IDENTIFICATION OF FUNCTIONAL REGIONS DEFINING DIFFERENT ACTIVITY BETWEEN CASPASE-3 AND CASPASE-7 WITHIN CELLS

Hirokazu Nakatsumi¹ and S Yonehara¹

Laboratory of Molecular and Cellular Biology, Department of Animal Development and Physiology, Graduate School of Biostudies, Kyoto University, Kyoto 606-8501, Japan

Running head: Analysis of differences between caspase-3 and 7

2 to whom correspondence should be addressed: Laboratory of Molecular and Cellular Biology, Graduate School of Biostudies, Kyoto University, SCRB/Building G, Yoshida Konoe-cho, Sakyu-ku, Kyoto 606-8501, Japan. Fax: +81 75 753 9235; E-mail: yonehara@lif.kyoto-u.ac.jp

1 Present address: Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8282, Japan

Caspases are central to apoptosis, and the principal executioner caspases, caspase-3 and caspase-7, were reported to be similar in activity, primary structure and 3-dimensional structure. Here we identified different activity between caspase-3 and caspase-7 within cells and examined the relationship between their structure and function by using human cells expressing almost equal amounts of exogenous caspase-3, caspase-7 and/or chimeric constructs after downregulation of endogenous caspase-3 and caspase-7 expression. Caspase-3, produced in human cells, showed much stronger cleaving activity than caspase-7 against a low molecular weight substrate in vitro dependent on four specific amino acid regions. Within cells, however, additional three regions were required for caspase-3 to exert much stronger protease activity than caspase-7 against cellular substrates. Three of the former four regions and the latter three regions were shown to form two different 3-dimensional structures that located at the interface of the homo-dimer of procaspase-7 on opposite sides. In addition, procaspase-3 and 7 revealed specific homo-dimer-forming activity within cells dependent on five amino acid regions, which were included in the regions critical to the cleaving activity within cells. Thus human caspase-3 and 7 exhibit differences in protease activity, specific homo-dimer-forming activity and 3-dimensional structural features, all of which are closely interrelated.

Apoptosis, or programmed cell death, plays an essential role in the development and homeostasis of metazoans. Deregulation of apoptosis leads to a variety of pathologic disorder including cancer, autoimmune diseases and neurodegenerative disorders. Caspases, part of the cysteine protease family, are central to the initiation and execution of apoptosis, acting to specifically cleave the carboxyl terminal side of an aspartate residue in substrates. In mammals, 14 kinds of caspases have been identified at least 7 of which are assumed to be involved in apoptosis (1,2). The apoptotic caspases are generally classified into initiator and executioner caspases. The former include caspase-8, 9 and 10, and the latter, caspase-3, 6 and 7 (3,4).

The activation of initiator caspases inevitably triggers the activation of caspase cascades. Activated initiator caspases are able to activate downstream executioner caspases through proteolytic cleavage. Once executioner caspases are activated, they execute apoptosis by proteolytically cleaving a variety of cellular death substrates. Executioner caspases form a homo-dimer both before and after activation by intra-chain cleavage. Caspases are also characterized by a preference for recognition sites in their substrates, as determined by studies using synthetic peptides and/or peptide libraries (5,6). The preferred substrate of caspase-3 and 7 is known as DEXD, whereas that of caspase-8, 9 and 10 is known as I/LEXD (2).

Among executioner caspases, caspase-3 and 7 share 54 percent identity in amino acid (aa) sequence (7) and exhibit a highly similar 3-dimensional (3D) structure. These two caspases, which are generally expressed in mammals, were reported to show similar functions in vitro and homology in their inherent substrate preference. The 3D structures of dimeric procaspase-7, active caspase-7, and substrate-bound forms of active caspase-7 have been resolved (8-10). The structure of substrate-bound forms of dimeric active caspase-3 was also reported and shows many similarities to that of active caspase-7 (11,12). Both caspase-3 and 7 were shown to have grooves for directly binding to their substrates, and these grooves bear a close resemblance in
structure (13). The groove is constructed of four surface loops, L1, L2, L3, and L4 (8). L2 contains a site of cleavage by initiator caspases in apoptosis.

In caspase-3 and 7 double-knockout mouse embryonic fibroblast cells, csapases-3 and 7 have been shown to have some overlapping, but some different, roles in apoptosis (14); however, the precise relationship between their structure and function in apoptosis has not been clear. Here we identified functional regions defining differences between caspase-3 and 7 in the selection of a partner for dimerization and execution of apoptosis within cells, indicating that the structural regions responsible for defining the two types of activity are essentially the same.

