Apoptosis (2006) 11:1331–1337
DOI 10.1007/s10495-006-6983-0

The apoptotic endonuclease DFF40/CAD is inhibited by RNA, heparin and other polyanions

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Published online: 9 May 2006

Abstract DFF40/CAD, the major apoptotic nuclease, is specific for double-stranded DNA. However, RNA and single-stranded DNA, though not substrates for the enzyme, compete with double-stranded DNA and inhibit its cleavage by the nuclease. In addition, other anionic polymers, like poly-glutamic acid and heparin also inhibit DFF40/CAD, the latter one being highly effective at nanomolar concentrations. The inhibitory poly-anions bind to the nuclease and impair its ability to bind double-stranded DNA. We propose that such poly-anions bind to the positively charged surface formed by α4 helices of the DFF40/CAD homodimer. This surface has been proposed recently to bind to either the major groove of DNA or poly (ADP-ribose), another inhibitor of the nuclease.

Keywords Apoptosis · CAD · DFF · Heparin · Nuclease · Poly(ADP-ribose) · RNA

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Introduction

The major nuclease primarily responsible for DNA fragmentation in cells undergoing apoptosis is DNA Fragmentation Factor (DFF), also termed Caspase-activated DNase (CAD), which preferentially attacks chromatin in the internucleosomal linker, generating oligonucleosomal DNA ladders [reviewed in: 1]. In its inactive form, DFF is a heterodimer composed of a 40-kD latent endonuclease subunit (DFF40/CAD) and a 45-kD chaperone and inhibitor subunit (DFF45/ICAD) [2–5]. More recently, formation of dimers of such DFF heterodimers has been reported [6]. DFF45 carries two caspase-3 recognition sites—aspartate residues 117 and 224. Upon caspase-3 cleavage of DFF, DFF45/ICAD is cut and released from DFF40/CAD, which forms homo-dimers [7] and higher order homo-oligomers [8, 9] that are forms of the enzymatically active nuclease. Several lines of evidence show that homo-oligomers of activated DFF40/CAD can further bind additional regulatory proteins, both activators and inhibitors. The *C*-terminal domain of histone H1 acts as an activator and binds to DFF40/CAD, which increases its ability to bind to DNA [10]. By contrast, nucleophosmin/B23, the nuclear receptor of PI(3,4,5)P_3, binds to DFF40/CAD and inhibits its enzymatic activity [11]. Similarly, a novel protein termed CIIA, which also regulates apoptosis signal-regulating kinase 1 of the MAPKK kinase family, binds to and inhibits DFF40/CAD [12].

Tight and precise control of the activity of pro-apoptotic proteins are essential for both the prevention of accidental suicide in normal cells and the appropriate execution of apoptosis when desired. In the present study, we have searched for new factors associated with inhibiting DFF40/CAD nuclease activity. We have detected a potent inhibitor of DFF40/CAD in the cytoplasmic fraction of several cell types, which works downstream of caspase-3 cleavage of the inhibitory subunits

DFF45/ICAD. Surprisingly, this inhibitor has been identified as RNA, which we show like other poly-anions compete with double-stranded DNA for binding to the nuclease.

Materials and methods

Nucleases and other reagents

Recombinant 6-His-tagged human DFF40 or mouse CAD was co-expressed with DFF45 using the pRSFDuet1 vector (Novagen). Synthetic genes were engineered to optimize codon usage and efficiency of expression in *E. coli*. DFF heterodimers were activated by pre-incubation with hamster recombinant caspase-3 (in an approximate molar ratio of 2:1) for 15 min at 20°C, and then the caspase was inhibited by 10 μM DEVD. Micrococcal nuclease (MNase) and RNase T1 were purchased from Worthington, DNase I and RNase A were from Roche, RNase H and RNase One™ were from Promega.

Poly-L-glutamic acid (poly-Glu; mol. weight 100,000) was purchased from Miles-Yeda, ϕX174 single-stranded DNA was from New England Biolabs, poly(ADP-ribose) (PAR; mol. weight 15,000) was from Biomol, heparin from porcine intestinal mucosa and yeast tRNA was from Sigma. Total RNA was purified from human lymphoblastoid HL60 cells by single-step guanidine isothiocyanate-phenol-chloroform extraction using RNA-STAT60 reagent from Iso-Tex Diagnostics. 18S rRNA and 28S rRNA were purified by electroelution from agarose gels.

