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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.

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.

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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 Sigma–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).

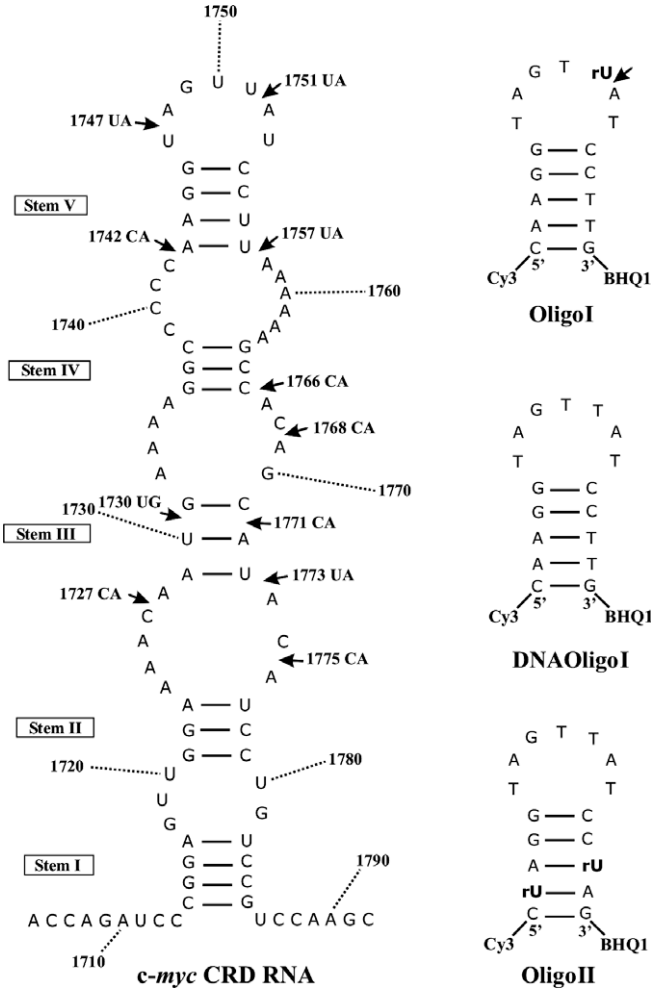

Fig. 1. Fluorogenic substrates. Shown are the sequence and structure of fluorogenic oligonucleotide substrates in comparison with the segment of c-*myc* CRD RNA. Cy3 was labeled at the 5′ end, and BHQ1 was labeled at the 3′ end, of OligoI, OligoII, and DNAOligoI. OligoI, OligoII, and DNAOligoI contain 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.

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