Human Apurinic/Apyrimidinic Endonuclease (Ape1) and Its N-terminal Truncated Form (AN34) Are Involved in DNA Fragmentation during Apoptosis*

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We previously isolated a 34-kDa nuclease (AN34) from apoptotic human leukemia cells. Here, we identify AN34 as an N-terminally truncated form of human AP endonuclease (Ape1) lacking residues 1–35 (Δ35-Ape1). Although Ape1 has hitherto been considered specific for damaged DNA (specific to AP site), recombinant AN34 (Δ35-Ape1) possesses significant endonuclease activity on undamaged (normal) DNA and in chromatin. AN34 also displays enhanced 3′-5′ exonuclease activity. Caspase-3 activates AN34 in a cell-free system, although caspase-3 cannot cleave Ape1 directly in vitro. We also found that Ape1 itself preferentially cleaves damaged chromatin DNA isolated from cells treated with apoptotic stimuli and that silencing of Ape1 expression decreases apoptotic DNA fragmentation in DFF40/CAD-deficient cells. Thus, we propose that AN34 and Ape1 participate in the process of chromatin fragmentation during apoptosis.

Apoptosis is an essential mechanism for maintaining cell number homeostasis in multicellular organisms. Both the morphological changes occurring in apoptotic cells and the molecular mechanism by which cells die are widely conserved (1–3). Degradation of DNA is an important biochemical hallmark of apoptosis and results from the activation of cellular nucleases. A number of candidate apoptotic nucleases, including DNase I (4), DNase II (5, 6), and Nuc 18 (7, 8), have been characterized. Recently, caspase-activated DNase (CAD, also known as DNA fragmentation factor 40 kDa (DFF40)) has been shown to produce apoptotic DNA fragmentation. CAD forms a heterodimer with ICAD (inhibitor of CAD, also known as DFF45), and ICAD reduce apoptotic DNA fragmentation. Recently, caspase-independent DNA degradation has been reported as an apoptotic endonuclease. Endonuclease-G is released from mitochondria during apoptosis and induces caspase-independent DNA degradation (17).

We previously purified an apoptotic nuclease 34 kDa (AN34) from human leukemia cells undergoing apoptosis (18). AN34 is a Mg2+-dependent nuclease, generating DNA breaks with 3′-hydroxy termini. In addition, it induces predominantly DNA single strand breaks but also produces internucleosomal DNA cleavage in isolated nuclei (18). In the present study, we have determined the origin of AN34 and found that it is an N-terminally truncated form of human apurinic/apyrimidinic (AP) endonuclease (Ape1) (19–22). Ape1 (also called HAP1 (20), Ref-1 (21), and APEX (22)) is a well characterized, 37-kDa DNA base excision repair protein, highly conserved among different species (23). It cleaves the DNA phosphodiester backbone immediately 5′ to an AP site. In addition, Ape1 acts as a reducing factor that stimulates the DNA binding activity of ubiquitous transcription factors such as Fos, Jun, NF-xB, HIF-1, and p53 (24). In this report, we show that AN34 (Δ35-Ape1) possesses significant endonuclease activity and cleaves chromatin DNA, although Ape1 itself has no such an activity. Using a cell-free system, we also show that caspase-3 causes accumulation and activation of AN34. In addition, we report that Ape1 selectively cleaves chromatin DNA from cells treated with apoptotic stimuli and that silencing of Ape1 expression decreased apoptotic DNA fragmentation in DFF40/CAD-deficient cells. This report is the first demonstration for a role for Ape1 in degrading DNA during apoptosis.

EXPERIMENTAL PROCEDURES

Materials—3H-Escherichia coli DNA (2.2 Ci/mg), α-[32P]cordycepin 5′-triphosphate, γ-[32P]ATP, and α-[32P]dCTP were purchased from PerkinElmer Life Sciences (Boston, MA). HiTrap-Q, HiTrap-Heparin, Mono S HR 5/5, Superdex-200 pg, and Superose 12 HR 10/30 columns were obtained from Amersham Biosciences (Piscataway, NJ). Hydroxyapatite columns were purchased from Bio-Rad (Hercules, CA). Anti-Ape1 polyclonal antibody from rabbit was purchased from Santa Cruz

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Ape1-induced Apoptotic DNA Fragmentation

Biotecnology Inc. (Santa Cruz, CA). Anti-PPF40 monoclonal antibody from mouse was purchased from Imgenex (San Diego, CA). Recombinant caspase-3 was from Upstate Biotechnology (Lake Placid, NY). Oligonucleotides were purchased from The Midland Certified Reagent Co. (Midland, TX).

**Measurement of Nuclease Activity by a 3H-DNA Fragmentation Assay**—Nuclease assays were carried out using 3H-E. coli DNA as substrate. Nuclease activity was measured by the formation of acid-soluble DNA fragments as described previously (18). Incubations were carried out at 37 °C for 60 min in 60 μl of 50 mM Tris-HCl buffer, pH 7.0, containing 1 mM DTT, 50 ng of 3H-E. coli DNA (1 × 105 dpm) in the presence of 5 mM MgCl2. Incubations were terminated by the addition of an equal volume of ice-cold 1 M perchloric acid:1 M NaCl and rapid chilling on ice. Nuclease activity was determined from the percentage of acid-soluble 3H-E. coli DNA released in the supernatant (percent DNA fragmentation). One unit of endonuclease activity was defined as the activity inducing 20% DNA fragmentation.

