Sequential activation of three distinct ICE-like activities in Fas-ligated Jurkat cells

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Abstract ICE family proteases have been implicated as important effectors of the apoptotic pathway, perhaps acting hierarchically in a protease cascade. Using cleavage of endogenous protease substrates as probes, three distinct tiers of ICE-like activity were observed after Fas ligation in Jurkat cells. The earliest cleavage detected (30 min) was of fodrin, and produced a 150 kDa fragment. The second phase of cleavage (50 min) involved PARP, U1–70kDa and DNA-PK$_{cs}$, all substrates of the CPP32-like proteases. Lamin B cleavage was observed during the third cleavage phase (90 min). Distinct inhibition profiles obtained using a panel of peptide-based inhibitors of ICE-like proteases clearly distinguished the three different cleavage phases. These studies provide evidence for a sequence of ICE-like proteolytic activity during apoptosis. The early fodrin cleavage, producing a 150 kDa fragment, identifies an ICE-like activity proximal to CPP32 in Fas-induced Jurkat cell apoptosis.

Key words: Apoptosis; Protease; CPP32; ICE

1. Introduction

Protease activity has been placed early in the final common pathway of apoptosis, and the importance of the interleukin 1β-converting enzyme (ICE) family of proteases has been emphasized [1,2]. In mammals, the extended ICE family includes at least seven members [3], which have been divided into several subfamilies with homology to the prototype proteases ICE, CPP32 and Ich-1 [4]. The effects of these proteases in apoptosis appear to be accomplished by the cleavage of key homeostatic cellular substrates including poly (ADP-ribose) polymerase (PARP) [5], the 70 kDa subunit of the U1 ribonucleoprotein (U1–70kDa) [6], the catalytic subunit of the DNA-dependent protein kinase (DNA-PK$_{cs}$) [7], sterol regulatory element binding protein [8], and the GDP dissociation inhibitor protein D4-GDI [9], as well as cleavage of several structural proteins including fodrin [10], lamin B [11], nuclear mitotic apparatus protein (NuMA) [12], and Gas-2 [13]. Recent studies have demonstrated that the protease subfamilies have distinct substrate specificities [14], and different kinetics of activation after delivery of an apoptotic signal [15]. Although all homologs studied to date have an absolute requirement for Asp in the P_1 position, ICE prefers a hydrophobic residue in P_4 [14], while CPP32 has a stringent requirement for another Asp at this position [16].

Because a large proportion of treated cells rapidly undergo apoptosis, Fas ligation of Jurkat cells has been a useful system for the biochemical analysis of apoptosis in intact cells [17]. In these studies, we demonstrate that substrates cleaved in apoptotic cells can be separated into at least three groups, based on time-course of cleavage and sensitivity to various inhibitors of ICE-like proteases. Study of these cleavage events will allow elucidation of the sequential proteolytic steps along the apoptotic pathway.

2. Materials and methods

Leupeptin, antipain, pepstatin A, chymostatin, and Nonidet P-40 (NP-40) were purchased from Calbiochem. Phenylmethylsulfonyl fluoride (PMSF) was obtained from Sigma. Monoclonal antibodies were purchased from the following sources: CH-11 anti-Fas antibody was from Panvera (Madison, WI); anti-fodrin (non-erythroid spectrin) antibody was from Chemicon International (Temecula, CA), anti-lamin B antibody 101-B7 and anti-NuMA antibody 107-7 were from Matritech (Cambridge, MA). Monoclonal antibody 18-2 that recognizes the intact form and the 250 kDa fragment of DNA-PK$_{cs}$ was a kind gift of Dr. Tim Carter (St. John's University, Jamaica, NY). The protease inhibitor zVAD-fmk was from Enzyme Systems Products (Dublin, CA); Ac-YVAD-CHO and Ac-DEVD-CHO were from Merck Research Laboratories (Rahway, NJ). The Supersignal CL-HRP substrate system used to visualize blotted proteins was purchased from Pierce. Nitrocellulose and PVDF membranes were purchased from Millipore. All other reagents and compounds were analytical grade.