Extract preparation

Cytoplasmic and nuclear extracts were obtained from human lymphoblastoid HL60 cells. Cells washed in PBS buffer at 4°C were suspended in cold buffer consisting of 0.5% Nonidet P40 (NP40), 20 mM Tris-Cl (pH 7.5), 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.5 mM EGTA and 2.5% glycerol, and incubated for 15 min on ice with mild agitation. Cells were centrifuged for 5 min at $600 \times g$ to remove nuclei, and then the supernatant was centrifuged for 15 min at $14{,}000 \times g$ to obtain a cytoplasmic extract (CE). Pelleted nuclei were suspended in buffer consisting of 20 mM Tris-Cl (pH 7.5), 400 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA and 2.5% glycerol, incubated for 15 min on ice and then centrifuged for 15 min at $14{,}000 \times g$ to obtain a nuclear extract (NE). The purity of the CE and NE fractions was Western-analyzed using fraction-specific antibodies; NE was nearly free of cytochrome *c*, while CE contained only small amounts of PCNA. Cytoplasmic extracts (10 μg of proteins) were incubated with trypsin (0.2 μg) for 10 min at 20°C, and then trypsin was inhibited by adding Soybean trypsin inhibitor. Similar amounts of extracts were incubated with RNase A (0.1 μg) for 10 min at 20°C.

Nuclease assay

One μg of plasmid double-stranded DNA (4.2 kb in length) was incubated with caspase-activated DFF (0.5–1 nmol), MNase (0.1 unit) and DNase I (0.001 unit) for 30 min at 33°C, in a final volume of 20 μl. Ten μg of protein, either BSA, cytoplasmic or nuclear extracts and different amounts of inhibitors were mixed with nucleases before DNA was added. The Reaction Buffer consisted of (final concentration) 4 mM $MgCl_2$, 1 mM DTT, 10 mM KCl, 50 mM NaCl, 0.1% Triton X-100, 20 mM Tris-Cl (pH 7.5) and 0.5 mM EGTA (EGTA was replaced with 1 mM $CaCl_2$ when MNase or DNase I were tested). To terminate the nuclease reaction 1/4 volume of stop solution was added (1% SDS, 50 mM EDTA, and 6 mg/ml proteinase K) followed by incubation for 1 h at 50°C. After addition of gel loading dye buffer, DNA samples were run on 1.5% agarose (1×TAE) gels and stained with ethidium bromide. To remove RNA gels were soaked over night at 4°C with RNase A (0.1 μg/ml). To analyze the reaction constants plasmid DNA was linearized with restriction enzyme, P^{32}-end-labelled by polynucleotide kinase and diluted with non-radioactive plasmid DNA, then various amounts of DNA (0.5–10 μg) were used as a substrate for caspase-activated DFF in the presence of either 1 μg of RNA/poly-Glu or 20 ng of heparin. Reaction mixtures were separated electrophoretically on 4% polyacrylamide (0.5 × TBE) gels, gels were dried and radioactivity in lane segments was counted using a PhosphoImager. The relative number of DNA cleavage events, taken as the reaction initial velocity, was calculated from the fold-size reduction in the DNA substrate.

DNA binding assay

One hundred ng of P^{32}-end-labelled plasmid double-stranded DNA was incubated with 5 nmol of caspase-activated DFF in the presence of different amounts of RNA, poly-Glu or heparin for 10 min at 20°C in 20 μl of buffer consisting of 10 mM KCl, 50 mM NaCl, 0.1% Triton X-100, 20 mM Tris-Cl (pH 7.5), 1 mM DTT, 1 mM EDTA, 5% glycerol and 0.25 mg/ml BSA. Reaction mixtures were then separated electrophoretically on 0.9% agarose (0.5 × TBE) gels, gels were then dried and autoradiographed.