**Purification of AN34 and Peptide Sequence Analysis**—AN34 was purified as described previously (18) with the following modifications. A hydroxylapatite column chromatography was additionally used to purify protein thoroughly prior to peptide sequence analysis. All chromatographic separations were performed at 4 °C using fast protein liquid chromatography (Amersham Biosciences). Cell lysates were prepared from etoposide-treated apoptotic HL-60 cells as described previously (18).

Cell lysates (3 × 10⁶ cells, ~3 liters of culture) were passed over a Q-Sepharose column (HiTrap-Q), equilibrated with buffer A (50 mM Tris-HCl, pH 7.0, 5 mM MgCl2, 1 mM DTT, 3 μg/ml leupeptin, and 1 mM PMSF). The column was washed with buffer A, and flow-through fractions were collected. Active fractions were concentrated by Centriprep-10 and applied to a heparin-Sepharose column (HiTrap-heparin) equilibrated with buffer A containing 0.2 M KCl. The column was washed with buffer A containing 0.2 M KCl, and bound proteins were eluted with a linear gradient (0.2 to 0.6 M KCl in buffer A). The nuclease activity eluted around 0.3 M KCl. The active fractions were pooled and loaded onto a Mono S HR5/5 column equilibrated with buffer A containing 0.1 M KCl, and bound proteins were eluted with a linear gradient (0.01 to 0.6 M potassium phosphate in buffer B). The nuclease eluted around 0.1 M potassium phosphate. The active fractions were concentrated by Centriprep-10 and Microcon-10 and applied to a Superose 12 HR10/30 gel filtration column equilibrated with buffer A containing 0.1 M KCl. Active fractions were pooled and loaded onto a Mono S HR5/5 column equilibrated with buffer A containing 0.1 M KCl. The column was washed with buffer A containing 0.1 M KCl, and bound proteins were eluted with a linear gradient (0.1 to 0.5 M KCl in buffer A). Nuclease activity eluted around 0.2 M KCl. Purified proteins were analyzed by SDS-polyacrylamide gel electrophoresis. Proteins were stained with Coomassie Blue (Novex, San Diego, CA) or silver. The major protein bands were excised from the gels and digested with trypsin. For matrix-assisted laser desorption ionization (MALDI) protein analysis, peptides derived from trypsin digestions were run on a Voyager-DE STR Biospectrometry workstation (Applied Biosystems, Foster City, CA). Data using electrospray mass spectrometry analysis were also obtained from collagen induced dissociation spectra with a Finnigan-MAT LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) after introduction via a polyamide-coated fused silica microcapillary high-performance liquid chromatography. N-terminal amino acid sequence analysis was performed using Peptide 49HT automated protein sequence (Applied Biosystems). Ape1 digestion of AN34 from Caspase-3-treated Cell Lysates—Cell lysates (cytoplastic extracts) were prepared as described previously (18). Cytoplastic extracts were incubated at 37 °C for 40 min with recombinant caspase-3 (final concentration, 10 μg/ml) in buffer A (50 mM Tris-HCl, pH 7.0, 5 mM MgCl2, 1 mM DTT, and 1 mM PMSF). Caspase-3-treated cell lysates were applied to a Superdex-200 gel filtration column equilibrated with buffer A containing 0.1 M potassium phosphate. Active fractions were further purified by Q-Sepharose, heparin-Sepharose, hydroxylapatite, Mono S column as described above.

**Expression and Purification of Recombinant Ape1, AN34 (Δ35-Ape1), and a Site-directed Mutant Form of Ape1**—Expression and purification of full-length Ape1 and of a site-directed mutant form of Ape1 in which Asp-210 was substituted by Asn (AN34 (Δ35-Ape1)) were performed as described previously (25). AN34 (comprising residues 36–318 and hence lacking the N-terminal 35 amino acids (Δ35-Ape1)) was expressed and purified as described previously (26). To create a mutant form of AN34 (Δ35-Ape1) containing an Aep-210 to Aas substitution (AN34 (D210N)), the DNA encoding full-length Ape1-D210N protein was used as a template for PCR amplification. EcoRI and HindIII sites were incorporated into the primers for directional cloning of the AN34 (D210N) cDNA into pT7-7, as described previously (26). The structure of the cloned fragment was verified by DNA sequencing. The AN34 (D210N) protein was expressed and purified as described previously (26).

3′-5′-Endonuclease Assays—A polyacrylamide gel assay was used to detect 3′-5′-exonuclease activity. Purified nuclease was incubated with a 20-mer oligodeoxynucleotide labeled with 32P at the 5′ or 3′ termini. 5′-end labeling was carried out with [γ-32P]ATP using T4 polynucleotide kinase. Labeling mixtures were subsequently centrifuged through a Sephadex G-25 column to remove unincorporated 32P. DNA substrates (~50 fmol/10μl reaction) were incubated with 1 μl of purified proteins for the indicated times at 37 °C in standard reaction buffer (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 15 μg/ml bovine serum albumin, 0.2 mM DTT). Reactions were stopped by the addition of EDTA to a final concentration of 50 mM. Samples were applied to 16% denaturing polyacrylamide gels (7 M urea). Gels were dried, and imaging was performed with a PhosphorImager (Amersham Biosciences).

