Isolation and partial characterization of a protease involved in Fas-induced apoptosis

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Abstract Protease involvement has been implicated in the signalling process of activation-induced apoptosis. Here we report the isolation of a protease from Jurkat T cells undergoing Fas-induced apoptosis. Although the protease probably is a serine protease, it seems to be distantly related to members of the ICE/ced-3/Ich-1(nedd-2) family. In a cell-free system using isolated thymocyte nuclei, the protease rapidly induces DNA fragmentation and morphological changes typically seen in apoptosis. Our results clearly show protease activation downstream to Fas-ligation and implicate an important role for the isolated protease in signalling of Fas-induced apoptosis.

Key words: Apoptosis; Fas/APO-1; Cell-free system; Protease inhibitor; DNA fragmentation; Interleukin 1-β-converting enzyme

1. Introduction

Fas (APO-1/CD95) is a member of the tumor necrosis/nerve growth factor receptor superfamily and mediates apoptosis in various cell types that express the Fas receptor. Mice carrying mutations in the Fas ligand (*gld* mice) or the Fas receptor (*lpr* mice) display a nonfunctional Fas ligand/Fas receptor pair and suffer from autoimmune diseases. The effects of these loss-of-function mutations indicate that the Fas-system plays an important role in the processes involved in T cell development (see [1] for recent review).

It has recently been shown that T cell receptor stimulation induces the expression of both Fas and its ligand on T cells, and that activation-induced apoptosis can be inhibited by blocking either the receptor or the ligand [2–4]. Despite the rapidly growing interest in Fas-induced apoptosis, very little is known about the molecular mechanism involved in its induction of apoptosis.

We have shown that several protease inhibitors can block Fas-induced apoptosis in Jurkat T cells (Weis, M., Schlegel, J., Kass, G.E.N., Holmstöm, T.H., Peters, I., Eriksson, J., Orrenius, S. and Chow, S.E. (1995) manuscript submitted). The involvement of proteases has also been implicated in the induction of apoptosis in other systems [5–11]. In a cell-free system adapted from [12] using extracts from Jurkat T cells undergoing Fas-induced apoptosis, a proteolytic activity inducing DNA fragmentation and morphological changes typical of apoptosis was detected (Chow, S.C., Weis, M., Kass, G.E.N., Holmström, T.H., Eriksson, J. and Orrenius, S. (1995) Manuscript submitted). We now describe the partial purification and characterization of this protease. Inhibitor studies with a variety of protease inhibitors reveal that the protease is probably a serine protease with some similarities to members of the ICE/ced-3/Ich-1(nedd-2) protease family. The importance of these findings in signalling of Fas-induced apoptosis is discussed.

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Abbreviations: CSOM, confocal optical scanning microscopy; DCI, 3,4-dichloroisocoumarin; DMSO, dimethylsulfoxide; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol-*O*,*O*′-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FIGE, field inversion gel electrophoresis; HMW, high molecular weight; ICE, Interleukin 1-β-converting enzyme; TLCK, *N*-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; PSI, Cbz-Ile-Glu-(Ot-Bu)-Ala-leucinal; VAD-FK, Val-Ala-Asn-fluoromethyl ketone.

2. Materials and methods

2.1. Reagents

Anti-human Fas antibody (IgM, clone CH-11) was purchased from AMS Biotechnology, Sweden. PSI was a gift from S. Wilk, Mount Sinai School of Medicine, New York, USA. DCI was obtained from Calbiochem, Pefabloc from Boehringer-Mannheim, TLCK and TPCK from SIGMA, and VAD-FK from Enzyme Systems (Dublin, USA). All other chemicals were of research grade and obtained from local sources.

2.2. Cell culture

The human leukemic T cell line, Jurkat, was obtained from ATCC (Maryland, USA) and grown according to ATCC guidelines.

2.3. Isolation of the Jurkat protease involved in Fas-induced apoptosis

Jurkat cells (15×10^6 cells/ml) were incubated at 37°C with anti-Fas antibody (750 ng/ml) in medium. After 75 to 90 min, when at least 60% of the cells showed typical apoptotic features as assessed by light microscopy, the cells were centrifuged, washed once with ice-cold medium and resuspended in S-buffer consisting of 50 mM NaCl, 2 mM $MgCl_2$, 40 mM β-glycerophosphate, 5 mM EGTA, 10 mM HEPES, pH 7.0 (500 μl per 300×10^6 cells). After four cycles of freezing and thawing, the cell lysates were centrifuged for 15 min at $20{,}000 \times g$ to pellet the membranous fraction. The supernatant was then centrifuged a further 30 min at $120{,}000 \times g$ to obtain a supernatant fraction consisting mainly of cytosolic proteins.

