Endoribonuclease activity of human apurinic/apyrimidinic endonuclease 1 revealed by a real-time fluorometric assay

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ABSTRACT

Apurinic/apyrimidinic endonuclease 1 (APE1) is a multifunctional enzyme with a well-established abasic DNA cleaving activity in the base excision DNA repair pathway and in providing redox activity to several well-known transcription factors. APE1 has recently been shown to cleave at the UA, CA, and UG sites of c-*myc* RNA in vitro and regulates c-*myc* messenger RNA (mRNA) in cells. To further understand this new endoribonuclease activity of APE1, we have developed an accurate, sensitive, and rapid real-time endonuclease assay based on a fluorogenic oligodeoxynucleotide substrate with a single ribonucleotide. Using this substrate, we carried out the first kinetic analysis of APE1 endoribonuclease activity. We found that the purified native APE1 cleaves the fluorogenic substrate efficiently, as revealed by a k_{cat}/K_m of $2.62 \times 10^6 \, M^{-1} \, s^{-1}$, a value that is only 71-fold lower than that obtained with the potent bovine pancreatic RNase A. Ion concentrations ranging from 0.2 to 2 mM Mg^{2+} promoted catalysis, whereas 10 to 20 mM Mg^{2+} was inhibitory to the RNA-cleaving activity of APE1. The monovalent cation K^+ was inhibitory except at 20 mM, where it significantly stimulated recombinant APE1 activity. These results demonstrate rapid and specific endoribonucleolytic cleavage by APE1 and support the notion that this activity is a previously undefined function of APE1.

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The role that endonucleolytic cleavage plays in the control of mammalian messenger RNA (mRNA)[1] degradation is poorly understood. For example, mRNA endonucleolytic decay intermediates for several mammalian genes have been described, but the responsible endoribonucleases are unidentified [1]. However, the recent discovery of endoribonuclease activity exhibited by a component of the eukaryotic exosome [2,3], and by a protein involved in the metazoan nonsense-mediated decay pathway [4,5], underscores the significance of endonucleolytic cleavage and suggests that this mode of RNA cleavage to control mRNA decay should be examined further. To fully understand the significance of endonucleolytic cleavage in the control of mRNA degradation and therefore mRNA abundance, both the mRNA target and the enzyme responsible must be identified and their role must be characterized. To this end, we recently purified and identified apurinic/apyrimidinic DNA endonuclease 1 (APE1) as a 35-kDa protein that is capable of cleaving c-*myc* mRNA in vitro as well as regulating c-*myc* mRNA levels and half-life in cells [6].

Our discovery of APE1 cleavage between the dinucleotides UA, CA, and to some extent UG in the single-stranded region of c-*myc* coding region determinant (CRD) RNA was unexpected [6]. APE1 endonucleolytically cleaves DNA at apurinic/apyrimidinic (AP) sites and is a key enzyme in base excision repair of eukaryotic cells [7]. It has also been shown to provide the major redox activity for AP-1, p53, HIF1, and other transcription factors [8]. In addition to the AP DNA endonuclease activity, APE1 has 3′–5′ DNA exonuclease [9], 3′ phosphodiesterase [7], and RNase H activities [10]. Apart from an initial study showing that the endoribonuclease activity of APE1 shares somewhat the same active site as its other nuclease activities [6], and the report that it possesses abasic RNA endonuclease activity [11,12], the biochemical RNA-cleaving properties of APE1 remain uncharacterized. For instance, it is not known which additional cofactors or sulfhydryl-modifying agents will influence the endoribonuclease activity of APE1.

Measuring kinetic parameters of ribonucleases with their natural substrates is difficult because of the multiplicity of potential cleavage sites and the kinetic interdependence of the cleavage events. To conveniently and rapidly characterize APE1 endoribonuclease kinetic parameters, a sensitive and continuous assay needed to be developed. Several laboratories have successfully developed and used fluorescence-based assays to study ribonuclease activities [13–16]. Based on APE1's ability to preferentially cleave in between the 1751 UA dinucleotide of c-*myc* CRD RNA [6], we designed a 17-nt fluorogenic/quench substrate with a

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  [1] *Abbreviations used:* mRNA, messenger RNA; APE1, apurinic/apyrimidinic endonuclease 1; CRD, coding region determinant; AP, apurinic/apyrimidinic; DTT, dithiothreitol; BHQ1, Black Hole Quencher 1; HPLC, high-performance liquid chromatography; DEPC, diethylpyrocarbonate; BSA, bovine serum albumin; ReAPE1, recombinant human APE1; RFU, relative fluorescence unit; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CoA, coenzyme A.

single ribonuclease-sensitive bond to monitor the endonucleolytic cleavage by APE1 under real-time conditions. Here we show that our fluorescence assay can be reliably and conveniently used to study the endoribonuclease activity of APE1. The kinetic parameters of both native and recombinant APE1, as well as RNase A and RNase I_f, are reported along with the effects of Mg^{2+}, K^+, RNasin, and the sulfhydryl-modifying agent dithiothreitol (DTT).