**AP Endonuclease Assays**—The upper strand of a 37-mer oligodeoxynucleotide containing a single tetrahydrofuran residue as a model for an AP site (27) (see Fig. 4a) was 3′-end labeled with [γ-32P]cordycepin using terminal deoxynucleotidyl transferase (Invitrogen). 5′-end labeling was carried out with [γ-32P]ATP using T4 polynucleotide kinase. Labeling mixtures were subsequently centrifuged through a Sephadex G-25 column to remove unincorporated 32P. DNA substrates (~50 fmol/10μl reaction) were incubated with 1 μl of purified proteins for the indicated times at 37 °C in standard reaction buffer (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 15 μg/ml bovine serum albumin, 0.2 mM DTT). Reactions were stopped by the addition of EDTA to a final concentration of 50 mM. Samples were applied to 16% denaturing polyacrylamide gels (7 M urea). Gels were dried, and imaging was performed using a PhosphorImager (Amersham Biosciences).

**In Vivo Chromatin Digestion Assays**—Nuclei were isolated from HL-60 cells as described previously (18). Nuclei (7 × 10⁶) were resuspended in STKM buffer (0.25 M sucrose, 50 mM Tris-HCl, pH 7, 25 mM KCl, and 5 mM MgCl₂) and mixed with purified Ape1 (30 units) in the presence of 1 μM DTT. Reaction mixtures were incubated at 37 °C for
the indicated times. DNA was extracted by standard phenol/chloroform/isoamyl alcohol extraction procedures, and analyzed by 1.3% agarose gels electrophoresis.

**Immunoblotting**—Cells were washed in cold PBS and lysed in 0.25% Triton X-100, 50 mM Tris-HCl (pH 7.0), 5 mM MgCl₂, 3 µg of leupeptin, 3 µg of aprotinin, and 2 mM PMSF. Lysates were centrifuged at 14,000 g for 15 min at 4 °C. Supernatants were collected and electrophoresed at 125 V in 12% SDS-polyacrylamide gels. After transfer to Immobilon-P membranes (Millipore, Bedford, MA), membranes were blocked overnight in PBS-Tween containing 5% nonfat dried milk, probed for 1 h with primary antibody and for 1 h with secondary antibody (1:1000 dilution). Visualization was achieved using enhanced chemiluminescence (Pierce, Rockford, IL) according to the manufacturer’s instructions.

**Immunodepletion Experiments**—Purified Ape1 and AN34 were incubated with anti-Ape1 antibody (6 µg) or normal rabbit immunoglobulin (6 µg) in buffer A at 4 °C for 4 h. Protein A-Sepharose was then added, and incubations were carried out at 4 °C for 2 h. After centrifugation at 3000 g for 3 min, the supernatant was collected as immunoadsorbed supernatant. Nuclease and chromatin digestion assays were performed as described above using 3H-E. coli DNA or isolated nuclei, respectively.

**Immunofluorescence and Confocal Microscopy**—HL-60 cells were incubated in the absence or presence of 50 µM etoposide for 3 h, acetone-fixed, and stained with anti-Ape1 antibody (Santa Cruz Biotechnology). The coverslips were incubated with secondary antibody (goat anti-rabbit IgG-fluorescein isothiocyanate). Propidium iodide was used for nuclear staining. Images were acquired by confocal laser scanning microscopy (eclipse TE300, Nikon, Tokyo, Japan).

**Fig. 2.** Domain structure of Ape1 and AN34. Underlined blue characters show N-terminal amino acid residues of AN34 identified by using Procise 494HT automated protein sequencer (Applied Biosystems). AN34 is an N-terminally truncated form of Ape1 (comprising residues 36–318 and hence lacking the N-terminal 35 amino acids (boxed sequence)).