Experimental Procedures

Cell lines and reagents- Human HeLa and Jurkat cells were cultured in Dulbecco's Modified Eagle's medium and RPMI 1640 medium (Nacalai Tesque), respectively, supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 mg/ml of streptomycin at 37°C in 5% CO2. Monoclonal anti-human Fas antibody (CH-11) was obtained from MBL Co. (Nagoya, Japan) (15). Cycloheximide (CHX) was purchased from Nacalai Tesque.

Western blotting and antibodies- Cells were suspended in ice-cold lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 0.5% sodium deoxycholate) containing a protease inhibitor cocktail (Roche Diagnostics). For immunoprecipitation, the cell lysate was incubated with anti-flag M2 antibody for 1 h and subsequently with protein G Sepharose (Amersham) for 1 h at 4°C. The precipitate was extensively washed with lysis buffer and eluted with 0.3 mg/ml of flag-peptide for 1 h at 4°C.

Cell lysates and immunoprecipitates were resolved by SDS-PAGE and analyzed by western blotting as described earlier (16). The antibodies used in this study were those for caspase-3 (Cell Signaling), caspase-7 (4G2; MBL), flag-tag (M2; Sigma), HA-tag (12CA5; Sigma), Lamin A/C (Cell Signaling), cleaved SETβ (MBL) and actin (C4; Chemicon).

cDNAs- For the expression of exogenous caspase-3, caspase-7 and chimeric caspases in DKD cells, we utilized cDNAs with silent mutations in the target nucleotides of the shRNAs used in the generation of DKD cells. All the cDNAs with silent mutations were generated with a QuikChange Site-Directed Mutagenesis Kit (Stratagene). cDNAs for chimeric caspases were generated using a PCR-driven overlap extension method as described (17), and/or a QuikChange Site-Directed Mutagenesis Kit (Stratagene). Primer sequences are listed in Supplementary Table S1 and S2.

Preparation of lentiviral vectors- Lentiviral vectors, provided by H Miyoshi (RIKEN), were prepared as described (18,19). For the expression of caspase-3, caspase-7 and the chimeric constructs, their cDNA fragments were inserted into pCSII-EF-MCS. For gene silencing with the RNAi system, we used a lentivirus-based shRNA expression vector, CSII-U6 (provided by Y Satou and M Matsuoka, Kyoto University) and CSII-U6-puro vectors. The target sequences of the shRNAs were as follows: GATCGTTGTAGAAGTCTAA for caspase-3 cDNA (GenBank accession number NM_004346), and GTACCGTCCCTTTCAGTA for caspase-7 cDNA (NM_001227). The shRNA-encoding DNA oligonucleotide inserts were generated using the Insert Design Tool for pSilencer Vectors (Applied Biosystems, http://www.ambion.com/techlib/misc/silencer_converter.html).

Transient Transfection of Expression Vectors- For transient transfection, HeLa cells were seeded at 1 x 105 cells per well in 6-well plates, cultured for 1 day and then transfected with various expression vectors (0.7 µg/each well) by using Lipofectamine plus (Invitrogen) according to the manufacturer's protocol.

Colorimetric assay of DEVDase Activity in vitro- DEVDase activity was measured using the APOPCYTO caspase 3 colorimetric assay kit (MBL) according to the manufacturer's directions.

Analyses of DNA fragmentation Cells were suspended in lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM EDTA, and 0.5% Triton-X 100), and the lysates were centrifuged at 15,000 rpm for 20 min. The resulting supernatants were incubated with 4 µl of RNase A (10mg/ml; Wako) for 1 h at 37°C followed by 4 µl of proteinase K (10 mg/ml; Wako) for 1 h at 56°C. After electrophoresis on a 2% agarose gel, DNA fragmentation was visualized by staining with ethidium bromide.
RESULTS

