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LDFF, the large molecular weight DNA fragmentation factor, is responsible for the large molecular weight DNA degradation during apoptosis in *Xenopus* egg extracts

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ABSTRACT

DNA degradation is a biochemical hallmark in apoptosis. It has been demonstrated in many cell types that there are two stages of DNA fragmentation during the apoptotic execution. In the early stage, chromatin DNA is cut into large molecular weight DNA fragments, although the responsible nuclease(s) has not been recognized. In the late stage, the chromatin DNA is cleaved further into short oligonucleosomal fragments by a well-characterized nuclease in apoptosis, the caspase-activated DNase (CAD/DFF40). In this study, we demonstrate that large molecular weight DNA fragmentation also occurs in *Xenopus* egg extracts in apoptosis. We show that the large molecular weight DNA fragmentation factor (LDFF) is not the *Xenopus* CAD homolog XCAD. LDFF is activated by caspase-3. The large molecular weight DNA fragmentation activity of LDFF is Mg^{2+}-dependent and Ca^{2+}-independent, can occur in both acidic and neutral pH conditions and can tolerate 45°C treatment. These results indicate that LDFF in *Xenopus* egg extracts might be a new DNase (or DNases) responsible for the large DNA fragmentation.

Keywords: apoptosis, caspase-activated DNase (CAD), large molecular weight DNA fragmentation factor (LDFF).

INTRODUCTION

Apoptosis, a morphologically distinct form of programmed cell death, has attracted considerable attention for its implicated roles in embryonic development, tissue homeostasis and protection against diseases. A cell in apoptosis undergoes cell shrinkage, cytoskeleton collapse, nuclear envelope disassembly, chromatin condensation into apoptotic bodies, DNA fragmentation, protein degradation, and so on. DNA fragmentation is considered to be the biochemical hallmark for this suicidal process. In most apoptotic cell types, there are two stages of DNA degradation. In the early stage, chromatin DNA is cleaved into large molecular weight chromatin DNA fragments. In the late stage, the fragmented chromatin DNA is digested further into short oligonucleosomal DNA fragments by the nucleases[1, 2]; however, in some cell types only large molecular weight fragments were observed[3, 4]. So far, more than twenty nucleases have been identified and implicated as being responsible for the late stage DNA degradation, such as DFF40/CAD/CPAN[5-7], DNase I[8], inducible-lymphocyte Ca^{2+}/Mg^{2+}-dependent endonuclease [9], cyclophilins[10], NUC-1[11], endonuclease G[12], DNA topoisomerase IIα and so on[13]. Among these implicated nucleases, DFF40 is one of the well-characterized. DFF40 was first identified as one subunit of DNA fragmentation factor in the cytoplasm of Hela cells[14], and subsequently proved to be the homolog of caspase-activated DNase (CAD) in mouse[6]. In non-apoptotic cells, DFF 40 was in complex with the inhibitory DNA fragmentation factor DFF45/ICAD. Besides its inhibitory function on CAD nuclease activity, DFF45/ICAD also acts as a CAD/DFF40-specific folding chaperone, which is essential for apoptosis[15]. During apoptosis, ICAD/DFF45 is cleaved by activated caspase-3, releasing CAD/DFF40 to digest chromosome DNA into oligonucleosomal fragments [5, 6, 16]. CAD and ICAD exist in the cells of a variety of species. In *Xenopus* egg extracts, we have identified the CAD and ICAD homologs and re-named them as XCAD and XICAD (previously termed as XAD and IXAD respectively) [17, 18]. The XCAD/XICAD complex performs the same acti-vation mechanism as the CAD/ICAD complex does in DNA fragmentation in apoptosis in *Xenopus* egg extract. Furthermore, human ICAD can inhibit XCAD activity and *Xenopus* XICAD can inhibit human CAD[17-19].

Another well-defined DNase for the late stage oligonucleosomal DNA digestion is endonuclease G[10, 20].