**Table I**

| Spot | Protein identified | [M+H]⁺ observeda | Δ, % | Residues | Sequence from databaseb |
|------|-------------------|------------------|------|----------|-------------------------|
| 37 kDa AP endonuclease 1 65% coverage | 659.3 | -0.1 | 136–140 | QCPLK |
| | 717.4 | 0 | 79–84 | GLDWVK |
| | 822.4 | 0 | 187–192 | WDEAFR |
| | 845.4 | -0.1 | 78–84 | KGLDWVK |
| | 865.4 | 1.0 | 181–186 | LEYQRQ |
| | 952.3 | 0.8 | 187–193 | WDEAFRK |
| | 1019.5 | 0 | 228–236 | NAGFTPQER |
| | 1131.5 | 0 | 7–17 | GAVAEDGDLR |
| | 1137.6 | 0 | 125–135 | EGYSGVGLSLR |
| | 1259.7 | 0.1 | 6–17 | KGAVAEDGDLR |
| | 1690.8 | 0.1 | 141–155 | VSTYGIDDEHDQEGR |
| | 1786.9 | 0.1 | 35–51 | EAAEGPAPLYEDPQK |
| | 1831.1 | 0.2 | 7–23 | GAVAEDGDLRTPEAK |
| | 1874.1 | 1.1 | 301–317 | SKALGHDHPITLYL |
| | 2267.4 | 0.2 | 156–176 | VIVAEFDYFVLTAYV_CMPAGR |
| | 2266.5 | 0.3 | 109–124 | IPAEQLEFLGLSHQWVSA_PDSK |
| 34 kDa AP endonuclease 1 45% coverage | 1848.3 | 0.3 | 237–253 | QGFGELQAVPLADSFR |
| | 2266.8 | 0.6 | 103–124 | IPAEQLEFLGLSHQWVSA_PDSK |
| | 1136.9 | -0.7 | 125–135 | EGVYSGVGLSLR |
| | 605.1 | -0.3 | 25–30 | SKTAAK |
| | 631.3 | 0 | 276–280 | NVQWR |
| | 657.3 | -0.1 | 221–226 | NPKGNK |
| | 659.4 | 0 | 136–140 | QCPLK |
| | 717.4 | 0 | 79–84 | GLDWVR |
| | 823.4 | 0 | 187–192 | WDEAFR |
| | 846.0 | 0.5 | 78–84 | KGLDWVK |
| | 890.6 | -0.4 | 194–201 | FLKGLASR |
| | 1007.5 | 1.3 | 177–184 | GLYRLEyr |
| | 1019.6 | 0.1 | 228–236 | NAGFTPQER |
| | 1109.5 | 2.0 | 185–192 | QRTDEAFR |
| | 1787.5 | 0.7 | 35–51 | EAAGEGPALYEDPPQ |

a Monoisotopic.
b The difference between experimental and calculated masses.
c Underlined residues were corroborated by MS/MS product ion spectra.
Ape1 Silencing by RNA Interference—We prepared siRNA duplexes for Ape1 from synthetic 21-nucleotide RNAs (Qiagen, Tokyo, Japan). The human glioblastoma cell line, U87-MG, was used for Ape1 silencing experiments (16). The sequences for siRNA for Ape1 were as follows: sense, 5’-H11032-GUCUGGUACGACUGGAGUACC-3’; antisense, 5’-H11032-UACUC-CAGUCGUACCAGACCU-3’. Chemically synthesized control (non-silencing) siRNA was purchased from Qiagen (Tokyo, Japan). Transfection of siRNA were carried out with TransMessenger reagent (Qiagen) in 6-well plates. siRNA was condensed with Enhanser R and formulated with 4 l/l of TransMessenger reagent, according to the manufacturer’s instructions. The transfection complex was diluted in 900 l/l of Dulbec-

![Fig. 3](image_url)

**Fig. 3.** AN34 and AN37 (Ape1) are recognized by Ape1 antibodies in Western blot analysis. Purified AN34/AN37 were subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and analyzed by immunoblotting using polyclonal anti-Ape1 antibodies (lane 1, C-terminal antibodies; lane 2, N-terminal antibodies).

![Fig. 4](image_url)

**Fig. 4.** The nuclease activity of purified AN34/AN37 can be immunodepleted with polyclonal anti-Ape1 antibodies. Purified AN34/AN37 was incubated with polyclonal anti-Ape1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) or normal immunoglobulin fraction (rabbit), followed by incubation with Protein-A-Sepharose. a, after centrifugation (as described under “Experimental Procedures”), supernatants were incubated with isolated nuclei for 2 h at 37 °C. DNA was extracted, subjected to 1.2% agarose gel electrophoresis, and visualized by ethidium bromide. b, nuclease activities of supernatants after immunoprecipitation were assessed using 3H-labeled E. coli DNA as a substrate.

![Fig. 5](image_url)

**Fig. 5.** Purified AN34/AN37 possesses AP endonuclease activity. a, an oligonucleotide containing a single abasic site (tetrahydrofuran, indicated by a caret) 3’-end-labeled with 32P-cordycepin (*A) was used as substrate. b, the labeled oligonucleotide was incubated with purified AN34/AN37 (1 ng, lane 2) or recombinant human Ape1 (5 ng, lane 3) at 37 °C for 15 min. Cleavage products were analyzed by DNA sequencing gels. Lane 1, DNA oligonucleotide only.

![Fig. 6](image_url)

**Fig. 6.** Recombinant AN34 (Δ35-Ape1) possesses sufficient endonuclease activity to generate nucleosomal DNA ladder. Native SV40 DNA was incubated with recombinant Ape1, AN34 (Δ35-Ape1), or mutated AN34 (D210N) for 60 min at 37 °C. a, reaction products were analyzed by 1% agarose gel. b, results were quantified by using a Fluorimager (Amersham Biosciences) TPE1 corresponds to AN34. c, isolated nuclei from HL-60 cells were incubated in the absence (lane 1) or presence of recombinant AN34 (Δ35-Ape1) (100 ng, lane 2 or 300 ng, lane 3) at 37 °C for 90 min. DNA was extracted and subjected to 1.2% agarose gel electrophoresis.

