Caspase 7-induced cleavage of kinectin in apoptotic cells

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Abstract Kinectin has been characterized as the first known receptor for the molecular motor kinesin, which is critically involved in microtubule-based vesicle transport and membrane trafficking. Here we identify kinectin as a target for caspase-mediated proteolysis during apoptosis. Treatment of cells with diverse apoptotic stimuli including TNF, anti-Fas, anticancer drugs, γ-radiation or ceramide leads to rapid proteolytic cleavage of the 160-kDa form of kinectin to a 120-kDa fragment. Evidence is provided that kinectin cleavage is mediated by caspase 7.

Key words: Kinectin; Caspase 7; Apoptosis

1. Introduction

Apoptosis, or programmed cell death, is a fundamental process for normal development of multicellular organisms, and is involved in the regulation of the immune system, normal morphogenesis, and maintenance of tissue homeostasis.

An important central mechanism in apoptotic cell death is proteolytic cleavage of key cellular substrates by cysteine proteases [1,2]. These proteases are related to mammalian interleukin-1β converting enzyme (ICE) [3]. The caspase protease family consists of at least 10 members which can be divided by their phylogenic relationship and substrate specificity into three subfamilies [4]. Those caspase members most closely related to the cleavage during apoptosis are caspase 3, caspase 6 and caspase 7.

The identified substrates of caspase 3 include poly(ADP-ribose) polymerase (PARP) [5,6], sterol-regulatory element-binding proteins (SREBPs) [7,8], the U1-associated 70-kDa protein [9], D4-GDI [10], huntingtin [11], the DNA-dependent protein kinase [12,13], the DNA fragmentation factor (DFF) [14], p21-activated kinase 2 (PAK2) [15], MEKK [16], gelsolin [17] and retinoblastoma protein (RB) [18] to list a few.

It is not known whether the cleavage of any of these substrates plays a causative role in apoptosis. Therefore, the identification and characterization of further physiological caspase substrates appears mandatory to further our understanding of the complex molecular and morphologic processes during apoptotic cell death. The cellular signaling pathways involved in controlling apoptosis remain poorly defined as well. In particular, little is known about the mechanisms underlying the dramatic cytoskeletal, morphological, and membrane changes that accompany cell death and that may be important in the subsequent recognition and disposal of apoptotic cells by phagocytic leukocytes.

Kinectin has been purified as a kinesin-binding protein of 160 kDa from the chick embryo brain [19]. A human homolog has been cloned as a 4.6-kb mRNA species and characterized as an integral membrane protein anchored in the endoplasmic reticulum [20]. The properties of this protein suggested that kinectin functions as a kinesin receptor that is required for kinesin-dependent organelle movement [21]. Kinesin is a member of an entire superfamily of ATP-driven motors in eukaryotes (reviewed in [22]) which binds to and moves along a microtubule substrate, powering a variety of transport processes, such as vesicle movement.

In this report we demonstrate that kinectin is cleaved by caspase 7 during apoptosis induced by different stimuli. This finding may be especially interesting because kinectin functions as a membrane anchor for kinesin and may therefore be relevant to the disruption of vesicle trafficking during apoptosis.

2. Material and methods

2.1. Cell culture and reagents

The human cervix carcinoma cell line HeLa, the human T-cell lymphoma cell lines Jurkat and HUT78 were obtained from the American Type Culture Collection (ATCC, Rockville, USA). Cells were maintained in RPMI 1640 supplemented with 5% fetal calf serum, 2 mM glutamine and 50 μg/ml each of streptomycin and penicillin in a humidified incubator containing 5% CO_2.

Highly purified recombinant human TNF (hTNF, 3×10^7 U/mg protein) was kindly provided by Dr. G. Adolf (Bender, Vienna, Austria). Anti-Fas antibody (IgM CH-11) was purchased from Coulter-Immunotech. Monoclonal anti-PARP antibody was obtained from Dr. G. Poirier (Quebec, Canada). Daunomycin, etoposide and *cis*-platin were obtained from Sigma (Munich, Germany), protease inhibitors PMSF, Pefabloc and Complete from Boehringer Mannheim (Germany) and DEVD-CHO and YVAD-CHO from Bachem (Heidelberg, Germany).