2.1. Cell culture and induction of apoptosis

Jurkat cells were maintained in RPMI containing 10% heat-inactivated fetal calf serum, using standard tissue culture procedures. When performing experiments, cells were washed and resuspended at 8×10^6 cells/ml in culture medium, in the absence or presence of 200 μM CH-11 anti-Fas antibody. After incubating for the indicated times at 37°C, the cells were immediately chilled to 4°C and washed with ice-cold phosphate-buffered saline.

2.2. Gel electrophoresis and immunoblotting for detection of DNA-PK$_{cs}$, U1–70kDa, PARP, fodrin, NuMA, and lamin B

Cells were lysed at 4°C in buffer containing 1% NP-40, 20 mM Tris, pH 7.4, 1 mM NaCl, 1 mM EDTA, and the following protease inhibitors: pepstatin A, leupeptin, antipain, chymostatin, and PMSF. 37.5 μg of the reduced gel samples were electrophoresed on 10% SDS-polyacrylamide gels containing 0.087% bisacrylamide, and proteins were transferred to nitrocellulose [6]. Proteins were immunoblotted with patient sera recognizing U1–70kDa, PARP, and NuMA (serum RW [7]) or monoclonal antibodies raised against DNA-PK$_{cs}$ and fodrin. Identical results were obtained using serum AG [7] to detect intact DNA-PK$_{cs}$ and the 160 kDa fragment (data not shown) and with monoclonal antibody 107-7 to detect NuMA (data not shown). Blotted proteins were visualized using an enhanced chemiluminescent substrate according to the manufacturer's instructions. Alternatively, the cells were lysed by freeze-thawing four times followed by centrifugation for 10 min at $230000 \times g$ in a TL100 ultracentrifuge (Beckman). Pellets were solubilized in an equal volume of lysis buffer [18],

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and aliquots of the pellet were separated by SDS PAGE on 16% (lamin B) or 4–20% (PARP) Novex gels. Gels were transferred to PVDF membranes, and immunoblots were performed as described above. The protein fragments observed in apoptotic cells were never seen in immunoblots of control cells.

2.3. Protease inhibitor experiments

Jurkat cells were pre-incubated for 15 min at 37°C in the absence or presence of the following protease inhibitors: 10 μM zVAD-fmk, 100 μM Ac-YVAD-CHO, or 100 μM Ac-DEVD-CHO. Cells were then induced to undergo apoptosis by incubating with 200 ng/ml CH-11 anti-Fas antibody for 90 min prior to chilling, lysing, and immunoblotting as described above. Incubation with the protease inhibitors in the absence of Fas ligation did not produce the apoptotic protein fragments (data not shown).

2.4. DNA extraction and gels

DNA from whole cell populations, harvested at the indicated times after Fas ligation, was extracted, processed, and electrophoresed on 1.5% agarose gels as described [19].