Materials and methods

Materials and general procedures

The oligonucleotides DNAOligoI with 5′-Cy3-CAA GGT AGT TAT CCT TG-1(BHQ1)-3′ (where BHQ1 is Black Hole Quencher 1), OligoI with 5′-Cy3-CAA GGT AGT rUAT CCT TG-BHQ1-3′, and OligoII with 5′-Cy3-CrUA GGT AGT TAT CCrU AG-BHQ1-3′ (Fig. 1) were custom synthesized and high-performance liquid chromatography (HPLC) purified on a 1-μmol scale by Integrated DNA Technologies (Coralville, IA, USA). The fluorogenic substrates were resuspended in diethylpyrocarbonate (DEPC)-treated water and stored at $-80\,°C$ (for long-term storage) or $-20\,°C$ (for short-term storage after daily use). RNase A (cat. no. R6513) and DTT were obtained from Sig-

ma–Aldrich (Oakville, Ontario, Canada). RNasin and DNase I were obtained from Promega (Madison, WI, USA). RNase III was obtained from Ambion (Austin, TX, USA), and RNase T1 was obtained from Roche Applied Science (Laval, Quebec, Canada). RNase I_f was obtained from New England Biolabs (Pickering, Ontario, Canada).

Protein purification

Native APE1 was purified from juvenile frozen rat livers as described previously [6]. The plasmid pET15b–hAPE1 containing human APE1 complementary DNA (cDNA) was used to express the recombinant His-tagged APE1 in BL21(DE3) *Escherichia coli* cells. The recombinant protein was first purified using nickel–nitrilotriacetic acid (Ni–NTA) column chromatography. Following removal of the His tag with thrombin [6], the recombinant protein was further purified using an SP-Sepharose High Performance column (GE Healthcare, Montreal, Quebec, Canada). Prior to use, the recombinant protein was dialyzed against 10 mM Tris–HCl (pH 7.4), 2 mM DTT, 2 mM magnesium acetate, 1 mM l-glutathione reduced, and 0.1 mM glutathione oxidized, with two buffer changes over 5 h [6]. For use in experiments assessing the role of metal ions, the recombinant protein was dialyzed in the same buffer but lacking magnesium acetate. During the initial part of this study, the recombinant APE1 was denatured with 2 M guanidine hydrochloride and then dialyzed in the buffer described above [6]. However, we later found no significant differences in the enzyme kinetics and enzyme responses to external agents such as K^+, Mg^{2+}, DTT, and RNasin whether or not it underwent the refolding step. Therefore, for most of the studies conducted here, we had omitted the denaturation and renaturation steps for the enzyme preparation. Our preparation of recombinant APE1 is free of RNase A or any bacterial RNase that could cleave oligos used in this study. This is supported by our observations that two APE1 mutants, H309N and E96A (which were prepared essentially in the same manner), did not exhibit any nuclease activity against 88-nt RNA substrate [6].

Fluorescence RNA cleavage assay

Unless otherwise indicated, assays were carried out in a total volume of 80 μl of 10 mM Tris (pH 7.4), 2 mM DTT, 2 mM magnesium acetate, 0.1 mM spermidine, and DEPC-treated water in a 384-well microplate, and the microplate was equilibrated at 20 °C. During optimization, substrate concentration was held at 20 nM. The fluorescence emission of Cy3 was measured at 565 nm with excitation at 535 nm using a Thermo Electron Varioskan (Milford, MA, USA). Reactions were initiated by the addition enzyme, followed by mixing. The plate was then placed in the flourimeter, and the reaction time was initiated immediately. The increase in fluorescence arising from substrate cleavage was measured from 1 to 13 min, with a data point taken every minute. Data analysis was carried out using Prism (GraphPad Software, San Diego, CA, USA) or KaleidaGraph (Synergy Software, Reading, PA, USA). Unless otherwise indicated, final enzyme concentrations or activities were as follows: 1.25×10^{-3} μg/μl RNase A, 6.25×10^{-3} μg/μl bovine serum albumin (BSA), 1.25×10^{-2} U/λ RNase I_f, 1.25×10^{-2} U/λ RNase T1, 1.25×10^{-2} U/λ DNase I, 1.25×10^{-3} U/λ RNase V1, 1.25×10^{-2} U/λ RNase III, 1.38×10^{-3} μg/μl recombinant human APE1 (ReAPE1), and 5.50×10^{-4} μg/μl native APE1. Relative fluorescence units (RFUs), fluorescent intensity measurements with background intensity (no protein added) subtracted, are used to report activity.