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Endonuclease G is normally situated in mitochondria in non-apoptotic cells and relocates into the nucleus during apoptosis, where it digests the chromatin DNA into oligonucleosomal fragments[21, 22].

Unlike that of the late stage apoptotic DNA fragmentation, the mechanism for the early stage DNA fragmentation largely remains to be elucidated, although some reports indicated that CAD also plays a role in large molecular weight DNA fragmentation[23]. Recent experiments with CAD-depleted sub-lines of chicken DT40 cells showed that the large molecular weight DNA fragmentation activity was not seriously affected by the CAD depletion, which suggested that CAD is not the responsible DNase for the early stage chromatin DNA fragmentation[24].

In this study, using exogenous cytochrome c to induce apoptosis in the *Xenopus* egg extract containing mouse liver nuclei, we found that a two-stage DNA fragmentation also existed in this cell-free system. During the apoptotic process, exogenous chromatin DNA was firstly digested into large molecular weight DNA fragments by an unknown large molecular weight DNA fragmentation factor (LDFF). The large molecular weight DNA fragments were then digested into short oligonucleosomal DNA fragments by XCAD. We partially characterized the LDFF and showed that LDFF is Mg^{2+}-dependent and Ca^{2+}-independent. LDFF remains active in an acidic optimal pH condition and could tolerate up to 45°C heat treatments.

MATERIALS AND METHODS

Preparations of mouse liver nuclei and *Xenopus* egg extract S-150

Mouse liver nuclei and crude egg extract were prepared as described[18, 25]. The high speed supernatant (termed extract S-150) was prepared by centrifuging the crude egg extract at 150,000 g for 2 h (HITACHI 55P-72, RPS 50-II rotor). Prior to centrifugation, separate additions of aprotinin and leupeptin (Sigma) were added to give final concentrations of 6 μg/ml and 8 μg/ml, respectively. After being stored in liquid nitrogen, frozen extract S-150 can be induced into apoptosis by addition of 1 μM cytochrome c after being thawed rapidly[17, 18].

Apoptotic chromatin DNA fragmentation assay

The final concentration of 1 μM cytochrome c (from horse heart, Sigma) was added into 50 μl extract S-150 with 10^6 mouse liver nuclei. The extract was incubated at 23 °C for 2 h, then mixed with 450 μl buffer D (0.1 M Tris CHCl, pH 8.5, 5 mM EDTA, 0.2 M NaCl, 0.2% w/v SDS, and 0.2 mg/ml protease K) and incubated at 37°C for another 3 h or overnight. NaCl was added to a final concentration of 1.5 M, centrifuged at 10,000 g for 20 min at room temperature and the resultant pellet was discarded. The chromatin DNA in the suspension was precipitated with equal volume of 100% ethanol, and then centrifuged at 14,000 g for 5 min. After washing once with 70% ethanol, the DNA pellet was dissolved at 37°C for 2 h in 20 μl TE containing 200 μg/ml RNase A. For nucleosomal fragments detection, 10 μl DNA was loaded onto a 1.5% agarose gel and subjected to electrophoresis. For large molecular weight DNA fragments detection, 10 μl DNA was loaded onto 0.4% agarose gel and subjected to electrophoresis in 0.5×TBE buffer for 8 h at 4°C.

Prokaryotic expression and purification of human DFF40 and DFF45, and *in vitro* nuclease activity assay of recombinant DFF40

The coding region of CAD/DFF40 and ICAD/DFF45 were amplified from the total RNA of Hela cells by RT-PCR, then were inserted into pET28a (+) (Invitrogen) respectively. For protein expression, the *E. coli* BL21 (pLys) were transfected with pET28a (+)-CAD/DFF40 or pET28a(+)-ICAD/DFF45. Protein expression was induced by adding IPTG into the medium to a final concentration of 1 mM and allow the bacteria to grow for additional 4 h at 30°C. The cells were then collected for protein purification.