2.2. Preparation of cytosolic extracts from human cell lines

Cell extracts from Jurkat cells were prepared as described [23]. Briefly, 2×10^8 cells were left untreated or treated with 100 μM etoposide for 3 h. Cells were washed twice with 50 ml PBS, followed by a single wash with 5 ml cell extraction buffer (CEB; 50 mM PIPES, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10 μM cytochalasin B, 1 mM PMSF, 2 mM Pefabloc). Cells were pelleted and after removal of supernatant, cells were transferred to a Dounce homogenizer. After addition of one volume CEB (100 μl/10^8 cells), cells were incubated for 20 min on ice. Cells were lysed by 20–30 strokes in a homogenizer (Braun, Germany). Cell lysates were transferred to 1.5-ml Eppendorf tubes and centrifuged at 4°C for 20 min at $22\,000\times g$. The clear cytoplasmic lysate was transferred to a new tube and diluted to 7.5 μg/μl protein using enzyme dilution buffer (EDB: 10 mM HEPES, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 2 mM ATP, 10 mM phosphocreatine, 50 μg/ml creatine kinase). Extracts were used either immediately or stored at −80°C. Western blot analyses were performed as described [24].

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Abbreviations: ER, endoplasmic reticulum; TNF, tumor necrosis factor

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2.3. In vitro cleavage of kinectin

The full-length cDNA of human kinectin was cloned into the *Eco*RI site of Bluescript KS II (Stratagene). Cell-free reactions (total volume: 15 μl) were performed with 1 μl of in vitro transcribed/translated ^{35}S-labeled kinectin (TNT kit, Promega) and 1.5 μl of recombinant caspases 3, 6, 7 and 8 (Pharmingen) diluted in the assay buffer (20 mM PIPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% (w/v) CHAPS and 10% sucrose, pH 7.2) [25]. The activity of caspases was tested prior to the experiments in a fluorogenic assay measuring the rate of Ac-DEVD-AFC hydrolysis in a Labsystems Fluoroskan 2, as suggested by the company providing the caspases. The concentrations of the caspases used were up to 130 nM for caspase 6, 5 nM and 16.5 nM for caspase 7, 82 nM for caspase 8 and 1.2 resp. 12 nM for caspase 3. After incubation at 37°C for one hour, samples were subjected to SDS-PAGE and further analyzed using a Fuji BAS 1000 bioimager or exposure to a Kodak Biomax film.

2.4. Annexin-V-binding assay

Cells were washed with PBS, and resuspended in 100 μl annexin-V-FITC labeling solution (Boehringer Mannheim, Germany). After a 15-min incubation period, annexin-V-FITC staining was determined by flow cytometry analysis, using a fluorescence activated cell sorter (Excalibur, Becton and Dickinson).