Kinetic assays

To determine the kinetic parameters of ReAPE1, native APE1, RNase I_f, and RNase A, OligoI substrate concentrations were varied from 20 to 4250 nM. The amount of enzyme and reaction time

Fig. 1. Fluorogenic substrates. Shown are the sequence and structure of fluorogenic oligonucleotide substrates in comparison with the segment of c-*myc* CRD RNA. Cy3 was labeled at the 5′ end, and BHQ1 was labeled at the 3′ end, of OligoI, OligoII, and DNAOligoI. OligoI, OligoII, and DNAOligoI contain deoxyribonucleotides except at specified positions where ribonucleotides (indicated by an "r") are present and nucleotides are bolded. Arrows on c-*myc* CRD RNA indicate sites cleaved by APE1. The principal cleavage site at 1751 UA on OligoI is marked with an arrow.

were optimized to ensure that the reaction rate of each enzyme falls within a linear range. The final concentrations of each enzyme for kinetic parameter determination were as follows: RNase A, 1.25×10^{-7} µg/µl; ReAPE1, 1.25×10^{-3} µg/µl; native APE1, 1.25×10^{-4} µg/µl; and RNase I_f, 1.00×10^{-2} µg/µl. The reaction for ReAPE1 was carried out for 30 min, the reaction for native APE1 was carried out for 15 min, the reaction for RNase A was carried out for 3 min, and the reaction for RNase I_f was carried out for 5 min. Nonlinear regression analysis of the data plotted was used to determine the kinetic parameters (K_m and k_{cat}) with Kaleida-Graph. The reported values are the means ± standard errors of six replicate measurements from at least two separate experiments. The k_{cat} values were converted to s^{-1} from $RFUs^{-1} M^{-1}$ by use of the Cy3 extinction coefficient of 150,000 $M^{-1} cm^{-1}$.

Results

Design of fluorogenic substrates and initial optimization studies

To allow precise measurement of the RNase activity of APE1 at a single position, we sought to design a DNA oligo substrate with a single ribonucleotide. The previously determined secondary structure of c-*myc* CRD RNA [17] showed that both purified native and recombinant APE1 have a strong preference for cleaving in between the 1751 UA dinucleotide located within the stem V–loop region(s) (Fig. 1). Therefore, the fluorogenic substrate was based on the sequence immediately surrounding the stem V–loop region (OligoI) and incorporating a fluorescent tag, Cy3, at the 5′ end and a fluorescence quencher, BHQ1, at the 3′ end of the oligonucleotide. OligoI sequences correspond to c-*myc* CRD DNA nt 1743 to 1757 with two additional nucleotides, a cytosine at the 5′ end and a guanine at the 3′ end, to increase the strength of stem V. In addition, a 2′ hydroxyl uridine was incorporated at nt 1751 (bolded nucleotide in Fig. 1). We also designed DNAOligoI, which has an identical sequence to OligoI except that deoxythymidylate was substituted for 2′ hydroxyl uridine (Fig. 1). A second oligonucleotide, OligoII (Fig. 1), was designed to incorporate two UA dinucleotide pairs within the stem region to test cleavage of UA in a double-stranded region.

Fluorescence optimization

Using OligoI as substrate and bovine pancreatic RNase A as a control, initial experiments were performed to optimize conditions for measuring maximal fluorescence signal on quencher cleavage.

Combinations of excitation (530–550 nm) and emission (560–580 nm) wavelengths were assessed initially. We found that using the dual-labeled substrate (Cy3 and BHQ1), a maximum of 8-fold enhancement of fluorescent signal can be achieved with an excitation wavelength of 535 nm and an emission wavelength of 565 nm (data not shown). Reaction parameters such as reaction temperature, volume, and substrate concentration were then varied individually to find the optimal reaction conditions. The optimal reaction conditions used in all experiments are as described in Materials and Methods.

Proof of concept for measuring APE1 endoribonuclease activity

The proof of concept that OligoI could indeed be used to measure endoribonuclease activity was carried out by using two known ribonucleases, RNase A and RNase I_f, and two known proteins expected to have no activity, BSA and RNase T1. Both RNase A and RNase I_f are known to cleave UA dinucleotide bonds, whereas BSA and RNase T1 have no endoribonuclease activity against UA dinucleotides. Both RNase A and RNase I_f significantly increased fluorescence signal over a 10-min period, whereas BSA and RNase T1 had no effect (Fig. 2A), indicating that an increase in fluorescent signal is indicative of cleavage between this dinucleotide. To further confirm the specificity for the ribonucleotide substrate, RNase A activity with DNAOligoI as the substrate, was compared with a known DNA nuclease, DNase I (Fig. 2B). Whereas a 10-fold higher concentration of RNase A had no effect on fluorescent signal with DNAOligoI, DNase I significantly enhanced the fluorescence signal (Fig. 2B). These results confirm the specificity of the fluorescent assay.