ICAD/DFF45 was expressed as a soluble fraction and was purified by His6×Talon-spin column (Clontech) according to the manual provided. Proteins were desalted using a G-25 column and then concentrated to 2 mg/ml by 10K cut-off centrifuges (Nanosap). Glycerol was added to a final concentration of 50% and the proteins were stored in aliquots at -20°C.

DFF40 was expressed almost exclusively in insoluble bodies. After sonication, the suspension was centrifuged at 12,000 g for 15 min. The pellets were washed twice with wash buffer (50 mM Sodium Phosphate, pH 7.0, 300 mM NaCl, 1% Triton X-100), and dissolved in denaturation buffer (10 mM HEPES-KOH, pH 7.2, 50 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 10 mM DTT, 20% glycerol, 6 M guanine-HCl). After incubation at 30°C for 1 h to completely denature the proteins, the solutions were diluted at 1:100 with renaturation buffer (10 mM HEPES-KOH, pH 7.2, 500 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 100 mM DTT, 20% glycerol, 50 μg/ml DFF45) and incubated for additional 2 h. A 2 μl-aliquot of the reaction was incubated with 0.1 μg caspase-3 and 10^6 mouse liver nuclei in a final volume of 50 μl adjusted with nuclease buffer (10 mM HEPES-KOH, pH 7.2, 50 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 20% glycerol, 0.1 mM PMSF, 1 mg/ml BSA) at 37°C for 4 h. The DNA was then extracted for nucleosomal and large molecular weight fragments detection.

RESULTS

Two-stage chromatin DNA degradation in the cell-free apoptotic system

It has been previously reported that cytochrome c could induce apoptosis in *Xenopus* egg extract[17, 18, 26]. To study the chromatin DNA degradation, we established the cell-free apoptotic system using egg extract and mouse liver nuclei with the induction of cytochrome c. After adding cytochrome c and mouse liver nuclei to the extract for a while, the nuclei soon underwent apoptosis with chromatin condensation, margination, and nuclear fragmentation, which was observed by fluorescence microscopy (Fig 1B). To determine the chromatin DNA fragmentation, we extracted the DNA from the fragmented nuclei at various time points and separated the DNA on 1.5% agarose gel.

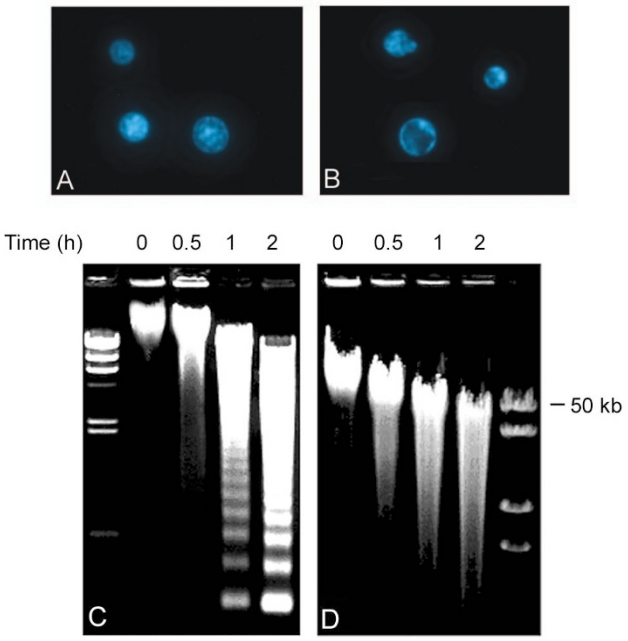

Fig 1. Two-stage chromosome DNA degradation exists in apoptosis of egg extracts. Egg extract S-150 containing mouse liver nuclei was induced to apoptosis by adding cytochrome c to a final concentration of 1 μM. After incubation at 23ºC for 2 h, the nuclei were stained with DAPI, and then observed by fluorescence microscopy. (**A**) Control; (**B**) Induction with cytochrome c. At the time indicated, the chromatin DNA was extracted and separated on 1.2% agarose for detecting the oligonucleosomal DNA (**C**) or 0.4% agarose for detecting the large molecular weight DNA fragments (**D**).