3. Results and discussion

3.1. Cleavage of endogenous substrates occurs in three phases in Fas-ligated Jurkat cells

To characterize the order of cleavage of endogenous macromolecular substrates in intact cells, apoptosis was induced in Jurkat cells with Fas ligation, and the time-course of cleavage of different substrates was evaluated by immunoblotting. Cleavage of PARP, U1–70kDa, DNA-PK_{cs} and NuMA was initially observed ~50 min after Fas ligation (Fig. 1A, Fig. 2), and reached completion 4 h after Fas ligation (data not shown). The time-course of cleavage of these substrates coincided with the activation of CPP32, detected by cleavage of the precursor into its mature form ([18], and data not shown). Of note, ADP ribosylation of PARP was also readily detected 50 min after Fas ligation, coincident with the appearance of the proteolytic fragments (Fig. 1A) and internucleosomal DNA degradation (Fig. 1B). In some experiments, minimal PARP cleavage was also observed at 30 min. In contrast, the time-course of cleavage of fodrin and lamin B differed significantly from the substrates described above. Fodrin cleavage was biphasic (Fig. 2): initial cleavage began at 30 min, generating a fragment of 150 kDa; a second phase of cleavage began at ~50 min, producing a second fragment of 120 kDa. This latter phase occurred co-incidently with the cleavage of CPP32 and its substrates (U1–70kDa, DNA-PK_{cs} and PARP [16]). Cleavage of lamin B (67 kDa) was significantly delayed, with the 46 kDa lamin B cleavage fragment only observed 90 min after Fas ligation (Fig. 2). This is consistent with previous studies which demonstrated that the lamin protease appears to be distinguishable from CPP32 by its later activation and distinct inhibition characteristics [11]. Thus, Fas-induced cleavage of these macromolecular protease substrates defines three discrete phases of proteolytic activity during apoptosis. The cleavage of fodrin, generating the 150 kDa fragment, is the earliest proteolytic event occurring in apoptotic cells after Fas-ligation and is clearly kinetically distinct from the cleavages catalyzed by CPP32 and similar homologues. This cleavage therefore provides a useful assay for proteolytic events that might be upstream of the activation of CPP32 in apoptotic cells. While the three distinct phases may reflect sequential activation of multiple protease activities, they may also result from differential access of the same proteases to different substrates as apoptosis progresses.

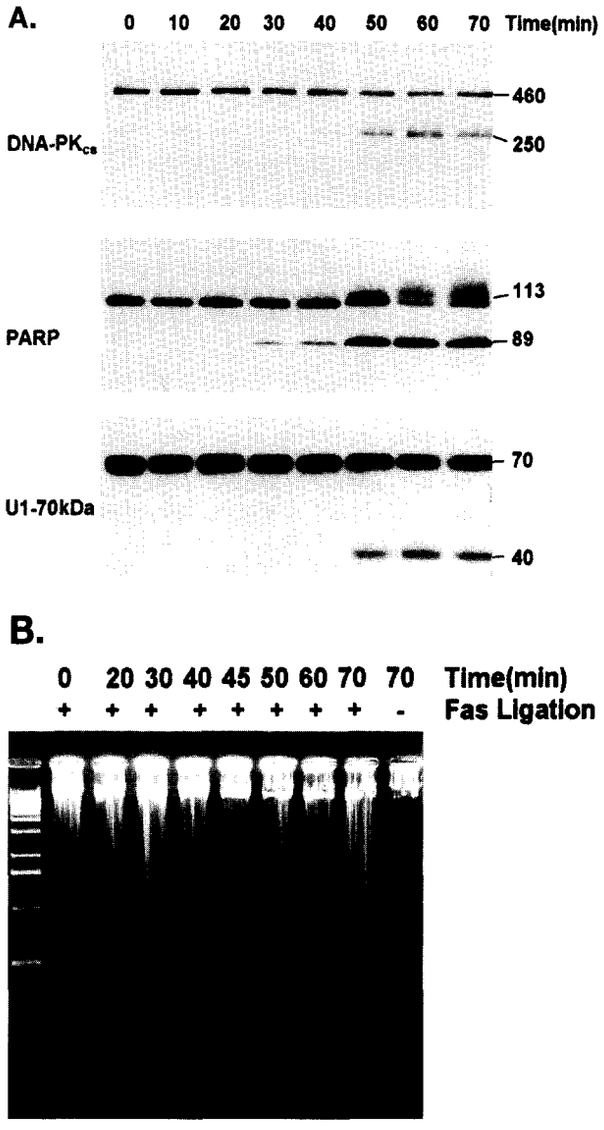

Fig. 1. Cleavage of homeostatic proteins occurs co-incident with the onset of DNA fragmentation in Fas-ligated Jurkat cells. Jurkat cells were incubated with 200 ng/ml of anti-Fas monoclonal antibody CH-11 for the indicated times. At each time, the cells were harvested and processed for immunoblotting (A) or for DNA gel analysis (B) as described in the methods section. Data shown are from a single experiment, and are representative of similar data obtained in four other experiments.