APE1 endoribonuclease activity

We next assessed both the native APE1 and recombinant APE1 for endoribonuclease activity using the fluorescent assay. The purified native APE1 contains three additional proteins as assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis [6]. As described in Materials and Methods, the recombinant APE1 was made in BL21(DE3) *E. coli* cells and its native activity might not be fully reconstituted.

To measure APE1's endoribonucleolytic activity, the ability of APE1 to cleave OligoI was determined and the specificity for single-stranded RNA was determined by comparison with OligoII as substrate. Both recombinant and native APE1 increase the fluorescence signal (Fig. 3A), consistent with their preferential cleavage at
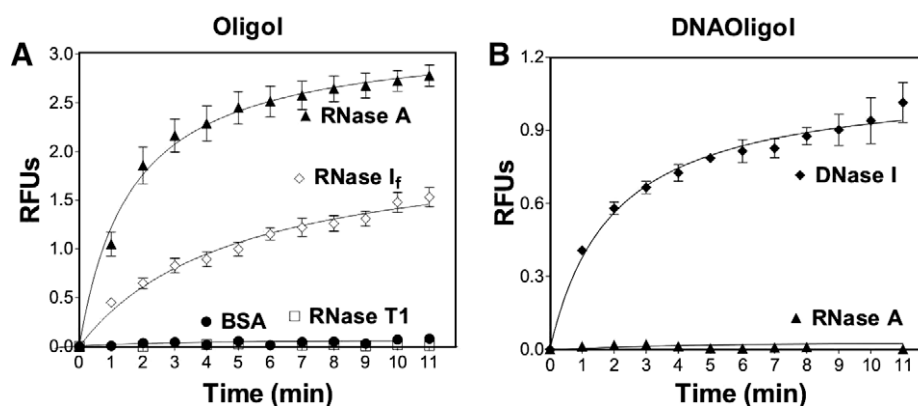

Fig. 2. Effect of nucleases on OligoI and DNAOligoI fluorogenic substrates. (A) OligoI (20 nM) was treated with RNase A (6.25×10^{-4} µg/µl), RNase T1 (1.25×10^{-2} U/µl), RNase I_f (1.25×10^{-2} U/µl), or BSA (6.25×10^{-3} µg/µl), followed by measurement of fluorescent signals at the indicated times. (B) DNAOligoI (20 nM) was treated with RNase A (6.25×10^{-3} µg/µl) or DNase I (1.25×10^{-2} U/µl) for the indicated times, followed by measurement of fluorescent signals. RFUs are measured fluorescent signals that had been subtracted from background controls (no protein added).
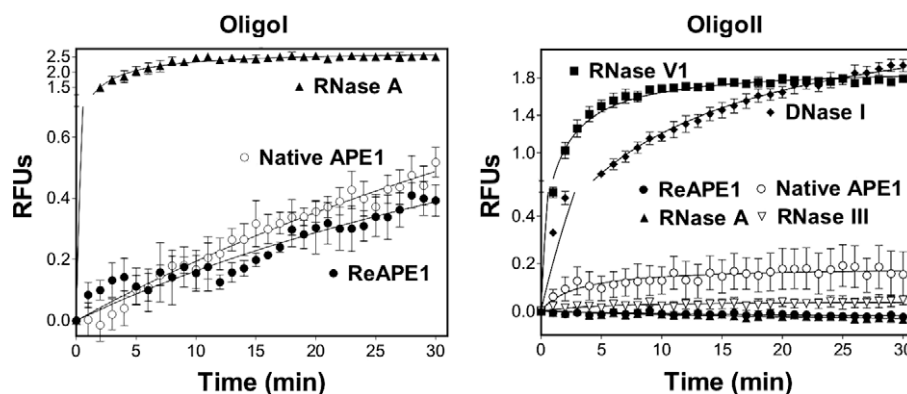
Fig. 3. Effect of nucleases on OligoI and OligoII fluorogenic substrates. (A) OligoI (20 nM) was treated with RNase A (1.25×10^{-3} µg/µl), recombinant APE1 (1.25×10^{-3} µg/µl), or native APE1 (5.50×10^{-4} µg/µl) for the indicated times. (B) OligoII (20 nM) was treated with RNase VI (1.25×10^{-3} U/µl), DNase I (1.25×10^{-2} U/µl), RNase A (1.25×10^{-3} µg/µl), RNase III (1.25×10^{-2} U/µl), recombinant APE1 (1.25×10^{-3} µg/µl), or native APE1 (5.50×10^{-4} µg/µl) for the indicated times, followed by measurement of fluorescent signals. Breakpoints in the RFUs on the *y* axis are indicated by two parallel horizontal lines.