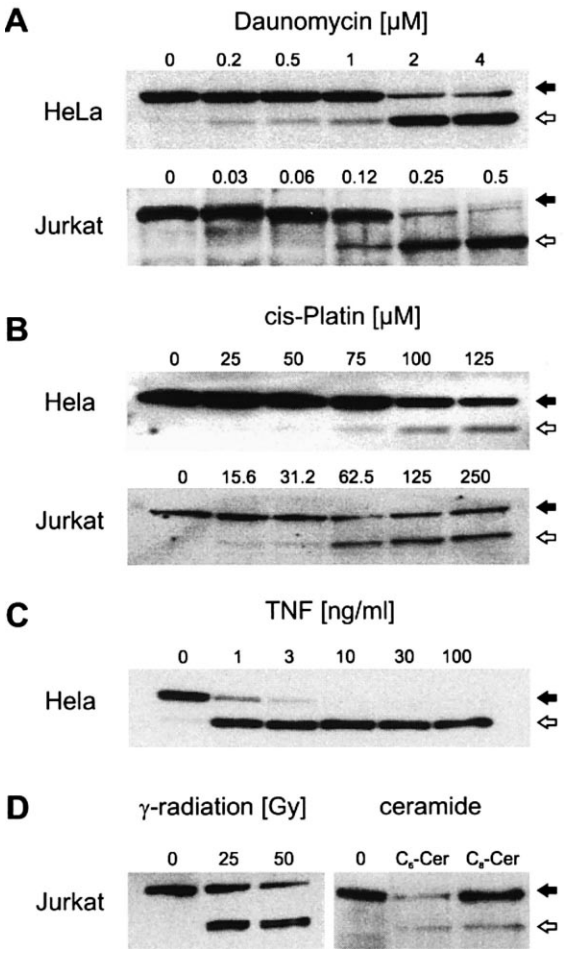

Fig. 1. Kinectin degradation is induced in different cell types by various apoptotic stimuli. HeLa or Jurkat cells were left untreated or treated for 12 h with indicated concentrations of daunomycin (A) or *cis*-platin (B). C: HeLa cells were left untreated or treated for 8 h with recombinant human TNF. D: Jurkat cells were left untreated or exposed to 25 Gy or 50 Gy γ-radiation and incubated for an additional 12 h. Jurkat cells were also incubated for 12 h with 50 μM of either C_6- or C_8-ceramide. Whole cell extracts were prepared and analyzed by Western blotting. The arrows indicate the 160-kDa (black arrow) and the 120-kDa (white arrow) forms of kinectin.

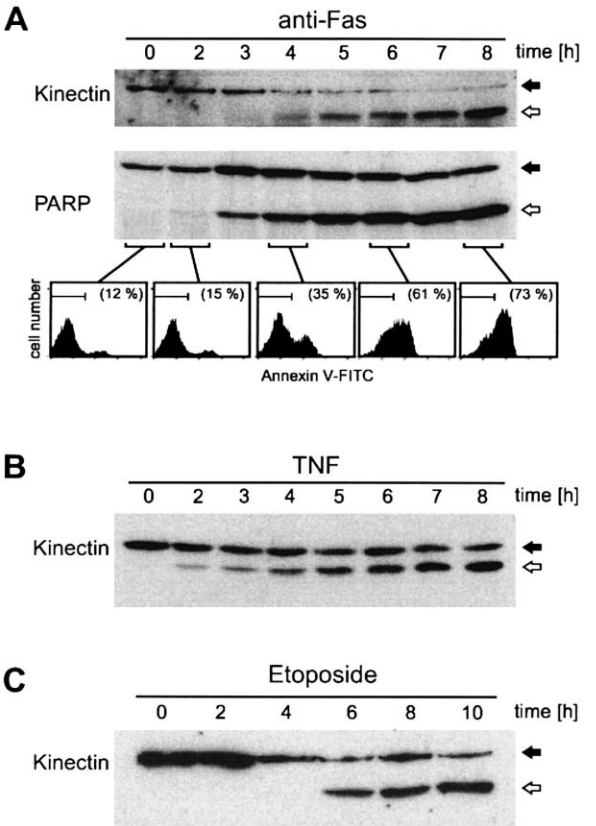

Fig. 2. Kinetics of kinectin cleavage during apoptosis. A: HUT78 cells were treated for the indicated times with 250 ng/ml anti-Fas mAb. Cell lysates were prepared and analyzed by Western blotting for the content of both kinectin and PARP. The percentage of apoptotic cells was determined by flow cytometry using annexin-V-FITC. Jurkat cells (B) or HeLa cells (C) were left untreated or treated for the indicated times with 200 μM etoposide or 100 ng/ml TNF, and cellular extracts were analyzed for kinectin degradation.

3. Results

3.1. Cleavage of kinectin is a general feature of apoptosis

HeLa or Jurkat cells were exposed to different apoptotic stimuli, such as antitumor agents daunomycin and *cis*-platin, TNF, ceramide or γ-radiation. As shown in Fig. 1 kinectin degradation was observed in every instance, suggesting that kinectin might serve as uniform target for proteases activated in different cell types exposed to different stimuli. Degradation of kinectin also occurs in primary leukemia cells treated with etoposide or daunomycin (data not shown).