For the isolation of the Jurkat protease a three-step chromatographical procedure was used. In brief, a gel filtration step on a SUPEROSE 12 HR 10/30 FPLC column (Pharmacia) to remove protein aggregates and low molecular weight contaminants was followed by an affinity chromatography step on a 5 ml HiTrap Blue column (Pharmacia), eluting bound proteins with a salt gradient from 0.05 to 1 M NaCl. The final isolation was on an anion exchange column (Mono Q HR 5/5, Pharmacia) with the protease activity eluting at 150 mM NaCl.

2.4. Isolation of nuclei and incubation conditions

Rat thymocyte nuclei were prepared as described [13] with two minor modifications: (i) before cell homogenization, the nuclei buffer was supplemented with 0.02% NP-40 and (ii) DTT and PMSF were not added. Using a recently published in vitro system [12] with a minor modification (no DTT present), isolated nuclei were incubated at 37°C with the different fractions containing the Jurkat protease in S-buffer. The total assay volume was normally 60 μl, consisting of 20–40 μl of the fraction containing the Jurkat protease (dialysed against S-buffer), $15–20 \times 10^6$ nuclei, and an ATP regenerating system [12]. After 60 to 80 min the reaction was stopped and the samples divided for analysis by CSOM, FIGE and conventional agarose electrophoresis (see below).

2.5. Analysis of oligonucleosomal and HMW DNA fragments

Analysis of oligonucleosomal DNA fragments was done with a

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10 μl aliquot of the terminated incubation using an agarose gel electrophoresis method described by Sorenson et al. [14]. After the run, the gel was stained with ethidium bromide, visualized using a 305 nm UV light source, and photographed using Polaroid 665 film. Boehringer Mannheim DNA Marker VI was used as a marker. The formation of HMW DNA fragments was analyzed using a 30 μl aliquot of the terminated incubation by FIGE as described in [15], with minor modifications: (i) instead of the whole agarose plugs, 1 mm slices of the plugs were used, and (ii) a vertical gel chamber was used in combination with a switcher program adapted for vertical gels. DNA was stained and visualized as described above. DNA marker for FIGE (0.1–200 kbp) was purchased from Sigma.

2.6. Assessment of nuclear morphology by CSOM

To monitor changes in nuclear morphology, 10 μl aliquots of the terminated incubation were incubated with propidium iodide (100 μg/ml) on ice for 2 min before viewing using a Bio-Rad MRC-600 laser confocal microscope with BHS filter (Bio-Rad).

3. Results

3.1. The supernatant of the cell lysate of anti-Fas treated Jurkat cells contains an activity leading to apoptotic changes in isolated nuclei

CSOM analysis showed that incubation of isolated rat thymocyte nuclei with the supernatant of the cell lysate prepared from anti-Fas treated Jurkat cells (Fas-extract) led to changes in nuclear morphology typically seen in apoptosis [16] (compare Fig. 1A,B). Incubation of the nuclei with supernatant prepared from untreated Jurkat cells did not cause these changes, thus indicating that the activity promoting the formation of apoptotic nuclei was only present in anti-Fas treated cells. Analysis of the DNA by FIGE (Fig. 1C) and conventional agarose gel electrophoresis (Fig. 1D) showed the formation of HMW and oligonucleosomal DNA fragments in the nuclei incubated with Fas-extract (Fig. 1C,D, lane 3). Neither incubation of the nuclei alone (Fig. 1C,D, lane 1), nor incubation of the nuclei in the presence of extract prepared from untreated Jurkat cells (Fig. 1C,D, lane 2) led to DNA fragmentation. Furthermore, anti-Fas antibody did not cause apoptotic changes when incubated directly with thymocyte nuclei (not shown). Incubation of nuclei from untreated Jurkat cells with Fas-extract led to similar changes in nuclear morphology and chromatin structure as seen with thymocyte nuclei (not shown).