3.2. Inhibition characteristics of substrate cleavage in Fas-ligated Jurkat cells indicate the involvement of multiple ICE-like proteases

To assess whether different ICE-like proteases might mediate the kinetically distinct cleavages described above, the inhibition characteristics of cleavage of the different substrates in intact Fas-ligated cells were determined, using the peptide inhibitors of the ICE-like proteases, zVAD-fmk, Ac-DEVD-CHO and Ac-YVAD-CHO. While Ac-YVAD-CHO is a potent and specific inhibitor of ICE (K_i ICE = 0.75 nM, K_i CPP32/Mch3 => 10 μM), Ac-DEVD-CHO has a broader spectrum of inhibitory activity, inhibiting CPP32-like enzymes (K_i CPP32/Mch3 = 0.2–2 nM) only approximately 20-fold more potently than ICE (K_i ICE = 17 nM) [20]. The inhibition profile of zVAD-fmk has not yet been fully determined,

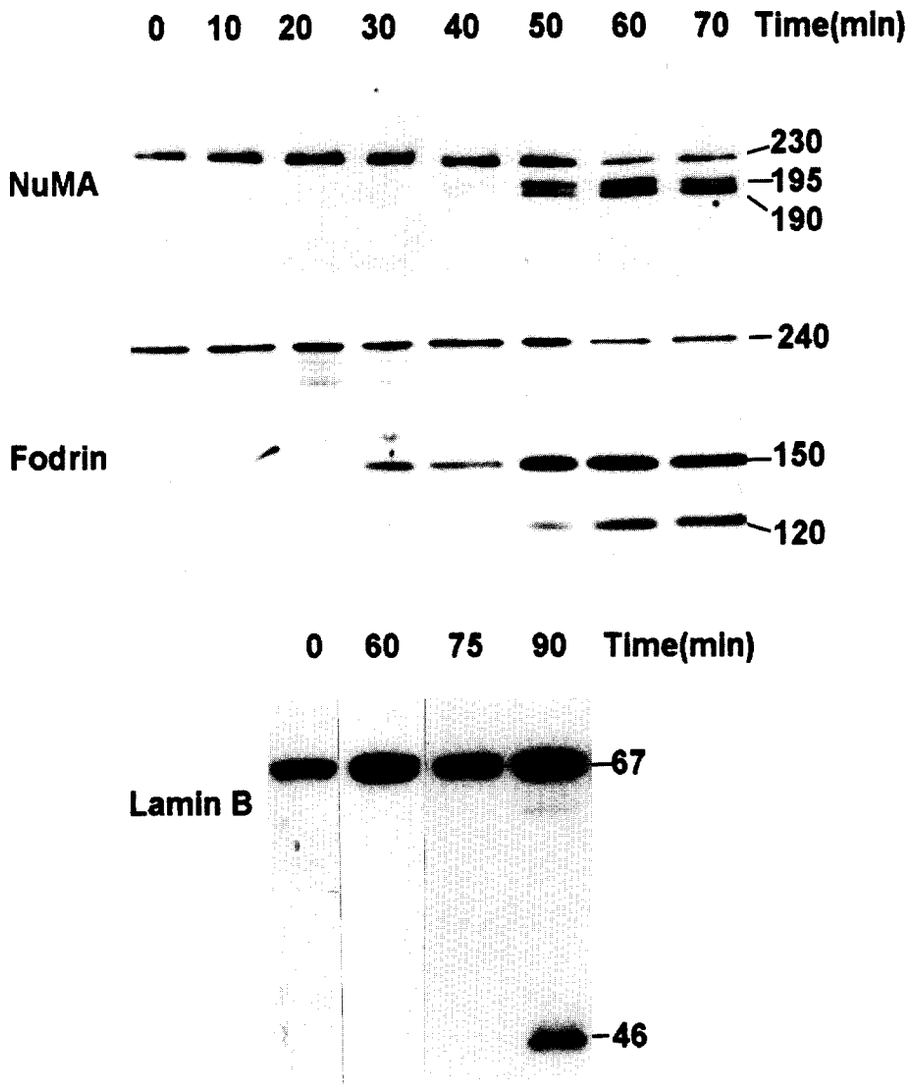

Fig. 2. Cleavages of structural proteins occur at different times in Fas-ligated Jurkat cells. Jurkat cells were Fas-ligated and processed for immunoblotting as described in the legend for Fig. 1. Data shown are from a single experiment, and are representative of similar data obtained in three other experiments.

although it is known to inhibit ICE-like proteases [21], and to prevent the processing of CPP32 to its active form [22]. Thus, the combined profile of inhibition obtained with these reagents is a useful approach with which to define the various ICE-like proteases active during different phases of apoptosis [20]. Jurkat cells were pre-incubated with the various protease inhibitors