1751 UA of c-*myc* CRD RNA [6]. Using OligoII, both DNase I and RNase VI (a double-stranded RNA cleaving enzyme) cause a substantial increase in the fluorescent signal, whereas the single-strand cleaving RNase A and long double-stranded RNA nuclease RNase III had no effect (Fig. 3B). Interestingly, the native APE1 (5.50×10^{-4} µg/µl) had a small but significant effect (Fig. 3B), consistent with earlier findings that both recombinant and native APE1 had weak activity against stem III of c-*myc* CRD RNA [6]. Although we have not assessed the effect of higher concentrations of recombinant APE1, at low concentrations it did not appear to have any effect on OligoII (Fig. 3B). Under similar conditions, both the recombinant and native APE1 had no effect on fluorescently labeled or ^{32}P-labeled DNAOligoI (data not shown), suggesting that

APE1 has no regular DNA endonuclease activity other than its established abasic DNA endonuclease function.

Kinetic parameters of APE1, RNase A, and RNase I_f

To further characterize the ribonuclease activity of APE1, and to initiate investigation into the mechanistic basis for apparent differences between native and recombinant APE1, we used the continuous fluorescent assay to determine the kinetic parameters of RNase A, RNase I$_f$, native APE1, and recombinant APE1. Concentrations of the OligoI substrate were varied in the presence of a limiting amount of enzyme, and the rate of RNA cleavage was determined. All enzymes fit Michaelis–Menten kinetics (Fig. 4),
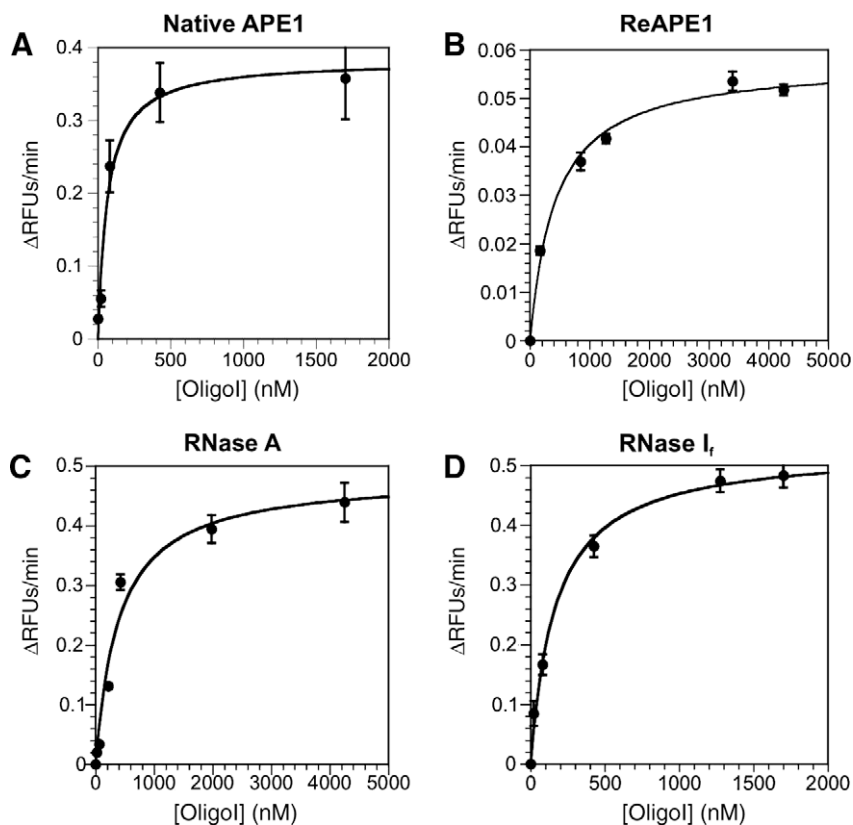
Fig. 4. Kinetic analysis of APE1 in comparison with RNase A and RNase I$_f$ using fluorescence assay. Various concentrations of OligoI as shown were treated with native APE1 (1.25×10^{-4} µg/µl) (A), recombinant APE1 (1.25×10^{-3} µg/µl) (B), RNase A (1.25×10^{-7} µg/µl) (C), or RNase I$_f$ (1×10^{-2} µg/µl) (D) for different times, as described in Materials and Methods. Michaelis–Menten plots were obtained after expressing the RFUs (ΔRFUs/min) over the various concentrations of OligoI.