Evaluation of physiological activities of caspase-3 and caspase-7 within human cell. To examine the role of caspase-3 and 7 within human cells, we generated double knockdown cells in which expression of both caspase-3 and 7 was down-regulated (DKD cells). The expression of caspase-3 and 7 in human HeLa and Jurkat cells was down-regulated by the expression of short hairpin RNAs (shRNAs) using lentiviral vectors (Fig. 1A). In Fas-stimulated DKD HeLa cells, apoptotic morphological change was significantly inhibited (Fig. 1B). In addition, activity to cleave a low-molecular-weight-compound, DEVD-p-nitroanilide (pNA), in vitro (in vitro DEVDase activity) as well as activity to cleave lamin A and SETβ within cells, observed during apoptosis, was also dramatically suppressed (Figs. 1C & D). Lamin A was reported to be directly cleaved by caspase-6, which is a direct target of caspase-3, while SETβ is a direct target of caspase-3 and/or caspase-7 (20,21). In DKD Jurkat cells, the formation of DNA ladder was also remarkably inhibited (Fig. 1E), indicating that Fas-mediated apoptosis is inhibited in DKD Jurkat cells. Taken together, not only cell death but also cleavage of the death substrates and the formation of DNA ladder were indicated to be dependent on caspase-3 and 7 in Fas-stimulated human cells.

To compare the activities of caspase-3 and 7 as an executioner caspase within cells, we next generated DKD cells expressing an almost equal amount of exogenous flag-tagged caspase-3 or caspase-7 with silent mutations in the target nucleotides of the shRNAs (Fig. 2A). These cells make it possible for the first time to avoid the influence of different expression levels of caspase-3 and 7 when comparing their enzymatic activities within human cells. In flag-caspase-3-expressing DKD HeLa cells, in vitro DEVDase activity recovered to relatively higher levels than in parental cells (Fig. 2B), and cleaving activity for the cellular death substrates within cells also recovered (Fig. 2C). In DKD Jurkat cells, the expression of flag-caspase-3 recovered the DNA ladder formation (Fig. 2D). In flag-caspase-7-expressing DKD HeLa cells, however, in vitro DEVDase activity did not significantly increase when compared to that in DKD cells, and was much weaker than that in flag-caspase-3-expressing cells (Fig. 2B). Cleaving activity for cellular death substrates within the Fas-stimulated cells was not sufficiently recovered in flag-caspase-7-expressing DKD HeLa cells, although both flag-procaspase-3 and flag-procaspase-7 disappeared after stimulation of Fas probably through auto-cleavage (Fig. 2C). In DKD Jurkat cells, the expression of flag-caspase-7 could not sufficiently recover the DNA ladder formation (Fig. 2D). All the results indicate that human caspase-3 and 7, produced and activated in human cells, differ in cleavage of cellular death substrates within cells, DNA ladder formation and in vitro DEVDase activity. These results essentially coincide with the previously reported results showing in vitro different activity of caspase-3 and 7 produced in bacteria (22).

Identification of aa regions involved in the selection of a specific partner. Caspase-3 and 7 differ distinctly in activities within cells, and the difference would be caused by different aa sequences. To identify the regions important for their different activities, we generated various chimeric constructs. When generating chimeric executioner caspases, however, it is necessary to keep intact the homo-dimer-forming activity of the precursor forms, because both procaspase-3 and 7 form homo-dimers and the dimerization is necessary to exert sufficient protease activity in their cleaved active forms (23,24). Thus, before examining their activities, it was necessary to reveal aa regions determining the specific partner for dimerization in procaspase-3 and 7 within human cells.

We first analyzed the accuracy of the homo-dimer’s formation in procaspase-3 and 7. HA-procaspase-3 and 7 were simultaneously expressed in DKD HeLa cells together with flag-caspase-3 or 7, and a co-immunoprecipitation analysis with anti-flag antibody was carried out. HA-procaspase-3 was co-immunoprecipitated with flag-procaspase-3 but not with flag-procaspase-7. Similarly, HA-procaspase-7 was co-immunoprecipitated with flag-procaspase-7 but not with flag-procaspase-3 (Fig. 3A). Thus, both procaspase-3 and 7 form only homo-dimers within cells.