Results

RNA is the component in cytoplasmic extracts that inhibits the nuclease activity of DFF40/CAD

To search for new activators or inhibitors affecting the enzymatic activity of DFF40/CAD, we mixed caspase-3-activated recombinant DFF with cytoplasmic (CE) or nuclear (NE) extracts from normal human lymphoblastoid

HL60 cells along with a plasmid DNA substrate, and also carried out DFF minus controls (Fig. 1(A)). After brief incubation we assayed for the activity of DNA fragmentation by gel electrophoresis. As expected, the nuclear extract largely stimulated DNA cleavage by DFF (lane 6), presumably because of the presence of high-mobility group HMGB proteins and histone H1 previously shown to be activators of DFF40/CAD [3]. Significantly, the corresponding cytoplasmic extract conferred potent inhibition of DFF40/CAD activity (lane 5). To analyze the nature of the inhibitor(s), cytoplasmic extracts were pretreated with either trypsin or RNase A (Fig. 1(B)). The trypsin pre-treatment did not reduce the ability of the extract to inhibit DNA cleavage by caspase-activated DFF (compare lanes 8 & 9). In marked contrast however, RNase pre-treatment fully abolished the inhibitory effects and such extracts now stimulated the DNase activity of DFF (lane 10). The pre-treatment of cytoplasmic extracts with "non-specific" RNases (RNase A and RNase One^{TM}) was more efficient in destroying the inhibitor as compared to the treatment with the sequence-specific RNase T1, while RNase H, a nuclease specific for RNA:DNA hybrids, was ineffective in inactivating the