3.2. The activity causing apoptotic changes in isolated thymocyte nuclei is a protease

In order to characterize the activity causing apoptotic nuclei, we have attempted to purify the supernatant obtained from the cell lysate of anti-Fas treated Jurkat cells (Fas-extract). Gel filtration chromatography of the Fas-extract on a SUPEROSE 12 HR 10/30 FPLC column calibrated with the marker proteins supplied by Pharmacia revealed that the fractions corresponding to a mol.wt. of 95 ± 15 kDa (mean ± S.D., $n = 5$) had the highest activity (not shown). The eluted protein was further purified by affinity chromatography and anion exchange chromatography as described in section 2. Comparison of this partially purified protein with the starting material showed that (i) the isolated protein still had the activity to induce apoptotic changes in nuclear morphology (Table 1) and DNA fragmentation (Fig. 2), and that (ii) the three-step chromatographical procedure resulted in a 66-fold enrichment of activity in the isolated protein as compared to the Fas-extract (Table 1). The partially purified protein fraction did not induce apoptosis when applied to intact Jurkat cells (not shown), implying that it acts on an intracellular target.

The isolated protein was further characterized by incubating it with thymocyte nuclei in the presence of inhibitors of different classes of proteases. The results obtained showed that

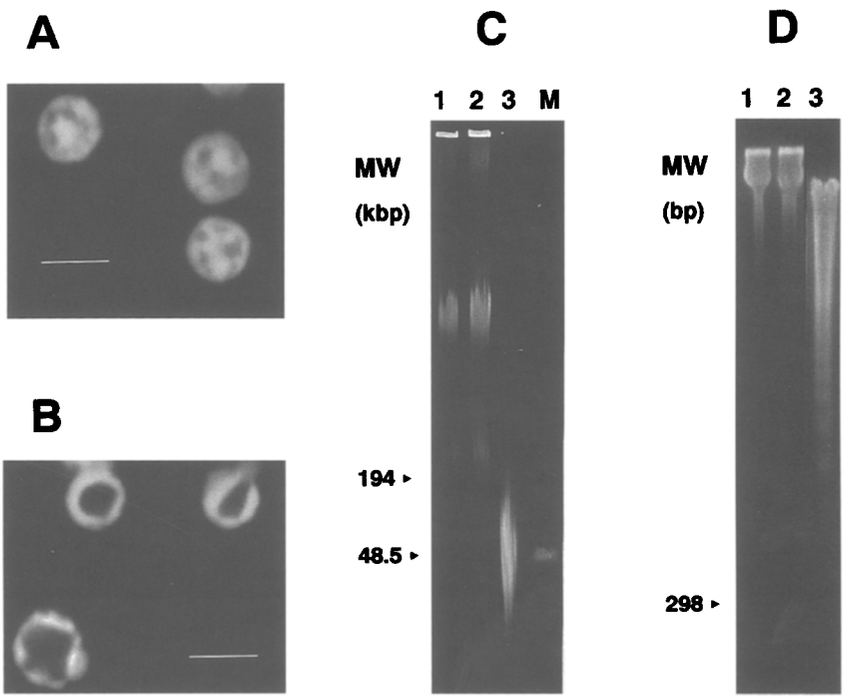

Fig. 1. Induction of apoptotic-like changes in isolated rat thymocyte nuclei by the supernatant of the cell lysate from anti-Fas treated Jurkat cells (Fas-extract). (A) and (B) are CSOM pictures showing the morphology of untreated nuclei and nuclei treated with Fas-extract, respectively. The scale bar corresponds to 5 μm. (C) HMW and (D) oligonucleosomal DNA fragmentation: nuclei in the absence of any extract (control, lane 1), nuclei in the presence of extract from untreated Jurkat cells (lane 2), and in the presence of Fas-extract (lane 3). M = Marker.