We then analyzed the regions important for determining the specificity with which procaspase-3 and 7 select a dimerization partner. We generated and analyzed various chimeric constructs by combining 21 separate blocks of aa sequences (Fig. 3B), and analyzed which blocks are necessary for the selection of a partner. We
identified five important regions named A, B, C, I and II (Fig. 3B). A procaspase-7-based chimera, in which regions A, B, C, I, and II were replaced by those of procaspase-3, designated procaspase-7 (ABCD I II), could form homo-dimer and a dimer with procaspase-3 but not with procaspase-7 (Fig. 3A). Interestingly, a procaspase-3-based chimera, designated procaspase-3 (I II), in which regions I and II of procaspae-3 were replaced by those of procaspase-7, could form a dimer with both procaspase-3 and 7. In contrast, procaspase-7 (ABC) could form a dimer with both procaspase-3 and 7, while procaspase-7 (I II) could form dimer with neither procaspase-3 nor 7 (Fig. 3A). We could analyze neither procaspase-7 (I II) nor procaspase-3 (ABCD I II) because expression levels of these molecules were too low to be detected. Taken together, procaspase-3 seems to be able to form a dimer with molecules containing regions A, B and C of procaspase-3, and procaspase-7 seems to be able to form a dimer with molecules containing regions I and II of procaspase-7.

Regions A, B, C, I and II are scattered throughout the primary aa sequence (Fig. 3B). Then, we highlighted these regions in the tertiary structure of procaspase-7 (PDB ID: 1GQF) (10) (Fig. 3C). Regions I and II, derived from both partners, are located near each other, and occupy a position straddling the homo-dimer interface. Similarly, regions A, B and C, derived from both partners, are also located close together, occupying a position straddling the homo-dimer interface on the opposite side of regions I and II. We designated the 3D structural regions composed of regions I and II, and regions A, B and C as structural region I II and structural region ABC, respectively. Interestingly, these two structural regions are located on the opposite sides of procaspase, separated by a curtain composed of 12 β-sheets.

We next analyzed which is most important among regions A, B and C of procaspase-3 in the formation of a dimer with procaspase-3. While procaspase-7 (ABC I II) can interact with procaspase-3 but not with procaspase-7, procaspase-7 (ABC) can bind to both procaspase-3 and 7. Then, we examined whether procaspase-7 (A I II), procaspase-7 (B I II) and procaspase-7 (C I II) can form a dimer with procaspase-3. Only procaspase-7 (B I II) could bind to procaspase-3 (Fig. 3D and data not shown), indicating region B to be most important. Procaspase-3 (B I II) could not bind procaspase-3 but could bind procaspase-7, while procaspase-3 (I II) could interact with both procaspase-3 and 7, supporting the idea that region B is most important in the formation of a homo-dimer with procaspase-3.

We then succeeded in narrowing down the range of regions B and II to b and ii, respectively. Procaspase-7 (b I ii) could form a dimer with caspase-3 but not with caspase-7 (Fig. 3E). Regions b and I ii were located at the center of structural regions ABC and I II, respectively, in the 3D structure of procaspase-7 (Fig. 3F). All the results indicate that the center of structural region ABC and structural region I II exhibits the most important effect on the selection of a partner for dimerization.

Identification of aa regions required for the strong DEVDase activity of caspase-3 in vitro

We generated caspase-7-based chimeric constructs with various regions of caspase-3, and analyzed their DEVDase activity in vitro. We exogenously expressed almost equal amounts of flag-caspase-3, flag-caspase-7 and the chimera in DKD HeLa cells (Fig. 4A), and identified important regions of caspase-3 required for strong DEVDase activity in vitro. Two caspase-7-based chimeric constructs, caspase-7 (D) and caspase-7 (ABC) (Fig. 3B), showed stronger DEVDase activity than flag-caspase-7 (Fig. 4B).

In the primary aa sequence, region D is located next to loop L3 which was previously reported to be spatially positioned near the active center (11,12) and to contain aa residues which make direct contact with the low molecular weight substrate DEVD (13). The difference in region D between caspase-3 and 7, which contains residues for directly binding to DEVD-pNA, would affect their distinct in vitro DEVDase activity.