Table 1
Endoribonuclease activity of various enzymes against OligoI

Enzyme	K_m (nM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	Activity (relative to native APE1)
RNase A	396 ± 104	73.70 ± 5.40	$(1.86 ± 0.50) \times 10^8$	71
RNase I$_f$	169 ± 24	$(1.60 ± 0.01) \times 10^{-2}$	$(9.47 ± 1.26) \times 10^4$	0.0036
Native APE1	64.2 ± 21	$(1.68 ± 0.12) \times 10^{-1}$	$(2.62 ± 0.88) \times 10^6$	1.0
ReAPE1	424 ± 66	$(2.72 ± 0.09) \times 10^{-2}$	$(6.42 ± 1.02) \times 10^4$	0.025

and their kinetic parameters were determined (Table 1). The native APE1 exhibited a K_m of 64.2 nM and a k_{cat}/K_m of 2.62×10^6 M^{-1} s^{-1}, whereas the recombinant APE1 had a K_m of 424 nM and a k_{cat}/K_m of 6.42×10^4 M^{-1} s^{-1}. The K_m values of RNase A and recombinant APE1 were remarkably similar, suggesting that the binding affinities of OligoI for both enzymes are similar, whereas RNase A had a much higher turnover number. In contrast, the native APE1 and RNase I$_f$ had approximately 6- and 2-fold lower K_m values than RNase A (Table 1). The catalytic efficiency of RNase A, as determined by k_{cat}/K_m, was approximately 71-fold greater than the native APE1 and 2884-fold greater than the recombinant APE1, whereas the catalytic efficiency of RNase I$_f$ was approximately 278-fold lower than native APE1 and 7-fold lower than recombinant APE1 (Table 1).

Characterization of APE1 endoribonuclease activity

To assess the degree to which the RNA-cleaving activity of APE1 is sensitive to ion concentrations, Mg^{2+} and K$^+$ concentrations were varied from 0.2 to 20 mM (Fig. 5). In agreement with the previous endonuclease assay [6], lower concentrations of Mg^{2+} (0.2–2 mM) were permissive for both the native and recombinant APE1 activity (Fig. 5A). The RNA-cleaving activity of the native and recombinant APE1 was enhanced approximately 2-fold (compared with the absence of Mg^{2+}) in the presence of 2 mM Mg^{2+}. However, at higher

Fig. 5. Effect of Mg^{2+} and K$^+$ on the endoribonuclease activity of APE1. Shown is endonuclease activity of recombinant APE1 (1.25×10^{-3} μg/μl) (●) and native APE1 (5.50×10^{-4} μg/μl) (○) against OligoI, expressed as RFUs (ΔRFUs/min), in the presence of various concentrations of Mg^{2+} (A) or K$^+$ (B). The x axis is broken to highlight the 0- to 5-mM ion concentration range.

concentrations of Mg^{2+} (10–20 mM), RNA-cleaving activities of both the native and recombinant APE1 were inhibited. Complete abrogation of RNA-cleaving activity of the native enzyme was observed (Fig. 5A), whereas 1.75- to 3.5-fold reduction (compared with the absence of Mg^{2+}) in RNA-cleaving activity was observed with the recombinant APE1 (Fig. 5A). The monovalent K$^+$ effect on RNA-cleaving activity of APE1 was inhibitory to the native APE1 at all concentrations, with approximately 5-fold inhibition (Fig. 5B). Although inhibitory at 0.2 to 10 mM to the RNA-cleaving activity of recombinant APE1, 20 mM K$^+$ surprisingly stimulated the RNA-cleaving activity of the recombinant APE1 by approximately 1.3-fold (compared with the absence of K$^+$) (Fig. 5B).

The dependence of the APE1 RNA-cleaving activity on disulfide bonds was tested by inclusion of the reducing agent DTT. Both the native and recombinant APE1 have enhanced activity at 2 mM DTT (Fig. 6A), whereas higher DTT concentrations were inhibitory.

Finally, we assessed the effect of the RNase inhibitor RNasin on APE1 activity using the assay. Fig. 6B shows that 3 to 40 U of RNasin was inhibitory to both the native and recombinant APE1. The inhibitory profile of RNasin on 6.25×10^{-4} μg/μl RNase A was very similar to that seen for APE1 (data not shown).

Discussion

We have developed a specific and rapid fluorescence-based assay to measure the endoribonuclease activity of APE1. The fluorescence assay developed in this study is highly sensitive. RNase A cleaves OligoI with a k_{cat}/K_m of 1.86×10^8 M^{-1} s^{-1}, whereas the native APE1 cleaves with a k_{cat}/K_m of 2.62×10^6 M^{-1} s^{-1} (Table 1). For comparison, using a tetranucleotide labeled with 5′,6-carboxyfluorescein and 3′,6-carboxytetramethylrhodamine, RNase A exhibited a k_{cat}/K_m of 3.6×10^7 M^{-1} s^{-1} and human angiogenin cleaves the tetranucleotide [14] with a k_{cat}/K_m of 3.3×10^2 M^{-1} s^{-1}. One of the major highlights of this study is the finding that the endoribonuclease activity of native APE1 that was isolated from rat liver polysomal fraction is approximately 71-fold lower than that of the potent RNase A (Table 1). Such strong endoribonuclease activity of the native APE1 suggests a biological role for the RNA-cleaving function of APE1 in vivo. Indeed, we have shown that APE1 regulates c-*myc* mRNA levels and half-life in cultured cells [6].