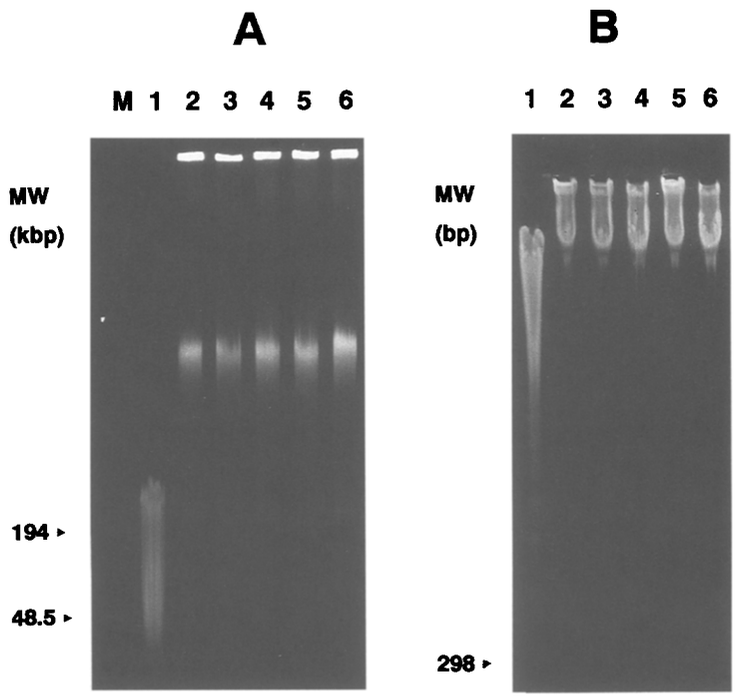

Fig. 2. (A) HMW and (B) oligonucleosomal DNA fragmentation in isolated thymocyte nuclei induced by the partially purified Jurkat protease and its inhibition by various agents. Jurkat protease pretreated with various agents (see below) for 30 min at room temperature and dialyzed against S-buffer was incubated with thymocyte nuclei for 70 min as described in section 2. (1) DMSO (vehicle, control), (2) 100 μM TPCK, (3) 200 μM TLCK, (4) 100 μM DCI, (5) 25 μM VAD-FK, (6) 250 μM iodoacetic acid. M = Marker.

several inhibitors acting preferentially on serine (but in part also on cysteine) proteases were effective (Fig. 2), while specific inhibitors of cysteine proteases and of the chymotryptic-like activity of the 20 S proteasome were ineffective (Table 2).

The agents used in Fig. 2 are known to irreversibly inhibit preferentially serine (TPCK, TLCK, DCI) or cysteine proteases (iodoacetic acid and VAD-FK). Therefore, the partially purified protein was treated with the different inhibitors (or the solvent) and then dialyzed against S-buffer before incubation with thymocyte nuclei, thereby avoiding the presence of inhibitors during incubation of the isolated protein with the nuclei. This allowed us to determine whether the inhibitors indeed acted on the isolated protein and not by blocking a later step involved in nuclear fragmentation. Fig. 2 shows that the dialyzed partially purified protein retained the activity to induce the formation of apoptotic nuclei, i.e. it induced HMW (Fig. 2A) and oligonucleosomal (Fig. 2B) DNA fragmentation in thymocyte nuclei (lane 1), whereas pretreatment of the isolated protein with TPCK, TLCK, DCI, VAD-FK, or iodoacetic acid (lanes 2–6) prior to dialysis irreversibly inhibited its activity. These results strongly suggest that the partially purified protein is a protease. In support of this we have also found that the partially purified protease has no endonuclease activity when incubated with λ-DNA under standard conditions (not shown).

Since loading different types of cells with the proteases trypsin and chymotrypsin has been shown to cause apoptotic cell death [11], we investigated if these nonspecific serine proteases could mimic the activity of the 'Jurkat protease' on isolated nuclei. Isolated thymocyte nuclei were therefore incubated under standard conditions with trypsin and chymotrypsin (final concentration ranging from 85 ng/ml to 0.85 mg/ml). Both proteases were found to digest the nuclei (mainly at the higher concentrations), but DNA fragmentation and apoptosis-like changes in nuclear morphology could not be detected (not shown).

Table 1
Comparison of the supernatant obtained from the cell lysate of anti-Fas treated Jurkat cells (Fas-extract) with the partially purified protein isolated from the Fas-extract

	Protein concentration	Activity[a]	Factor of purification
Fas extract	22.6 mg/ml	59 AU/mg	1
Partially purified protein	57 μg/ml	3,900 AU/mg	66

Protein concentration and activity measurements of a typical isolation are shown. The isolation described in section 2 has been repeated three times with similar results.
[a]1 AU (arbitrary unit) corresponds to the amount of protein needed to lead to apoptotic changes in the morphology of > 50% of the nuclei after one hour, as judged by CSOM.