CAGUCGUACACGUACC-3’. Chemically synthesized control (non-silencing) siRNA was purchased from Qiagen (Tokyo, Japan). Transfection of siRNA were carried out with TransMessenger reagent (Qiagen) in 6-well plates. siRNA was condensed with Enhanser R and formulated with 4 µl of TransMessenger reagent, according to the manufacturer’s instructions. The transfection complex was diluted in 900 µl of Dulbe-

Ape1-induced Apoptotic DNA Fragmentation
co's modified Eagle's medium and was added directly to the cells. It was replaced with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum after 3 h. Cells were analyzed 48 h after transfections.

Other Methods—Protein concentrations were determined by a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Silver staining was performed using silver stain plus kit (Bio-Rad).

RESULTS

Identification of AN34 as a N-terminal Truncated Form of Ape1—To clarify the origin of AN34, we purified the protein from etoposide-treated apoptotic HL-60 cells using five chromatographic steps as described under “Experimental Procedures.” The results of the last step of purification by Mono S column chromatography are shown in Fig. 1. The fractions 20–25 positive for DNA fragmentation were subjected to 12% SDS-polyacrylamide gel electrophoresis (PAGE), and the proteins were silver-stained. Two protein bands with molecular masses of 34 (AN34) (18) and 37 kDa were apparent, and their intensities correlated with the level of nuclease activity in the DNA fragmentation assay (Fig. 1). For consistency with our previous report (18), we refer to the 37-kDa endonuclease band as AN37.

To identify AN34 and AN37, the two protein bands were excised from the gel and digested with trypsin. Protein identification was then performed using MALDI and electrospray mass spectrometry. Peptide analysis of both AN37 and AN34 showed matched sequences from the major AP endonuclease, Ape1 (Table I). In addition, N-terminal analysis of the 34-kDa (AN34) protein indicated that AN34 lacks the first 35 amino acid residues of Ape1 (Fig. 2). To further test the identity of the purified proteins, a polyclonal antibody recognizing the C-terminal region of human Ape1 was used in Western blot analysis.

![Fig. 7. Purified AN34/Ape1 from apoptotic cells possesses 3'-5' exonuclease activity. a, sequence of the oligodeoxyribonucleotide used. b, purified AN34/Ape1 (3 ng, lane 2), recombinant AN34 (Δ35-Ape1) (50 ng, lane 5), or recombinant Ape1 (50 ng, lane 6) were incubated with the oligodeoxyribonucleotide labeled with 32P at the 5' terminus (*) for 15 min at 37 °C. Lane 3 shows a positive control reaction using 3'5'-phosphodiesterase I (10⁻³ units at 37 °C for 5 min), which released a deoxycytidine monophosphate (dCMP). Lanes 1 and 4, oligonucleotide only. c, purified AN34/Ape1 from apoptotic cells (3 ng, lane 2), recombinant AN34 (Δ35-Ape1) (50 ng, lane 4), or recombinant Ape1 (50 ng, lane 5) were incubated with the oligodeoxyribonucleotide shown in panel A and labeled with 32P at the 5’ terminus (*) for 10 min. Lanes 1 and 3, oligonucleotide only. Samples were applied to 16% denaturing polyacrylamide gels (7 M urea). Imaging was performed using a PhosphorImager (Amersham Biosciences).](image1)

![Fig. 8. Accumulation of AN34 after treatment of cytosolic fraction with caspase-3. Cell-free reactions were comprised of cytoplasmic extracts and recombinant proteins as described under “Experimental Procedures.” Cell extracts were then incubated at 37 °C for 40 min in the absence (b) or in the presence (a) of caspase-3. Cell lysates were fractionated by a Superdex-200 gel filtration column chromatography. Nuclease activity of each fraction was examined by 3H-E. coli DNA fragmentation assays as described under “Experimental Procedures.” Aliquots of (20 µl) were subjected to 12% SDS-PAGE followed by Western blot analysis using a polyclonal antibody against Ape1 and a monoclonal antibody against DFF40. The elution positions of protein standards of different molecular masses run in parallel are shown above.](image2)
The Ape1 antibody reacted both with purified AN37 and AN34 as shown in Fig. 3 (lane 1). Another polyclonal antibody that recognizes the N terminus of human Ape1 reacted with Ape1 but did not react with AN34 (Fig. 3). Furthermore, immunodepletion experiments using the C-terminal region-specific Ape1 antibody demonstrated that the nuclease activities of AN34 and AN37 were strongly reduced in both the 3H-DNA fragmentation and chromatin digestion assays (Fig. 4). To test whether purified AN34 and AN37 had AP endonuclease activity, an oligonucleotide containing a single abasic site was incubated with purified AN34 and AN37, and the cleavage products were analyzed by DNA sequencing gels. As shown in Fig. 5, purified AN34 and AN37 possessed AP endonuclease activity. Taken together, these experiments demonstrate that AN34 is an N-terminally truncated form of Ape1 (∆35-Ape1, comprising residues 36–318) and that AN37 in apoptotic cells corresponds to Ape1.