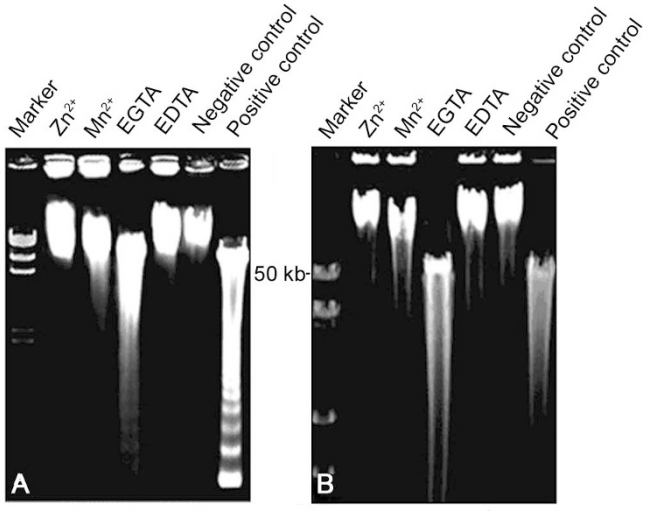

Fig 2. The sensitivities of XCAD and LDFF to diverse ions. Egg extract S-150 was induced to apoptosis by adding cytochrome c to a final concentration of 1 μM. After induction for 2 h, 10^5 mouse liver nuclei were added to apoptotic extract with a final concentration of 5 mM Zn^{2+}, 5 mM Mn^{2+}, 5 mM EGTA or 5 mM EDTA, respectively. After incubation at 23ºC for additional 2 h, total chromatin DNA was extracted and separated on 1.5% agarose gel for detection of the oligonucleosomal fragmentation (**A**) and 0.4% agarose gel for the large molecular weight DNA fragmentation (**B**).

It showed clearly that apoptotic nucleosomal DNA ladders appeared after 0.5 h of incubation. To see if chromatin DNA was degraded into large molecular weight fragments, we separated the same batch of DNA on 0.4% agarose gel. We found that chromatin DNA was also degraded into 50 kb fragments progressively (Fig 1C and D) and that the large molecular weight DNA fragmentation took place prior to nucleosomal fragmentation (Fig 1C and D, 0.5 h lane). These results suggest that in the egg extract, apoptosis also occurs as a two-stage DNA fragmentation process, similar to what was found in somatic cells.

Biochemical features of the large molecular weight DNA fragmentation factor (LDFF)

Since we observed large molecular weight DNA fragmentation, we presumed that there must be at least one factor responsible for this DNA fragmentation activity. We called this factor the large molecular weight DNA fragmentation factor (LDFF). Previously we had identified a 27 KD DNase in egg extract, termed XCAD, which was activated during apoptosis. XCAD could digest chromosome DNA into nucleosomal fragments[17, 18]. To identify if LDFF was XCAD, we compared the biochemical features of LDFF to those of XCAD.

First, we tested the sensitivities of LDFF and XCAD to diverse ions. We observed that EDTA, Zn^{2+} or Mn^{2+} could inhibit not only oligonucleosomal DNA fragmentation as reported[18] but also the large molecular weight DNA fragmentation activity (Fig 2A and B). However, EGTA could only extensively inhibit XCAD activity for oligonucleosomal fragmentation, but not LDFF for the large molecular weight DNA fragmentation (Fig 2A and B), indicating XCAD was a Ca^{2+}, Mg^{2+}-dependent DNase, and LDFF was Mg^{2+}-dependent and Ca^{2+}-independent.