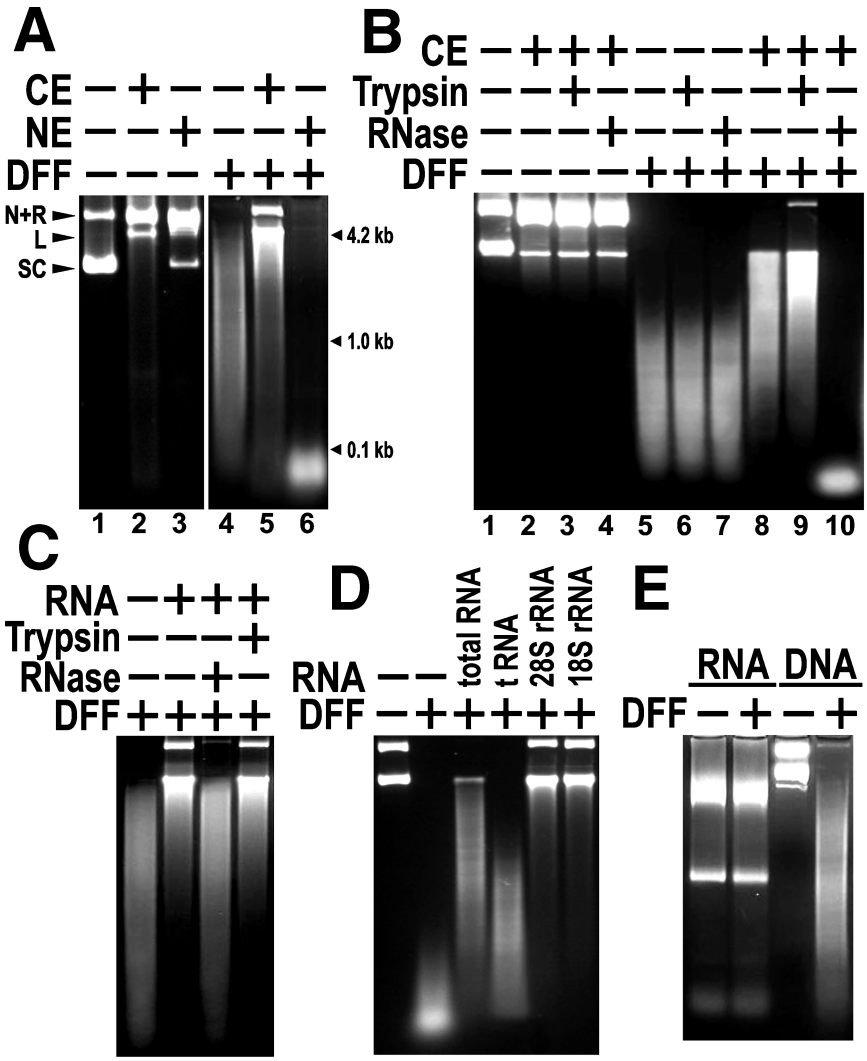

Fig. 1 RNA present in cytoplasmic extracts inhibits the DNase activity of DFF40/CAD. Plasmid DNA (1 μg) was incubated with caspase-3-activated recombinant DFF in the presence of either cytoplasmic (CE) or nuclear (NE) extracts from HL60 cells, and then reaction products were separated by agarose gel electrophoresis (A). Arrowheads denote the positions of different topological forms of the plasmid: *N*-nicked, *R*-relaxed, *L*-linear, SC-supercoiled. Control incubations in the absence of DFF revealed that extracts induced relaxation, nicking and/or linearization of the supercoiled plasmid DNA that may be attributed to the activity of DNA topoisomerases and endonucleases. Plasmid DNA was incubated with activated DFF in the presence of cytoplasmic extracts (B) or purified RNA (C), either untreated, pretreated with trypsin or pre-treated with RNase A. Plasmid DNA was incubated with activated DFF in the presence of total RNA, tRNA, 28S and 18S rRNA, 2 μg each (D). Either RNA or plasmid DNA was incubated with activated DFF (in 4 mM $MgCl_2$, 1 mM DTT, 50 mM NaCl, 20 mM Tris-Cl (pH 7.5) prepared using RNase-free water) (E)

DFF inhibitor(s) (data not shown). To verify whether RNA present in cytoplasmic extracts was indeed responsible for inhibition of DNA cleavage by caspase-activated DFF, RNA was purified from such extracts and tested at corresponding concentrations (∼1 mg of RNA could be isolated from the amount of extract corresponding to 10 mg of protein). In fact, purified RNA inhibited the DNase activity of caspase-activated DFF to a similar degree as complete extracts did, and pre-treatment of RNA with RNase A but not trypsin abolished its inhibitory potency (Fig. 1(C)). We also compared inhibitory potencies of different fractions of cellular RNA (tRNA, 18S rRNA and 28S rRNA) and found that ribosomal RNA was significantly more efficient than tRNA (Fig. 1(D)). We have previously shown that DFF40/CAD is a nuclease specific for double-stranded DNA (dsDNA) [13]. Here we confirmed that RNA isolated from the cytoplasmic extract was not cleaved by DFF40/CAD (Fig. 1(E)).

The inhibition of dsDNA cleavage by RNA and other poly-anions is specific for DFF40/CAD

In order to determine whether the inhibition of DNA cleavage by RNA was specific for DFF40/CAD, we examined the influence of RNA upon the cleavage of DNA catalyzed by two other DNases: micrococcal nuclease (MNase) and DNase I (Fig. 2(A)). The presence of RNA largely did not affect the activity of these nucleases. Strikingly, poly-L-glutamic acid (poly-Glu) and heparin also inhibited the DNase activity of DFF40/CAD but not that of MNase or DNase I (Fig. 2(A)). Interestingly, dextran sulfate and poly-L-aspartic acid, other poly-anions, also inhibited DFF40/CAD, while small anionic compounds like lysolecithin or ATP (used in excess of magnesium) did not (data not shown). The presence of single-stranded DNA, which is not a substrate of DFF40/CAD [13], also inhibited dsDNA cleavage by caspase-activated DFF (Fig. 2(B)).

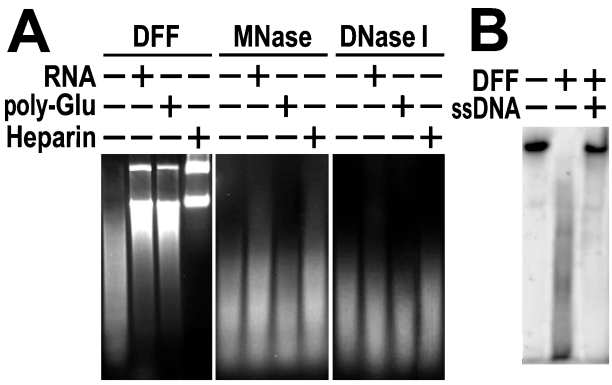

Fig. 2. RNA and other poly-anions inhibit dsDNA cleavage specifically by DFF40/CAD. Plasmid DNA (1 μg) was incubated with caspase-activated DFF, MNase or DNase I in the presence of 2 μg of RNA or poly-L-glutamic acid (poly-Glu), or 0.1 μg of heparin (A). One μg of radioactively-labeled dsDNA was incubated with activated DFF in the presence of 1 μg of ϕX174 ssDNA and reaction products were separated by 4% polyacrylamide gel electrophoresis (B)