Recombinant AN34 (∆35-Ape1) Possesses Endonuclease Activity Independently of the Presence of AP Sites, and Produces Internucleosomal DNA Fragmentation—Our data clearly show that purified Ape1 (AN37) and AN34 from apoptotic cells induce internucleosomal DNA cleavage (Fig. 4a). However, it is known that Ape1 itself has no endonuclease activity toward undamaged DNA (i.e. non-AP site-containing DNA). Thus, we analyzed whether AN34 possesses endonuclease activity toward undamaged (normal) DNA substrates. Recombinant full-length Ape1 and recombinant AN34 (∆35-Ape1) were used for this experiment and tested in a DNA cleavage assay using supercoiled SV40 DNA. As shown in Fig. 6a, recombinant full-length Ape1 did not cleave SV40 DNA. By contrast, recombinant AN34 levels increased during apoptosis. Cell lysates from untreated (lanes 1 and 3), staurosporine-treated (1 μM, 2 h and 30 min) HL-60 cells (lane 2) or camptothecin-treated (1 μM, 36 h) HT29 cells (lane 4) were subjected to 12% SDS-PAGE followed by Western blot analysis using a polyclonal antibody against Ape1.
DNA fragmentation.

**Purified AN34/Ape1 from Apoptotic Cells Possesses 3’-5’-Exonuclease Activity**—We next examined whether purified AN34/Ape1 from apoptotic cells possessed exonuclease activity. The 20-mer duplex oligodeoxynucleotide shown in Fig. 7a was either 5’- or 3’-32P-end-labeled. Incubation of purified AN34/Ape1 with the 3’-end-labeled oligodeoxynucleotide released a radiolabeled mononucleotide (Fig. 7b, lane 2). Similarly, recombinant Ape1 released this same mononucleotide (Fig. 7b, lane 5). When the 5’-end-labeled oligonucleotide was used, AN34/Ape1 from apoptotic cells generated oligonucleotide bands between 9 and 20 nucleotides in length (Fig. 7c, lane 2). Longer oligonucleotide bands (17–20) were observed in the case of recombinant Ape1 (Fig. 7c, lane 4), indicating that the 3’-5’-exonuclease activity of recombinant Ape1 was either weaker than that of AN34/Ape1 (AN37) purified from apoptotic cells or less processive. These results demonstrate that recombinant AN34 (∆35-Ape1) has 3’-5’-exonuclease activity (~2.5-fold greater than recombinant Ape1).

**Recombinant Caspase-3 Induces Accumulation of Active AN34**—Next, we investigated whether the activation of AN34/Ape1 could be regulated by caspase-3. A cytosolic fraction prepared from untreated human leukemia HL-60 cells was incubated with recombinant caspase-3 and applied to a Superdex-200 gel filtration column. Nuclease activity within each fraction was examined using the 3H-DNA fragmentation assay. Figure 8a shows that the DNA fragmentation activity eluted in a single peak with molecular mass of ~38–40 kDa. Western blotting with Ape1 antibodies revealed that this nuclease activity peak contained both Ape1 (AN37) and AN34 (Fig. 8a). The same fraction (#20) also reacted with DFF40 antibody (Fig. 8a). The reactivity to the DFF40 antibody was also observed in fractions 2 and 3, although no 3H-DNA fragmentation activity was detected in these fractions.

In the absence of caspase-3 treatment, the cytoplasmic fractions prepared from untreated HL-60 cells and fractionated by Superdex-200 column chromatography showed only a weak 3H-DNA fragmentation activity (Fig. 8b). In addition, only a small amount of AN34 was observed in the absence of caspase-3 activation (Fig. 8b). These data indicate that Ape1 has only a weak nuclease activity in HL-60 cytoplasmic extracts in the absence of caspase-3 treatment.

The peak of nuclease activity (corresponding to fractions 19–22 in Fig. 8a) was further purified using HiTrap-Q column chromatography. Nuclease activity and Ape1 immunoreactivity (AN34/Ape1) were both detected in the flow-through fraction (Fig. 9a). However, the DFF40-containing fractions (fractions 2, 3, and 20 in Fig. 8a), when applied to the HiTrap-Q column chromatography, eluted at ~0.2 M KCl, as detected by Western blotting, and lacked 3H-DNA fragmentation activity (Fig. 9a). Purified DFF40/CAD also failed to show 3H-DNA fragmentation activity under our assay conditions, although purified DFF40/CAD showed significant nuclease activity when SV40 DNA was used as substrate (data not shown). Importantly, the 3H-DNA fragmentation assay can detect only small DNA fragments of less than 13 nucleotides (28), suggesting that exonuclease activity can be preferentially measured by this assay.

The caspase-3-stimulated nuclease activity was further purified in three steps using heparin–Sepharose, hydroxypatite, and Mono S chromatography (for details see “Experimental Procedures”). The elution profiles for nuclease activity in each column were similar to those of AN34/Ape1 from apoptotic cells. The molecular masses of the purified nuclease was 37 kDa and 34 kDa (Fig. 9b, lane 1), and Western blotting (Fig. 9b, lane 2) demonstrated that the nuclease corresponded to AN34/Ape1. Furthermore, MALDI mass spectrometer analysis showed that the mass fingerprinting of the digested peptides matched those of Ape1 (data not shown). Increased endonuclease activity (to non-AP sites) of purified AN34/Ape1 from caspase-3-treated cell lysates was also observed (Fig. 9c). These experiments suggest that active caspase-3 induces accumulation of AN34, resulting in cleavage of undamaged (normal) DNA. We also examined whether the amount of AN34 may increase during apoptosis by Western blotting. An increase of AN34 was observed during apoptosis both in HL-60 cells treated with staurosporine (Fig. 9d, lanes 1 and 2) and in human colon carcinoma HT29 cells treated with camptothecin (Fig. 9d, lanes 3 and 4).