Secondly, the optimal pH for XCAD and LDFF activities in apoptotic DNA fragmentation were examined. We found that the optimal pH for XCAD for oligonucleosomal fragmentation were 7.4 and 8.0, while the optimal pH for LDFF for large molecular weight DNA fragmentation were 5.0, 7.4 and 8.0 (Fig 3A, B). These results indicated that LDFF, but not XCAD, could keep active under acidic condition.

Finally, the sensibility of LDFF and XCAD to the temperatures was also tested. The results showed that the optimal temperature for both XCAD and LDFF was 23ºC (Fig 4A, B), a good temperature for *Xenopus laevis*.

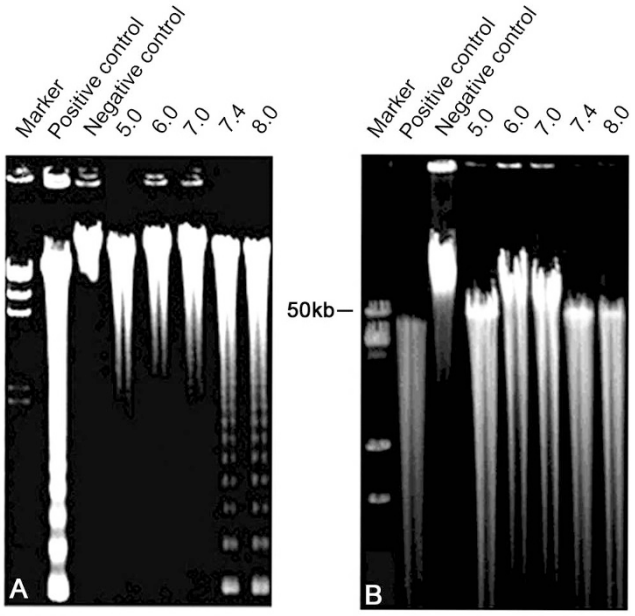

Fig 3. The sensitivities of XCAD and LDFF to different pH. The extract S-150 was induced to apoptosis by 1 μM final concentration of cytochrome c. After induction for 2 h, the apoptotic extract were mixed with 100 mM citrate-sodium citrate buffer in different pH as indicated, and 10^5/ml mouse liver nuclei were added, then the mixture was incubated for 2 h. The total chromatin DNA was extracted and separated on 1.5% agarose gel for the detection of oligonucleosomal fragmentation (**A**) and 0.4% gel for the large molecular weight DNA fragmentation (**B**).

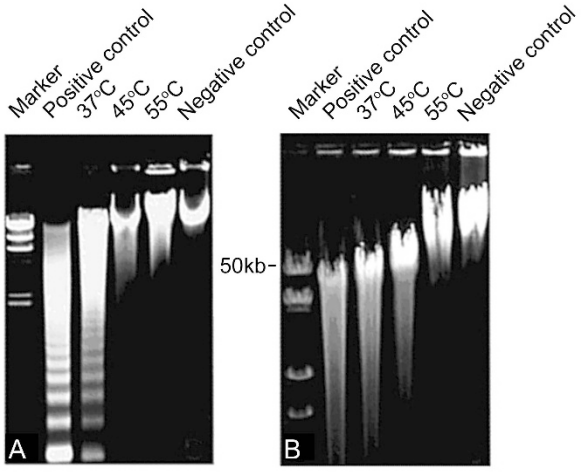

Fig 4. The sensitivities of XCAD and LDFF to diverse temperatures. The extract S-150 was induced to apoptosis by 1 μM final concentration of cytochrome c. After induction for 2 h, the apoptotic extract was treated at varies temperatures for 10 min and centrifuged at 14,000 rpm for 10 min.Then the mouse liver nuclei were added to the supernatant, and after incubated at 23°C for 2 h, the total chromatin DNA was extracted and separated on 1.5% agarose gel for the detection of oligonucleosomal fragmentation (**A**) and 0.4% gel for the large molecular weight DNA fragmentation (**B**).