3.2. Degradation of kinectin during etoposide- and fas-induced apoptosis

The fate of kinectin during apoptosis was analyzed by Western blotting analysis. Treatment of the T-cell lymphoma cell line HUT78 with anti-Fas monoclonal antibody (anti-Fas mAb) over a period of 12 h resulted in a dose-dependent conversion of kinectin from a 160-kDa form to a discrete fragment with an approximate molecular weight of 120 kDa (data not shown). Treatment of cells with 250 ng/ml anti-Fas results in almost complete degradation of the 160-kDa form of kinectin. A similar dose-dependent proteolytic degradation of kinectin was also observed with the T cell-lymphoma cell line Jurkat exposed for 12 h to the anticancer drug etoposide

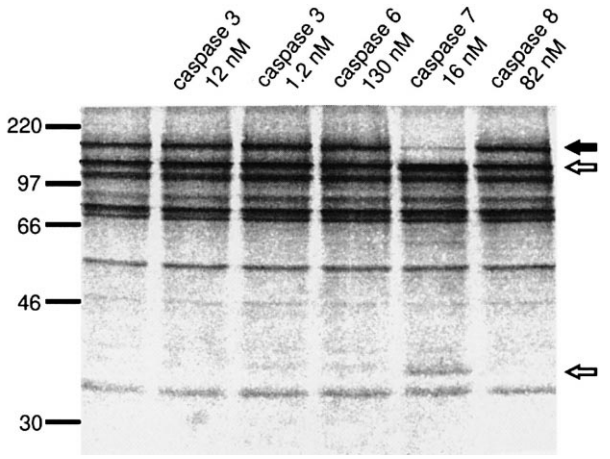

Fig. 3. Specific degradation of kinectin in a cell-free system by recombinant caspases. A: A full-length cDNA of human kinectin was in vitro transcribed/translated (TNT-kit, Promega) using [^{35}S]methionine (NEN) for radioactive labeling. The ^{35}S-labeled 160-kDa translation product of kinectin is shown in lane 1. The in vitro translated kinectin was incubated for indicated times with caspase 3, 6, 7 or 8 in the indicated concentration (lanes 2–6). Reaction products were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography using a Fuji BAS1000 imager. The 160-kDa (black arrow) and the 120-kDa (white arrow) forms of kinectin are indicated.

(data not shown). The extent of kinectin degradation corresponds well with the degree of apoptosis, as measured by the annexin-V-binding assay.

3.3. Kinetics of kinectin degradation

Proteolytic degradation of caspase targets such as PARP has been observed as early as 2 h after induction of apoptosis [12]. We have examined the time course of kinectin proteolysis during cell death induced by Fas, etoposide or TNF. The 120-kDa cleavage fragment of kinectin was discerned within 2–3 h after treatment of HUT78 or HeLa cells with either anti-Fas mAb or TNF, respectively (Fig. 2A,B), similar to the degradation kinetics of the well defined caspase substrate PARP (Fig. 2A). The time course of kinectin degradation corresponds well with annexin-V binding (Fig. 2A). These results demonstrate that kinectin degradation is an early event in the development of apoptosis. Notably, unlike Fas and TNF, etoposide induced degradation of kinectin only after 6 h (Fig. 2C). This delay of kinectin proteolysis, however, corresponded well with the delayed time course of annexin-V binding (data not shown).

3.4. Degradation of kinectin is inhibited by caspase inhibitors

To examine the role of caspases in kinectin proteolysis we employed YVAD-CMK, a specific tetrapeptide inhibitor of ICE (caspase 1) and DEVD-CHO, which preferentially inhibits caspase 3. Pretreatment of cells with YVAD did not inhibit Fas-induced kinectin degradation and only partially blocked etoposide at the highest concentrations used (200 μM) (data not shown). In contrast, DEVD treatment leads to a dose-dependent inhibition of both Fas- or etoposide-induced cleavage of kinectin with a close to complete inhibition at 200 μM DEVD indicating the involvement of caspase 3-like proteases. This distinct inhibitory activity of YVAD-CMK and DEVD-CHO on Fas-induced kinectin degradation correlated with the extent of inhibition of apoptosis (data not shown).

3.5. Degradation of kinectin in a cell-free system with recombinant caspases

To investigate whether kinectin serves as a direct substrate for caspases we employed a cell-free system, where in vitro transcribed and translated kinectin was exposed to recombinant caspases 3, 6, 7 and 8. As shown in Fig. 3A, proteolysis of kinectin could be observed within 60 min when incubated with caspase 7. The concentrations used and reaction conditions had been described previously [25]. Even at higher concentrations other caspases showed no cleavage of kinectin. The cleavage pattern seems to be identical when compared to the results with intact cells (Figs. 1 and 2), suggesting that proteolysis of kinectin occurs at a distinct cleavage site.