**Subcellular Localization of AN34/Ape1 in Apoptotic Cells**—It is known that Ape1 is localized prevalently in the cytoplasm in cell types such as macrophages, spermatocytes, hippocampal cells, hepatocytes, hypoglossal motor neurons, and breast cells (24). Indirect immunofluorescence analysis using anti-Ape1 antibodies and confocal laser microscopy were carried out in untreated HL-60 cells, and the results showed that Ape1 was mainly detected in the cytoplasm and the perinuclear region. In apoptotic HL-60 cells, the anti-Ape1 antibody also stained the nucleus, suggesting that AN34/Ape1 accumulates in the nucleoplasmic compartment in apoptotic cells (Fig. 10).

**Recombinant Ape1 and AN34 Can Induce DNA Degradation in Isolated Nuclei from Staurosporine-treated HL-60 Cells**—It...
induced internucleosomal DNA cleavage (Fig. 11, recombinant Ape1 also induced DNA degradation (Fig. 11, treated U87-MG cells. Fragmentation, although staurosporine clearly induced internucleosomal DNA cleavage (Fig. 11, isolated nuclei prepared from untreated HL-60 cells at 37 °C for 90 min (lane 2), recombinant Ape1 (500 ng, lane 3) or AN34 (Δ35-Ape1, 500 ng, lane 4) at 37 °C for 90 min. Recombinant Ape1 (500 ng) was also incubated with nuclei prepared from untreated HL-60 cells at 37 °C for 90 min (lane 2). DNA was extracted and subjected to 1.2% agarose gel electrophoresis.

Fig. 11. Chromatin DNA degradation induced by recombinant Ape1 or AN34. Isolated nuclei from staurosporine-treated (1 μM, 60 min) HL-60 cells were incubated in the absence (lane 1) or presence of recombinant Ape1 (500 ng, lane 3) or AN34 (Δ35-Ape1, 500 ng, lane 4) at 37 °C for 90 min. Recombinant Ape1 (500 ng) was also incubated with nuclei prepared from untreated HL-60 cells at 37 °C for 90 min (lane 2). DNA was extracted and subjected to 1.2% agarose gel electrophoresis. has been reported that apoptosis is associated with the accumulation of reactive oxygen species in a variety of systems (29–31). Because AP sites are among the most frequent DNA lesions induced by reactive oxygen species, we examined whether Ape1 might convert these AP sites into DNA breaks and contribute to chromatin fragmentation in apoptotic cells. Recombinant Ape1 and AN34 (Δ35-Ape1) were incubated with isolated nuclei prepared from HL-60 cells treated with staurosporine (1 μM, 60 min). The recombinant AN34 (Δ35-Ape1) induced internucleosomal DNA cleavage (Fig. 11, lane 4). Recombinant Ape1 also induced DNA degradation (Fig. 11, lane 3), although less than that observed with Δ35-Ape1 and without detectable DNA laddering. The ability of Ape1 to produce chromatin DNA degradation was not found when Ape1 was incubated with nuclei prepared from untreated HL-60 (Fig. 10, lane 2), suggesting that Ape1 can induce apoptotic DNA fragmentation by two mechanisms: cleavage of normal DNA by AN34 and cleavage of oxidatively damaged DNA by Ape1.

Silencing of Ape1 Expression Suppresses Apoptotic DNA Fragmentation in DFF40/CAD-deficient Cells—It has been reported that U87-MG cells lack DFF40/CAD expression (16). We confirmed this report by Western blotting (Fig. 12a). Next, we examined a requirement for Ape1/AN34 in apoptotic DNA fragmentation in U87-MG cells using RNA interference. Twenty-one nucleotide short interfering RNA (siRNA) duplexes for Ape1 were transfected into U87-MG cells. After 48 h, expression of Ape1 was strongly suppressed (Fig. 12b, lane 3), whereas cells transfected with a control siRNA showed unchanged expression of Ape1 (Fig. 12b, lane 2). As shown in Fig. 12c, silencing of Ape1 expression suppressed apoptotic DNA fragmentation, although staurosporine clearly induced internucleosomal DNA cleavage in control (non-silencing) siRNA-treated U87-MG cells.