RNA and poly-glutamic acid compete with DNA and prevents its binding by the enzyme

To examine further the nature of the DNA cleavage inhibition we determined the effects of RNA and poly-Glu on the K_m value and the velocity of the reaction. The K_m and V_{max} values were determined by plotting 1/velocity against 1/substrate concentration as shown in Fig. 3(A). The presence of 1 μg of RNA resulted in a ∼3-fold increase in the K_m value, without affecting significantly the V_{max} value. Essentially the same effects were observed for poly-Glu (data not shown). These results indicate an apparent reduction of the affinity of DFF40/CAD to bind to DNA and suggest a competitive mode of action of the inhibitor. The rate of DNA cleavage was proportionally reduced with increasing inhibitor/DNA ratios (Fig. 3(B)), confirming the competitive action of both RNA and poly-Glu upon DNA cleavage by DFF40/CAD.

The ability of RNA or poly-Glu to reduce binding of DFF40/CAD to DNA was examined using an electrophoretic mobility-shift assay. Radioactive dsDNA was incubated with a molar excess of caspase-activated DFF (in the absence of magnesium required for enzyme activity) in the presence of either RNA or poly-Glu, and then reaction mixtures were separated electrophoretically (Fig. 3(C)). In the absence of inhibitors radioactive DNA could be detected only in a form of retarded complexes with the nuclease. However, the mobility of complexes containing radioactive DNA was progressively increased in the presence of increasing concentrations of either RNA or poly-Glu, which reflects a reduction in the amount of DFF40/CAD molecules bound to DNA. In an excess of either RNA or poly-Glu (inhibitor/DNA w/w ratio ∼10), all DNA migrates with a mobility typical for free DNA, which indicates complete inhibition of DNA binding by the enzyme. Importantly, at a given concentration of RNA (or poly-Glu), the ability to disrupt interactions between DNA and the nuclease closely correspond to the potency of inhibition of DNA cleavage.

Heparin is a strong inhibitor of DFF40/CAD

Heparin, a negatively charged sulfated polysaccharide, specifically inhibits the DNase activity of DFF40/CAD (Fig. 2(A)). To examine further the nature of the DNA cleavage inhibition by this polysaccharide we also determined its effects on the K_m value and the velocity of the reaction (Fig. 4(A)). Significantly, the presence of 20 ng of heparin (that correspond to 50–100 nM concentration) resulted in a ∼10-fold increase in the K_m value and 2–3-fold reduction in the V_{max} value. Interestingly, at a slightly higher concentration (30 ng) heparin almost completely inhibited