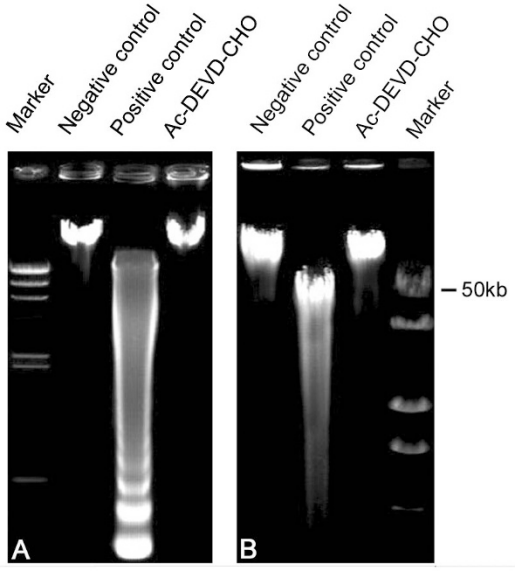

Fig 5. LDFF was activated by caspase-3 during apoptosis. 1 μM final concentration of caspase-3 inhibitor, Ac-DEVD-CHO, together with 1 μM cytochrome c and 10^5 mouse liver nuclei were adde to the egg extract S-150. After incubation at 23 °C for 2 h, DNA was extracted and separated on 1.5% agrose gel to examine the nucleosomal fragmentation (**A**) and on 0.5% agarose gel to examine large DNA fragmentation (**B**). Extract without cytochrome c was used as the negative control.

However, it was surprising that LDFF could tolerate up to 45°C heat treatment for 10 min (Fig 4A, B).

From the above biochemical analysis, we concluded that LDFF was not XCAD, but a different DNase (s).

LDFF was activated by caspase-3.

Caspase-3 plays a key role in apoptosis. During apoptosis, active caspase-3 cleaves ICAD/DFF45, releasing CAD/DFF40 to digest chromatin DNA into olignucleosomal fragments. To test if LDFF is also activated by caspase-3, we added caspase-3 inhibitor, Ac-DEVD-CHO, to the egg extract S-150 together with cytochrome c and mouse liver nuclei. We found that both the large molecular weight and the oligonucleosomal DNA fragmentation activities were inhibited (Fig 5A and B), suggesting LDFF was also activated by caspase-3.

Effects of DFF40/CAD and DFF45/ICAD in the large molecular weight DNA fragmentation

What kind of roles do DFF40/CAD and DFF45/ICAD play in large molecular weight DNA fragmentation is still under controversial. Some reports showed that DFF40/CAD is also responsible for large molecular weight DNA fragmentation[23], while some other reports indicated it does