DISCUSSION

In the present study, we identified and characterized a novel apoptotic nuclease, AN34, as an N-terminally truncated form of Ape1 (Δ35-Ape1 comprising residues 36–318). Moreover, we found that recombinant N-terminally truncated Ape1 (Δ35-Ape1) possesses both endonuclease and 3'–5’-exonuclease activities on DNA substrates lacking AP sites. The endonuclease activity of recombinant AN34 is consistent with the characteristics of AN34 purified from apoptotic cells (18). The known structural homology between N-terminally truncated Ape1 (D35-Ape1 = AN34) and Dnase I (26) may explain the observed endonuclease of AN34. The 3’–5’-exonuclease activity of normal Ape1 in 2–4 orders of magnitude less than the AP endonuclease activity of Ape1 (19, 20). However, Chou et al. (32, 33) recently demonstrated that Ape1 possesses a specialized 3’–5’-exonuclease activity that preferentially removes mismatches deoxyribonucleotides at the 3’ termini of nicked DNA. We find that AN34/Ape1 purified from apoptotic cells possesses enhanced exonuclease activity, which hydrolyzes the 3’-DNA terminus from normal deoxyribonucleotides more efficiently than does normal Ape1.

Although DFF40/CAD is a well-established apoptotic endonuclease (10), its expression in human tissue is limited. Mukae et al. (16) reported a lack of DFF40/CAD mRNA expression in the brain, lung, liver, skeletal muscle, thymus, testis, and small intestine. Our study also confirms that human glioblastoma U87-MG cells lack DFF40/CAD (16), although U87-MG cells remain able to induce internucleosomal DNA fragmentation in response to staurosporine, a ubiquitous inducer of apoptosis (34, 35) (Fig. 12). In U87-MG cells, we find that silencing of Ape1 expression strongly suppresses apoptotic DNA fragmentation, indicating that AN34 and Ape1 can contribute to chromatin degradation.

Previous work has demonstrated that the Ape1 gene is essential, because inactivation of the gene is lethal at early stages of embryogenesis (36). The human Ape1 gene encodes a protein of 319 amino acids with a calculated molecular mass of 35.5 kDa and an electrophoretic mobility of around 37 kDa (19, 20). Several studies have established that human Ape1 is a multifunctional DNA repair enzyme, which belongs to the class II AP endonuclease family and is the major cellular enzyme repairing AP sites in DNA (19, 20, 23). Ape1 also possesses a 3’–5’-exonuclease activity, a 3’-phosphodiesterase activity, a 3’-phosphatase activity, and an RNase H activity (19, 20, 23, 37). Moreover, human Ape1 functions as a redox factor that en-
hances the DNA binding of several important transcription factors, including p53 (38), c-Fos (21), c-Jun (39), AP-1 (40), Myb (21), and NF-xB (21). The redox regulatory function is localized in the N-terminal domain of Ape1 (21, 26) (see Fig. 2).

Our data demonstrate that AN34 is generated during apoptosis induced by etoposide staurosporine in human leukemia HL-60 cells and by camptothecin in human colon carcinoma HT29 cells (Fig. 9c). Cell-free system analyses revealed that there was a significant increase of AN34 protein after incubation of cytosol with caspase-3. We also examined whether caspase-3 can directly cleave Ape1 in vitro. However, caspase-3, even in molar excess, did not cleave recombinant Ape1 (data not shown). Using reverse transcription-PCR, we investigated whether AN34 might result from an alternative splicing of the Ape1 mRNA. However, no such splicing was observed. Based on these observations, it is likely that AN34 arises by cleavage of Ape1 by protease(s) other than caspase-3.

Indeed, it has been reported that trypsin preferentially cleaves Ape1 after Lys-35 to generate a stable globular protein comprising amino acid residues 36–318 (26). Thus, it is likely that AN34 is a cellular processing product of Ape1 after cleavage of Ape1 by a trypsin-like enzyme activated by caspase-3 during apoptosis. At present, we have not identified the one or more proteases that may specifically bind Ape1 and prevent the AN34 activation in normal cells.

AB 68 (34) is among the most common lesions that arise in cellular DNA. They are produced through a variety of mechanisms, including the generation of reactive oxygen species and exposure of cells to ionizing radiations and alkylating agents (23, 44, 45). Recently, it has been reported that reactive oxygen species play a critical role during apoptosis (29–31). Many apoptotic stimuli, including tumor necrosis factor, ionizing radiation, ceramide, and DNA-damaging agents, produce reactive oxygen species in a variety of cells (46–48). It is likely that these apoptotic stimuli generate AP sites in cellular DNA and that AN34/Ape1 cleaves these AP sites. This process may play an important role during apoptosis when reactive oxygen species induce multiple AP sites in cellular chromatin. Indeed, our data demonstrate that recombinant Ape1 can digest preferentially (albeit less than AN34) the chromatin isolated of cells treated with the ubiquitous apoptosis inducer staurosporine (34).

In conclusion, we report two novel functions for Ape1 in relationship with apoptosis. First, truncation of the first 35 amino acids of Ape1 (∆35-Ape1) generates AN34, an endonuclease that can induce both single and double strand breaks in purified DNA and DNA fragmentation in isolated nuclei. Second, we find that Ape1 can cleave chromatin in cells treated with apoptotic stimuli and that Ape1 silencing prevents internucleosomal DNA fragmentation. Thus, Ape1/AN34 is likely to participate in the process of chromatin DNA fragmentation in concert with other apoptotic nucleases such as DFF40/CAD (10, 15) and endonuclease G (17).

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