Although the traditional electrophoretic techniques for monitoring RNA cleavage are highly useful in visualizing RNA cleavages and mapping cleavage sites [6,17], they do have limitations. For instance, electrophoretic monitoring of cleavage is a discontinuous assay and is inadequate for performing the initial rate measurements required for kinetic analysis of enzyme function. Furthermore, because endoribonucleases often cleave RNA substrates at multiple sites, it is difficult to objectively measure endonucleolytic activity of enzymes that often take into account the intensity of decay products. To accurately measure enzyme activity and perform kinetic studies on the recently identified endoribonuclease activity of APE1, we developed a fluorescence-based assay.

Using a dual-labeled fluorogenic oligonucleotide based on c-*myc* CRD sequences, we showed that our fluorescence assay can be reliably used to quantify endoribonuclease activity of APE1. For instance, neither BSA nor RNase T1 caused an increase in the
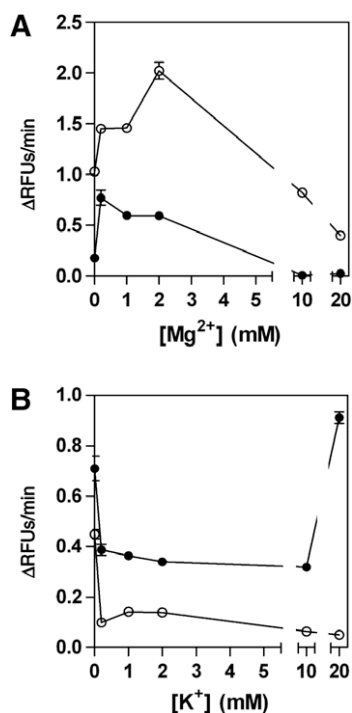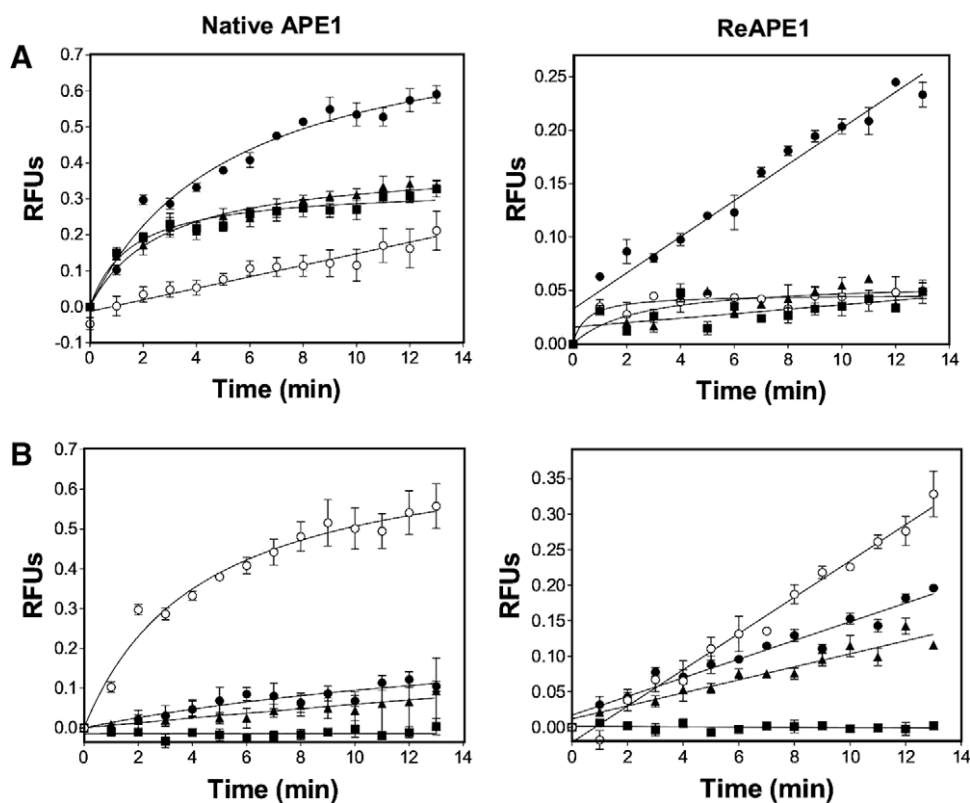
Fig. 6. Effect of DTT and RNasin on the endoribonuclease activity of APE1. Endonuclease activity of recombinant APE1 (1.25×10^{-3} µg/µl) (right panel) and native APE1 (5.50×10^{-4} µg/µl) (left panel) against OligoI was tested in the presence of various concentrations of DTT (A) or RNasin (B) for the indicated times. (A) The concentrations of DTT tested were 0 mM (○), 2 mM (●), 10 mM (▲), and 20 mM (■). (B) The concentrations of RNasin tested were 0 U (○), 3 U (●), 6 U (▲), and 40 U (■). RFUs are measured fluorescent signals that had been subtracted from background controls (no protein added).