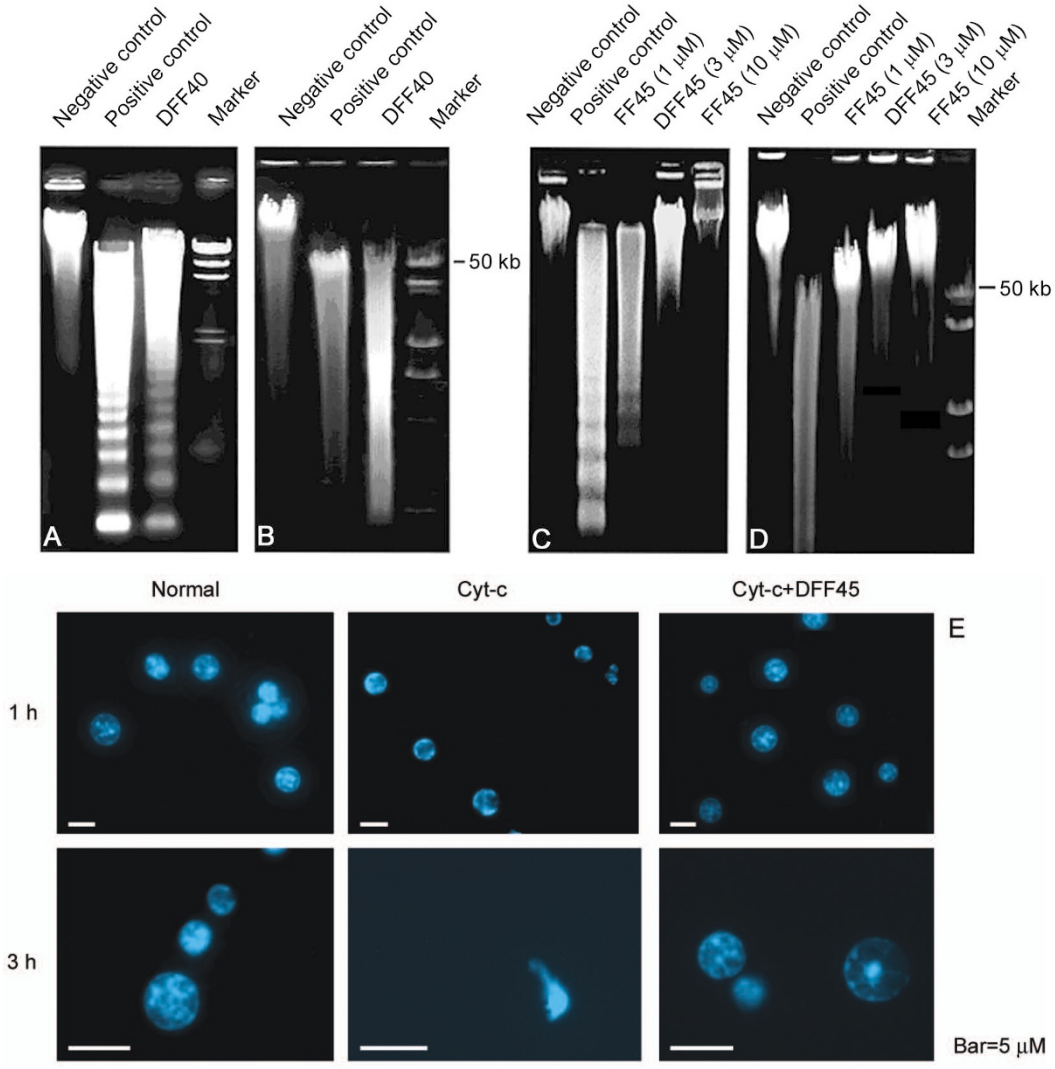

Fig 6. Effect of CAD/DFF40 and ICAD/DFF45 on the large molecular weight DNA fragmentation. (**A**) and (**B**): 1 μM renatured human CAD/DFF40 was added to 10^5/μl mouse liver nuclei with 100 ng/ml caspase-3 and incubated at 37°C for 4 h. Total chromatin DNA was extracted and separated on 1.5% agarose gel for oligonucleosomal (**A**) and 0.4% agarose gel for large molecular weight DNA fragmentation detection (**B**). (**C**, **D**) and (**E**): Purified human CAD/DFF45 was added to the egg extract S-150 with the absence of 1 μM cytochrome c and incubated at 23°C for 2 h. Total DNA was extracted and separated on 1.5% agarose gel for oligonucleosomal (**C**) and large molecular weight DNA fragmentation detection (**D**). Also, the nuclei were stained with DAPI at the indicated time, and observed by fluorescence microscopy (**E**).

not[24]. To confirm whether DFF40/CAD was responsible for the large molecular weight DNA fragmentation, we incubated the purified active human DFF40 with mouse liver nuclei. We found that with DFF40 the chromatin DNA could only be degraded into oligonucleosomal ladders, but not the large molecular weight DNA fragments (Fig 6A, B). This observation confirmed that CAD/DFF40 does not have the large molecular weight DNA fragmentation activity.