4. Discussion

4.1. The involvement of proteases in Fas-induced apoptosis

We have described the isolation and partial chracterization of a protease from Jurkat cells undergoing apoptosis after treatment with anti-Fas antibody. Using a cell-free system adapted from Lazebnik et al. [12] we found that incubation of rat thymocyte nuclei with supernatants obtained from lysates of anti-Fas treated Jurkat cells resulted in nuclear changes typically seen in apoptosis, i.e. chromatin condensation and abutting towards the nuclear membrane, and fragmentation of the DNA into HMW and oligonucleosomal fragments. Extracts from untreated Jurkat cells obtained by the same procedure did not

show this activity. The activity leading to apoptotic nuclei had no endonuclease activity but was blocked by a variety of protease inhibitors. Therefore, these results provide strong evidence for the involvement of proteolysis in Fas-induced apoptosis.

4.2. *The protease involved in Fas-induced apoptosis in Jurkat cells is a novel protease distantly related to ICE*

To characterize the 'Jurkat protease' it was necessary to first remove low molecular weight contaminants and as many other proteins as possible. Therefore, we enriched the Fas-extract more than 60-fold using a three-step chromatographical procedure. SDS-polyacrylamide gel electrophoresis analysis of this partially purified protease fraction revealed 6 bands by silver staining (not shown). We have not been able to determine which band corresponds to the protease activity. Therefore, work is underway to establish a sensitive assay for protease activity measurement that would allow purification of the protease to homogeneity.

Leupeptin and E64, two well-known cysteine protease inhibitors, did not affect the 'Jurkat protease'. The same held true for inhibitors of calpains as well as for a novel inhibitor of the chymotrypsin-like activity of the proteasome. However, incubation of the 'Jurkat protease' with iodoacetic acid led to its irreversible inhibition. The inhibition pattern obtained corresponds to the inhibitor profile originally published for ICE [18]. The observation that the 'Jurkat protease' has similarities with ICE is further supported by the finding that it was irreversibly inhibited by VAD-FK, a specific inhibitor for ICE. However, the isolated protease was also inhibited by TPCK, TLCK and DCI, agents belonging to two different classes of protease inhibitors acting preferentially on serine proteases, while ICE is most probably a cysteine protease. Furthermore, Pefabloc, a member of a third class of serine protease inhibitors, also showed partial inhibition of the 'Jurkat protease' at high concentrations (2 mM, not shown). These results suggest that the 'Jurkat protease' is a serine protease and distinct from ICE, although it is important to remember the difficulty of distinguishing cysteine and serine proteases only on the basis of inhibitor studies. In this context it is worth noting that the amino acid sequence of *crmA*, a specific inhibitor of ICE [8], is homologous to that of members of the serpin superfamily which usually inhibit serine proteases by acting as pseudosubstrates ([19] and references therein), providing further evidence for possible cross-reactivities between inhibitors of serine and cysteine proteases.

Table 2
Agents that failed to inhibit HMW and oligonucleosomal DNA fragmentation mediated by the isolated Jurkat protease

Addition[a]	Concentration range tested
Inhibitors of Cystein Proteases	
Leupeptin[b]	10–50 μM
E64[c]	10–50 μM
N-Acetyl-Leu-Leu-norleucinal[d]	5–50 μM
N-Acetyl-Leu-Leu-methional[d]	5–50 μM
Inhibitor of the 20 S proteasome[e]	
PSI[d]	5–100 μM

[a]Thymocyte nuclei were incubated with the isolated Jurkat protease in the presence of the different inhibitors as described in section 2. The agents were prepared as 200 times stock solutions in water[b], 50% ethanol in water[c] or DMSO[d]. [e]Specific inhibitor of the chymotryptic-like activity of the proteasome [17].

The two serine proteases trypsin and chymotrypsin, recently shown to induce apoptotic cell death when loaded into different types of cells [11], failed to induce any apoptotic changes in isolated thymocyte nuclei in the cell-free system described here. This provides strong evidence for the necessity of a specific proteolytic step for the induction of the nuclear changes seen in Fas-induced apoptosis.

In summary, our results show the involvement of the 'Jurkat protease' in Fas-induced apoptosis. This enzyme differs in size and inhibition pattern from other proteases implicated in apoptosis, such as Granzyme [20], 24 kDa U937 protease [10], CPP32 [21], ICE [18], and prICE [9]. It is therefore most probably a novel protease, that, as judged by its inhibition profile, is distantly related to ICE.

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