for 15 min, prior to activation of apoptosis by Fas-ligation for 90 min. Three distinct groups of cleavages could be defined, which correlated closely with those observed in the time-course studies. Firstly, the early cleavage of fodrin, which generated a fragment of 150 kDa, was insensitive to 100 μM Ac-DEVD-CHO and 100 μM Ac-YVAD-CHO, but was entirely inhibited by 10 μM zVAD-fmk (Fig. 3). Secondly, the cleavages of PARP, U1–70kDa, DNA-PK_{cs}, NuMA, and the 120 kDa fragment of fodrin were inhibited by 100 μM Ac-DEVD-CHO or 10 μM zVAD-fmk, but only minimally by 100 μM Ac-YVAD-CHO (Fig. 3, and data not shown). These proteins are thus formed with identical kinetics after Fas ligation, and share identical inhibition characteristics, implying that similar (CPP32-like) activities are involved, directly or upstream, in all these cleavages. Lastly, cleavage of lamin B was abolished not only by 100 μM Ac-DEVD-CHO and 10 μM zVAD-fmk, but was also inhibited significantly by 100 μM Ac-YVAD-CHO, making it the only Ac-YVAD-CHO sensitive cleavage among these substrates (Fig. 3).

3.3. A cascade of ICE-like proteases participate in apoptosis

Several recent studies have indicated that an additional proteolytic activity is activated upstream of the CPP32-like proteases during Fas-induced apoptosis. For example, Dixit and co-workers have presented evidence that a crmA-inhibitable proteolytic activity is activated upstream of CPP32 in Fas-induced apoptosis of Jurkat cells [23]. Cohen's group has demonstrated in Fas-ligated Jurkat cells that zVAD-fmk but

