinogen (DENA, 3'-MeMAB) exposure was tested. Ten-fold serial dilutions of the culture were made, and 0.1ml of each diluted culture was added to 2.0 ml of 0.7% molten agar(at 46°C) containing 2.0μM of DENA. The mixture was poured on top of a LB agar plate and incubated overnight. A, 1 X dilution; B, 10 X dilution; C, 100 X dilution; D, 1000 X dilution.

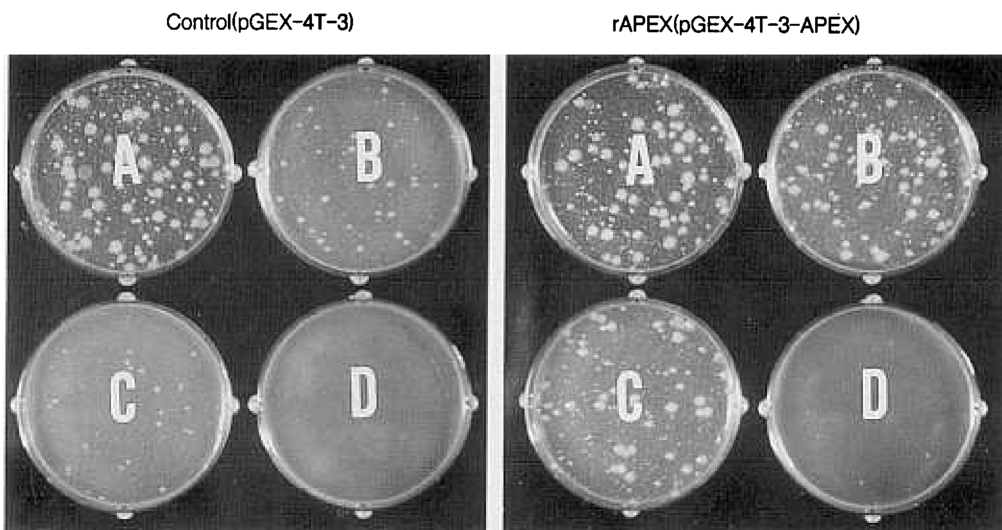

Fig. 7. Survival of E. coli transformed with pGEX-4T-3-APEX under exposure to chemical carcinogens. The resistance of E. coli cells expressing GST-APEX to carcinogens (DENA, 3'-MeMAB) exposure was tested. Ten-fold serial dilutions of the culture were made, and 0.1ml of each diluted culture was added to 2.0 ml of 0.7% molten agar(at 46°C) containing 2.0μM of 3'-MeMAB. The mixture was poured on top of a LB agar plate and incubated overnight. A, 1 X dilution; B, 10 X dilution; C, 100 X dilution; D, 1000 X dilution.

endonuclease IV, and the former is homologous to APEX in the primary structure and in biological activity. GST-APEX fusion protein induced in this report exhibited AP endonuclease activity and thrombin-cleavage of it did not abolish the enzyme activity, indicating that the catalytic domain of the rAPEX sequence was not damaged by thrombin digestion. rAPEX purified from thrombin-digested GST-APEX fusion protein through Sephacryl S-100 column chromatography had a molecular weight of 36 KDa which is consistent with the expected whole protein sequence of rAPEX, to which extra two amino acids are added at the N-terminal of the protein coded from cDNA of APEX inserted into the translation vector (pGEX-4T-3). It has been known that the sequence of APEX may be devided into two domains of which the N-terminal region (6 KDa) may be involved in nuclear location of the enzyme and the C-terminal region (29 KDa) may be involved in the catalytic function.[27] AP DNA endonuclease is widely

distributed,[5,28] and the presence of at least two kinds of AP DNA endonucleases have been reported.[5,8,29] The molecular weight of two kinds of AP DNA endonuclease purified from human fibroblasts were reported as 25−40 KDa,[8] and the heterogeneity in the molecular weight of AP endonuclease present in different cells or tissues was thought to be due to the partial incision at the N-terminal region of unprocessed enzyme molecules.[27]

The processed peptides that contained the C-terminal region were thought to exhibit DNA repair activity and to be localized at different sites of cells. The purified rAPEX in the present study showed a Km value of 0.022 μM, which is much lower than that of previously reported APEX purified from HeLa cells[30] and of rat chromosomal AP endonucleases.[22] The different Km values of AP endunucleases observed by each investigators might be due to the different sources of the enzyme and to the different substrates used. Inhibition of rAPEX by N-ethylmaleimide in the present study implicates the involvement of the sulfhydryl group of the enzyme in the catalytic reaction, and the inhibition of rAPEX by AMP indicates that rAPEX has a similar property in responding to nucleotide inhibitors such as NAD^+, ADP-ribose, hypoxanthine and adenine to that of native APEX.[10,31] AMP inhibited AP DNA endonucleases in rat chromatin in uncompetetive modes of reaction in which AMP binds to the enzyme-substrate complex, and the Ki values for AMP were reported to be 0.35−0.54 mM. The Ki value of rAPEX for AMP in this report was 0.48 mM, which is somewhat higher than that of the rat chromatin enzymes.[22] Exposure of cells to alkylating agents or proximate carcinogens was used to investigate presumed DNA repair in a variety of cell types.[32] A large number of carcinogens activated by generation of unstable electrophilic species may react in specific ways with DNA, RNA and protein in cells.[33] The induction of neoplasia by chemical carcinogens appears to require some type of interactions between the ultimate active forms of these carcinogens and critical macromolecules in the target cells.[34] APEX is responsible for the excision repair of AP DNA which may be generated by N-glycosylation of modified bases such as alkylated purines or pyrimidines, and many alkylating agents are recognized as electophilic reactants that react nonenzymatically with nucleophilic sites in nucleic acids and protein under physiological conditions.[35-38] Carcinogens of a variety of types are converted in vivo into alkylating agents through enzymatic or nonenzymatic means and these potential alkylating agents include nitrosamines, dialkylaryltriazenes and nitrosamides which may be nonenzymatically activated.[33] In the present study, the induction of GST-APEX in *E. coli* exposed to chemical carcinogens such as DENA or 3'-MeMAB caused the increased cell survival rate. This result indicates that rAPEX induced in the cells could protect the cells from genotoxic cell death by an efficient repair of chromosomal AP DNA generated by these carcinogens (alkylating or adduct forming). Although the precise mechanisms of AP DNA generation by DENA or 3'-MeMAB are not yet fully understood, the beneficial effect of rAPEX on cell survival implicates the essential role of APEX in cells exposed to genotoxic agents. Further studies are required for the evaluation of the role of APEX in the repair of DNA damaged by various kinds of genotoxins including chemical carcinogens in eukaryotic cells.

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