We also tested the effect of ICAD/DFF45 on the large molecular weight DNA fragmentation. We added the purified bacteria-expressed human ICAD/DFF45 to the extract S-150 with cytochrome c and mouse liver nuclei and found that human ICAD/DFF45 can only inhibit XCAD activity for the oligonucleosomal DNA fragmentation (Fig 6C) but not LDFF activity for the large molecular weight DNA fragmentation (Fig 6D). At the same time, it was found that DFF45 could only delay, but not inhibit, the occurrence of chromatin condensation and margination (Fig 6E).

These findings suggested that DFF40/CAD and DFF45/ICAD were not the critical factors for the large molecular weight DNA fragmentation.

DISCUSSION

In this paper we reported that there exists a two-stage

DNA fragmentation processe during apoptosis in the cell free extract from *Xenopus* eggs, which is similar to what has been reported in somatic cells[1-4]. In the first stage the chromatin DNA is cut into large molecular weight fragment (about 50 kb) and in the second stage into oligonucleosomal DNA ladders. However, there is an over-lap within the two different stages (Fig 1). We also confirmed that there are at least two different kinds of nucleases responsible for the DNA fragmentations: XCAD, a homolog of CAD, and LDFF. XCAD, which was identified previously[17, 18], digests the chromatin DNA into oligonucleosomal ladders, while LDFF will cut the chromatin DNA into the large molecular weight fragments. We have partially characterized LDFF with biochemical assays although we have not identified it.

It was further proved that LDFF was clearly not XCAD, but maybe a totally different kind of DNase(s). CAD/DFF40, the human homolog of XCAD, can substitute for XCAD in digesting chromatin DNA into oligonucleosomal DNA pieces; while it could not induce large molecular weight DNA fragmentation (Fig 6B). ICAD/DFF45, the specific inhibitor of CAD, which could also inhibit XCAD specifically[17-19], could not inhibit LDFF activity for the large molecular weight DNA fragmentation (Fig 6D). LDFF was sensitive to Mg^{2+}, but not to Ca^{2+} (Fig 2B), whereas XCAD was both Mg^{2+}- and Ca^{2+}-dependent (Fig 2A). LDFF could also resist up to 45ºC treatment and low pH down to 5.0 (Fig 4 and 5). These results showed LDFF and XCAD functioned differently in large molecular weight and nucleosomal DNA fragmentation.

According to the biochemical features, the best candidate for LDFF might be DNase II, an acid lysosomal nuclease. During apoptosis, DNase II relocates to nucleus[27] and like LDFF, DNase II was also Ca^{2+}-independent (Fig 3A) [27]. It was found that overexpression of DNase II could induce apoptosis in Chinese hamster ovary cells and this process could not be blocked by the caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-(O-methyl)-fluoromethylketone[28]. These results suggest that DNase II functions downstream of caspase, which is in accordance with our result that LDFF was activated by caspase-3 (Fig 3B). In *C. elegans*, NUC-1, the DNase II homolog, plays an important role in complete DNA digestion during apoptosis. Mutation of NUC-1 could lead to DNA degradation slow-down[29]. All these suggested that DNase II might get involved in the process of apoptotic DNA fragmentation, and that DNase II is very similar to LDFF identified in our study.

In conclusion, there are at least two kinds of DNases in egg extract, XCAD and LDFF, responsible for the two-stage chromatin DNA fragmentation in apoptosis. XCAD is the homolog of CAD/DFF40 and is responsible for nucleosomal DNA molecular weight chromatin fragmentation. But LDFF cuts chromatin DNA into large pieces. Although the activity of LDFF in digesting the chromatin DNA was partially characterized in this work, a lot remains to be done such as the cloning of LDFF gene.

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