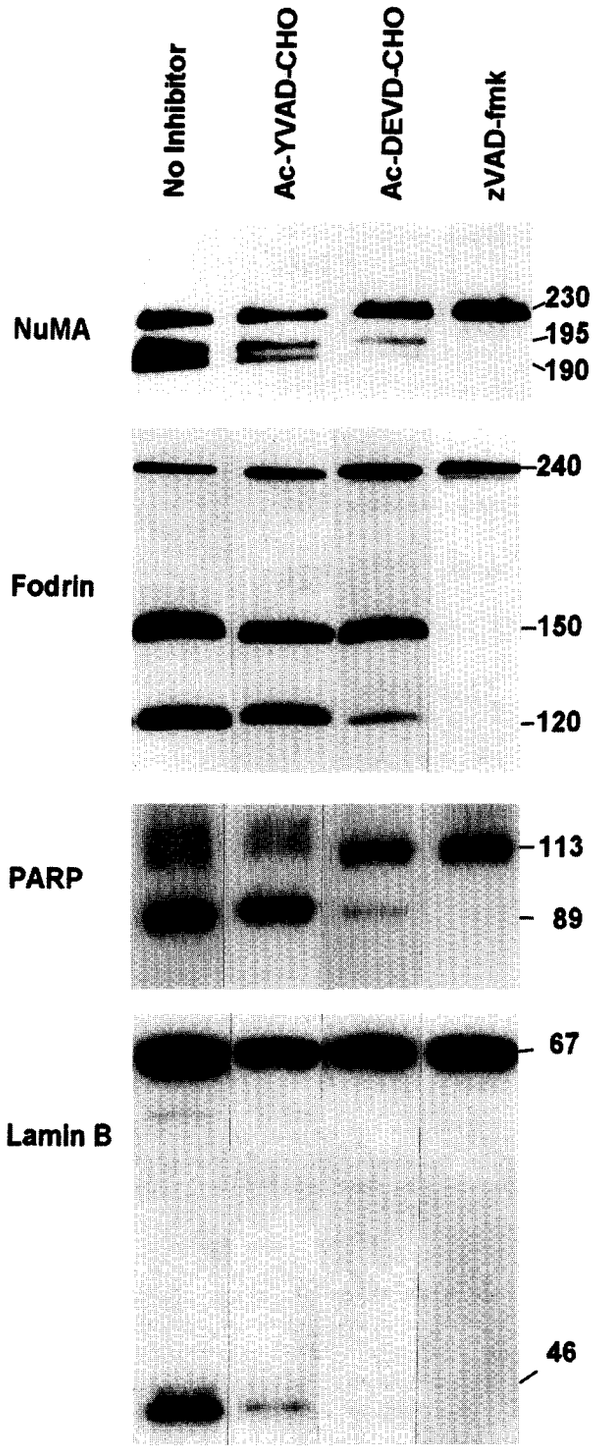

Fig. 3. Inhibition of protein cleavages by ICE family inhibitors in Fas-ligated Jurkat cells. Jurkat cells were preincubated for 15 min at 37°C with 10 μM zVAD-fmk, 100 μM Ac-DEVD-CHO, or 100 μM Ac-YVAD-CHO. Fas ligation was subsequently induced by addition of 200 ng/ml CH-11 antibody, and further incubation at 37°C for 90 min. The cells were harvested and processed for immunoblotting as described in Section 2. Data from a single experiment are shown, and are representative of similar data obtained in three other experiments.

not Ac-DEVD-CHO abolishes the processing of CPP32, suggesting that an additional zVAD-fmk-sensitive activity is upstream of CPP32-like proteases in a protease cascade [22]. Furthermore, Enari et al. [15] have recently demonstrated that Fas ligation results in the early and transient activation of a YVAD-cleaving activity, which clearly precedes the appearance of an DEVD-cleaving activity in W4 cells. Using several of the known macromolecular substrates cleaved during apoptosis as probes, our studies demonstrate that the earliest cleavage event detected to date in Fas-induced apoptosis is the proteolysis of fodrin which generates the 150 kDa fragment. This event is clearly distinct in terms of kinetics after Fas ligation, and has unique inhibition characteristics that separate it from all the other cleavages observed during Fas-induced apoptosis of Jurkat cells. Since this fodrin cleavage is resistant to inhibition by Ac-DEVD-CHO, it is likely that the responsible protease is upstream of, and does not belong to, the CPP32 subfamily of proteases. The kinetic studies also clearly identify the cleavage of lamin B as a later proteolytic event, consistent with previous observations [11]. These studies therefore provide direct evidence for an apoptotic protease cascade of at least three tiers following Fas ligation. Formation of the 150 kDa fodrin fragment will provide a useful assay to identify and purify the upstream ICE family activity in the Fas-induced apoptotic pathway. It will be important to determine whether a similar proximal activity is activated in all forms of apoptotic death.

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