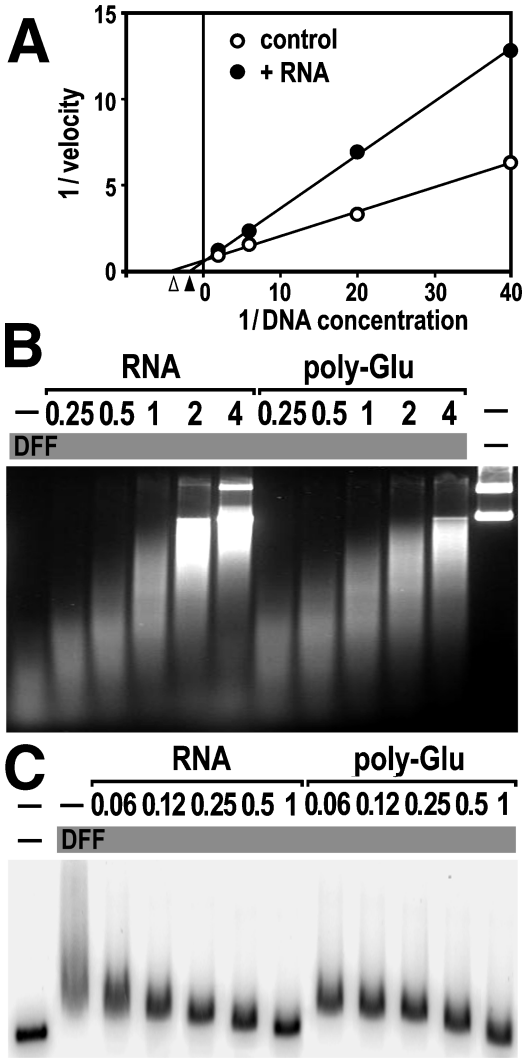

Fig. 3 Competitive mechanism of inhibition of DNA cleavage by RNA and poly-L-glutamic acid. Velocity of DNA cleavage by caspase-activated DFF (expressed in arbitrary units) determined as a function of DNA concentration (g/l) in the presence or absence of RNA (A). To obtain the K_m value 1/velocity was plotted against 1/DNA concentration. Arrowheads below the X-axis represent $-1/K_m$ values. The line obtained in the presence of poly-Glu essentially overlapped with one obtained with RNA and is not shown for simplicity. Plasmid DNA (1 μg) was incubated with activated DFF in the presence of increasing amounts of either RNA or poly-Glu (shown as μg) (B). Radio-labeled dsDNA (100 ng) was incubated with an excess of activated DFF in the presence of EDTA and increasing amounts (shown as μg) of either RNA or poly-Glu, and then DNA and protein-DNA complexes were separated by agarose gel electrophoresis (C)

DNA cleavage, while at a slightly lower concentration (3 ng) the enzyme was barely affected (Fig. 4(B)). The potency of heparin to disrupt interactions between the nuclease and DNA was also examined using the electrophoretic mobility-shift assay (Fig. 4(C)). The complete inhibition of DNA binding by the enzyme was observed in the presence of 100 ng of heparin, while lower concentrations of heparin caused gradual changes in the mobility of the complexes containing radioactive DNA. Most importantly, the nuclease-DNA complexes were detected in the presence of 10–30 ng of heparin, a concentration where DNA cleavage was largely inhibited. Thus, heparin is a strong inhibitor of DFF40/CAD activity even though the enzyme can still bind to DNA. Moreover, heparin almost fully inhibited DFF40/CAD at low concentrations where either RNA and poly-Glu (Fig. 3(B)) or poly(ADP-ribose) (PAR) (Fig. 4(D)), a previously reported inhibitor of the nuclease [14], were ineffective We conclude that mechanisms beyond competing with DNA for binding to the nuclease also exist to account for the inhibition of DFF40/CAD activity by heparin.

Discussion

It has been recently reported that PAR, an abundant cellular poly-anion, inhibits DNA cleavage by DFF40/CAD, although the enzyme is not poly(ADP-ribosyl)ated [14]. *In vitro*, purified PAR reduces binding of radioactive DNA to the nuclease, though at a lower level as compared to non-radioactive dsDNA [14]. It has been proposed that the putative PAR-binding domain overlaps with the DNA-binding domain of DFF40/CAD [14, 15]. Both domains map to α4 helices of the DFF40/CAD homodimer located in close proximity to the enzyme active site, and such helices have been proposed to interact with the DNA major groove [15]. The physiological significance of interactions between PAR and DFF40/CAD is not clear at the moment. However, one could propose that accumulation of PAR after the failure of a caspase-catalyzed inactivation of poly(ADP-ribosyl) polymerase-1 may eventually inhibit chromatin cleavage by DFF40/CAD and result in a necrotic-type cell death instead of apoptosis. Here we show that in addition to PAR other numerous poly-anions, including RNA, ssDNA, poly-glutamic acid and heparin interact with DFF40/CAD and disrupt DNA binding by the enzyme. These interactions are likely mediated by the positively charged surface formed by α4 helices of the DFF40/CAD homodimer that are involved in DNA binding. Data shown here suggest that the ability of PAR to inhibit the DNase activity of DFF40/CAD might simply reflect its anionic nature.

Some poly-anions, here exemplified by heparin, are indeed very powerful inhibitors of the nuclease, and certain cellular poly-anions are possibly involved in the regulation of DFF40/CAD activity *in vivo*. Internalized heparin is cytotoxic and it is generally thought to induce apoptosis by different mechanisms [16]. It is interesting to note however, that in several studies heparin has been reported to reduce apoptotic DNA fragmentation [17, 18]. RNA is the most abundant cellular polyanion, but no *in vivo* evidence for RNA-mediated regulation of DFF40/CAD is available yet. However, we can