The in vitro translated kinectin shows amongst other, non-specific bands, one at a similar molecular weight as the cleavage product (120 kDa). This could be an alternatively spliced variant as well.

4. Discussion

Although the precise biochemical pathways involved in the execution of cell death during the apoptotic process are still poorly understood it is now clear that proteases play an essential role in the downstream processes resulting in the apoptotic phenotype. However, a cause-relationship between the proteolysis of these targets and the morphological and biochemical alterations associated with apoptosis remain uncertain.

Here we identify kinectin, the receptor for the molecular motor protein kinesin as a substrate for proteolytic degradation during apoptosis. In TNF- or Fas-treated cells, degradation of kinectin could be detected within 2 h after induction of apoptosis, at the same time when phosphatidylserine translocation became measurable. We could demonstrate with long-term cultured cell lines and primary cells, that kinectin is degraded specifically and rapidly during the execution of apoptosis induced by a number of different stimuli, such as anti-Fas mAb, TNF, anticancer drugs, UV radiation and ceramide. These different stimuli probably use distinct molecular pathways for the initiation phase of apoptosis, indicating that kinectin may represent one point of convergence for different pathways leading to apoptosis.

Treatment of intact cells with DEVD-CHO prevented Fas-induced kinectin cleavage, whereas YVAD-CMK showed only marginal inhibitory effects. Because DEVD preferentially inhibits CPP32-like caspases these findings would suggest that a caspase 3-like enzyme may be involved in the proteolytic cleavage of kinectin. To further specify the caspase involved in the cleavage of kinectin we tested in a cell free system the degradation of kinectin with the recombinant caspases 3, 6, 7 and 8. Caspase 7 is the only caspase among these cleaving kinectin with the same pattern shown previously in the cells during apoptosis.

Caspase 7 was identified by the cloning of a cDNA detected by PCR amplification of conserved caspase cDNA and called MCH-3 [26]. It was also separately cloned from an EST and called ICE-LAP3 (ICE-like apoptotic protease 3 [27] or CMH1 (CPP32/Mch2 homolog 1) [28]. Overexpression of caspase 7 induces apoptosis, and this protease is activated during apoptosis induced by Fas, TNF or cytostatic agents. Although caspases 3 and 7 have a closely related structure [29] and almost identical tetrapeptide substrate specificity

[30,31], kinectin is cleaved only by caspase 7 and not by caspase 3 suggesting that caspase 7 may play a role distinct from caspase 3. Detailed analysis of Fas-induced signaling using novel synthetic tetrapeptides selectively inhibiting caspase 6 and caspase 3 provided evidence that the protease cascade bifurcates into a caspase 7 arm and a caspase 3 arm each inducing distinct downstream intracellular events [32]. Both caspase 6 and 3 are supposed to play major roles in nuclear apoptosis whereas caspase 7 is linked to cell shrinkage [32]. Further elucidation of cytoplasmic substrates specific for caspase 7 may clarify its role in the apoptotic process.

The molecular mechanism by which the caspases become activated in the apoptotic process is currently not known. The differential sensitivity of Fas- and etoposide-induced kinectin degradation might be explained by the different pathways these stimuli use to induce apoptosis and to activate caspase 7.

The uniform cleavage of kinectin induced by diverse apoptotic stimuli supports the idea that kinectin cleavage is an important event in apoptosis. The ER membrane integral 160-kDa protein kinectin has been recently molecularly cloned as the first kinesin receptor protein [33]. Kinesin is a member of an entire superfamily of ATP-driven motors acting in the microtubule-based motilities of organelles in eukaryotic cells. More than 30 members of murine kinesin superfamily proteins (KIFs) have been identified so far [34] whose detailed identification of the cargo organelles is not yet fully understood. It has been shown that microtubule-based motors play an important role in membrane trafficking pathways such as ER formation, membrane recycling from transitional ER back to ER or vesicle transport from the Golgi region to the plasma membrane. Recent findings that anti-kinectin antibodies can inhibit microtubule-based vesicle movement and kinesin binding and its localization to the ER suggest that kinectin is one essential membrane anchor used to connect vesicles to the microtubule-based transport machinery [21]. It is conceivable that the cleavage of kinectin will result in disruption of the highly regulated membrane trafficking pathways by disconnecting the membrane vesicles from the microtubule-based transport mechanisms.

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