fluorescence signal, whereas RNase A and RNase I_f, which are expected to cleave at the 1751 UA site of OligoI, significantly enhanced the fluorescence (Fig. 2). Similarly, RNase A, predicted to have no activity with DNAOligoI, had no effect on fluorescence response, whereas DNase I significantly enhanced the fluorescent signal (Fig. 2).

Using this fluorescence assay, we showed that the RNA-binding affinity of RNase A and recombinant APE1, as reflected by K_m, is quite similar for OligoI, whereas the RNA-binding affinity of RNase I_f is approximately 2-fold lower (Table 1). On the other hand, we found that the RNA-binding affinity of the native APE1 is about approximately 6-fold lower than its recombinant counterparts (Table 1). Such differences could be attributed to posttranslational modification of the native APE1 that is absent in the recombinant APE1 or to the presence of three other copurified proteins in the native APE1 [6].

Comparative biochemical studies of the native and recombinant APE1 were conducted, and the sensitivities of these enzymes to Mg^{2+}, K^+, and RNasin were very similar (Figs. 5 and 6) with the exception that 20 mM K^+ significantly enhanced the endoribonuclease activity of recombinant APE1 (Fig. 5). At low concentrations (0.2 and 2 mM), Mg^{2+} had a stimulatory effect on endoribonuclease activity of both the native and recombinant APE1, whereas 20 mM Mg^{2+} had an inhibitory effect on the endoribonuclease activity of APE1. This is consistent with a recent report that simultaneous occupancy of the two metal binding sites does not support the DNA endonuclease function of APE1 [18]. Inhibitory effects on endoribonuclease activity by increasing K^+ concentrations also agree with the previous finding of the AP DNA endonuclease function of APE1 [19,20].

We currently do not understand the stimulatory effect of high concentrations of K^+ on the endoribonuclease activity of the re-

combinant APE1 but not of the native APE1. It is possible that a high amount of K^+ was able to shield the generally negative charge of RNA and allows the highly pure ReAPE1 to bind tighter to the substrate to enhance catalysis. On the other hand, L-3-hydroxyacyl-CoA dehydrogenase (where CoA is coenzyme A), which binds c-*myc* CRD RNA (data not shown), and annexin III, which is predicted to bind RNA, were among the three proteins copurified with the native APE1 [6] and, thus, may prevent the charge-shielding function of K^+. In addition, the endoribonucleolytic mechanism is probably dependent on the dynamics of the RNA substrate sequences and secondary structures, as has been shown for APE1's AP DNA endonuclease activity [20,21]. Increasing concentrations of K^+ were found to be inhibitory to AP DNA endonuclease activity of APE1 against 34-mer single-stranded DNA, whereas it was stimulatory against 34-mer double-stranded DNA [20]. Our current knowledge of the mechanism of APE1 endoribonuclease activity is still very limited and will require in-depth structural and functional studies.

Our data indicated that 2 mM DTT is required for optimal endoribonuclease activity of APE1, suggesting that sulfhydryl groups are required to be in their reduced state. Although higher concentrations of DTT still enhanced native APE1 activity, they had no stimulatory effect on endoribonuclease activity of the recombinant APE1. We currently do not know the reason for such differences. Redox modification of Cys310 has been shown to be critical for AP endonuclease activity [22], whereas Cys65 and Cys93 are important for redox function of APE1 in the regulation of transcription factor activity [23,24]. Interestingly, it has been suggested that redox-related modification of cysteine thiols on Cys93 and Cys310 affects the process of cytoplasmic distribution of APE1 [24]. In addition, a recent study showed that Cys99 is critical for APE1's DNA substrate binding and catalysis [25]. Therefore, it would be

interesting to investigate whether any of these Cys residues are important in the endoribonuclease activity of APE1.

In summary, we have demonstrated the utility of a novel fluorescence assay that employs a fluorogenic *c-myc* oligonucleotide substrate to study the kinetics and biochemical properties of the endoribonuclease activity of APE1. We found that the polysomal native APE1 has relatively strong endoribonuclease activity and that the activity is highly dependent on the sulfhydryl state of the enzyme. The established assay can now be used to investigate the structure and RNA-cleaving function relationship of APE1 in a quantitative manner and allow specific comparison of endoribonuclease activity of the enzyme under differing treatments as well as specific site-directed modification.

Acknowledgments

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