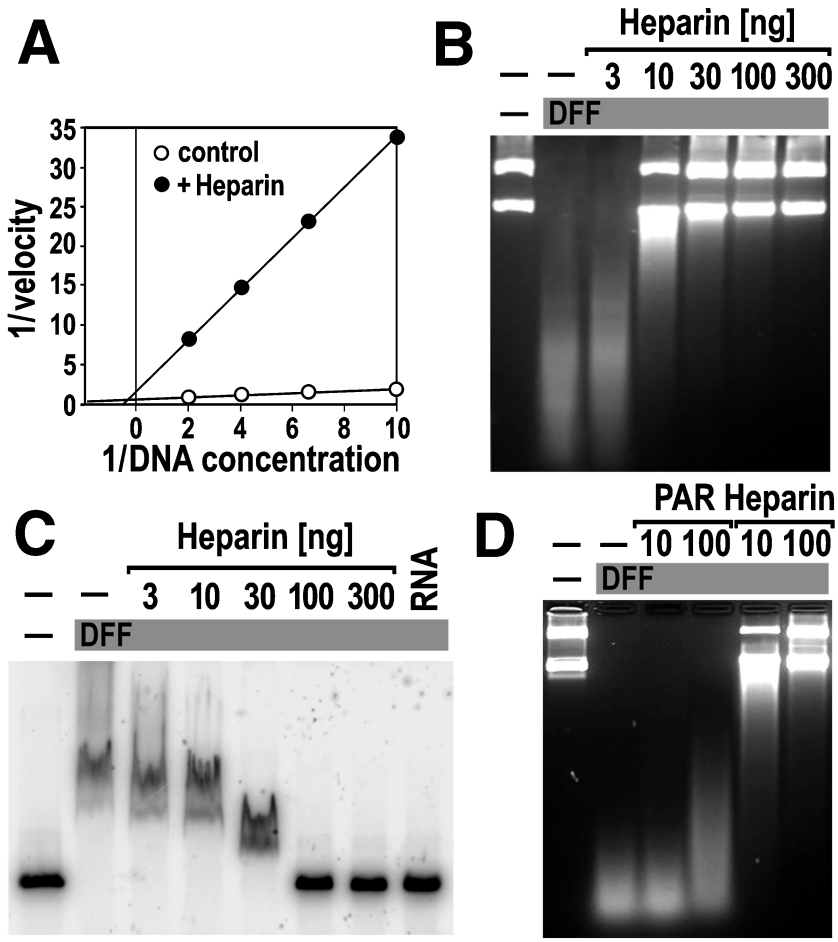

Fig. 4 Heparin is a potent inhibitor of DFF40/CAD. Velocity of DNA cleavage by caspase-activated DFF (expressed in arbitrary units) determined as a function of DNA concentration (g/l) in the presence of 20 ng of heparin (A). Plasmid DNA (1 μg) was incubated with activated DFF in the presence of increasing amounts of heparin (B). Radio-labeled dsDNA (100 ng) was incubated with an excess of activated DFF in the presence of EDTA and different amounts of heparin (or 2 μg of RNA), and then DNA and protein-DNA complexes were separated by agarose gel electrophoresis (C). Plasmid DNA was incubated with activated DFF in the presence of 10 and 100 ng of either PAR or heparin (D)

speculate on the physiological role of RNA in the regulation of DFF40/CAD. RNA might repress this DNase to avoid premature apoptotic DNA breakdown that would inhibit the induction of genes encoding pro-apoptotic factors. Also, if DNA breakdown needs to be co-ordinated with RNA degradation, then DFF activity would be kept in check until RNA levels fall below an inhibitory threshold. Endonuclease G, as we have proposed [19], may be an apoptotic RNase that functions to co-ordinate the proper timing of DNA and RNA breakdown.

Conclusion

Taken together, our results show that (i) RNA and other cellular polyanions inhibit DNA cleavage by the apoptotic DNase DFF40/CAD and (ii) such non-protein inhibitors compete with dsDNA and prevent its binding by the enzyme. Although the physiological significance of RNA in the regulation of DFF40/CAD remains unclear, our finding suggests a role for apoptotic RNase(s) to co-ordinate the proper timing of DNA and RNA breakdown during apoptosis.

Acknowledgments This work was supported in part by Grant KBN 3P05A10424 from the Polish Ministry of Education and Science (to P.W.), and Grants GM59809 and GM29935 from the National Institutes of Health and Grant I-0823 from the Robert A. Welch Foundation (to W.T.G.)

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