Interestingly, regions ABC are identical to those important for selecting a partner for dimerization. Region B contains one of the cleavage sites of the procaspase and both cleaved parts of region B were reported to change their spatial allocation during activation. Caspase-7 (AbC) shows almost equal activity to caspase-7 (ABC) (Figs. S1A & B), indicating that regions A, b and C are important for caspase-3 to reveal strong DEVDase activity in vitro. Region b is located on the small subunit side of region B which was reported to move and make loop bundles together with previously reported loops L2 and L4 of the other subunit in the pro-form during caspase activation. To
examine whether formation of the loop bundles is important for caspase-3 to effectively cleave DEVD-pNA, we generated caspase-7 knockdown HeLa cells expressing a much larger amount of mutant caspase-3 than endogenous caspase-3, in which both Cys 163 and Arg 207 at the active center and Asp 175 at the site of cleavage between the large and small subunits are replaced by Ala and Ala, and by Glu, respectively (Fig. S2A) (25). In these cells, expression of the mutant caspase-3 is expected to inhibit the formation of a homo-dimer of endogenous caspase-3 and to form a hetero-dimer between endogenous wild-type and mutant caspase-3. The hetero-dimer showed much weaker DEVDase activity in vitro than another hetero-dimer between wild-type and mutant caspase-3 in which the active center is inactivated but the inter-cleavage site is intact (Fig. S2B). These results suggest that the sufficient in vitro DEVDase activity of caspase-3 is provided through formation of the loop bundles with the small subunit side of region B (region b) in the partner in the pro-form. Taken together, region b would be important for caspase-3 in not only selecting its partner but also exerting strong DEVDase activity depending on the formation of the loop bundles.

To kinetically reveal the different enzymatic activities of caspase-3, caspase-7 and the chimera, we analyzed their DEVD-pNA-cleaving activity in vitro using the Michaelis-Menten equation and calculated Km and Vmax parameters with Eadie-Hofstee plots (Figs. S3A & B). Vmax values differed significantly among caspase-3, caspase-7, caspase-7 (D) and caspase-7 (ABC), consistent with their DEVDase activity, while Km values were similar. The different levels of DEVDase activity were indicated to depend on different reaction rates.

We then generated DKD HeLa cells expressing caspase-7 (ABCD) and analyzed DEVDase activity in vitro (Figs. 4C & D). Caspase-7 (ABCD) showed much higher and sufficiently higher levels of activity than caspase-7 and caspase-3, respectively, indicating the difference in DEVDase activity between caspase-3 and 7 to depend on the difference in regions A, B, C, and D.

Important aa regions for the strong activity of caspase-3 in cleaving its substrates within cells Next, we examined whether the chimeric constructs, caspase-7 (D), caspase-7 (ABC) and caspase-7 (ABCD), cleave cellular substrates in a similar manner to caspase-3 within cells. Surprisingly, all the chimera showed much lower activity levels than caspase-3 for the cleavage of SETβ and caspase-6-mediated cleavage of LaminA (Fig. 4F). These results suggest that in vitro DEVDase activity is not equivalent to intracellular cleaving activity and regions other than A, B, C, and D must be involved in the cleavage of cellular substrates within cells.

To identify regions important for the difference in activity to cleave cellular substrates between caspase-3 and 7, we generated new chimeric constructs based on caspase-7 (ABCD). Finally, we found that caspase-7 (ABCD I II III) expressed in DKD HeLa cells showed caspase-3-like activity to cleave lamin A and SETβ within cells as well as the cleavage of DEVD-pNA in vitro (Figs. 5A-C). In addition, caspase-7 (ABC I II III) was shown to induce the formation of a DNA ladder as well as caspase-3 in Jurkat cells (Fig. 5D). Taken together, regions A, B, C, D, I, II, and III of caspase-3 are all required for caspase-7 to exert strong caspase-3-like activity to cleave not only DEVD-pNA in vitro but also cellular substrates within cells (Fig. 5B).

**DISCUSSION**

We investigated the molecular basis for the difference in activity of executioner caspases using DKD cells expressing almost equal amounts of caspase-3, caspase-7, or chimeric constructs. This is a powerful way to analyze the different activities of analogous molecules within cells. Hence, we could closely compare caspase-3, caspase-7 and the chimera and found that the caspases differ in protease activity, and the difference depends on 7 regions designated A, B, C, D, I, II and III. In addition, it is worth noting that the regions important for the selection of specific partners are among those crucial to the difference in protease activity between caspase-3 and 7.

Caspase-3 and 7 differed in activity to cleave cellular substrates within cells as well as a low molecular weight substrate, DEVD-pNA, in vitro. To our astonishment, although replacing regions A, B, C and D of caspase-7 with those of caspase-3 was sufficient to convert the in vitro DEVDase activity of caspase-7 to that of caspase-3, it was not sufficient to convert the cellular substrate-cleaving activity of caspase-7 to that of caspase-3 within cells. The difference in substrate-cleaving activity within cells was
clearly shown to be dependent on regions I, II and III in addition to regions A, B, C and D. While regions A, B, C and D would be responsible for determining the preference for peptides such as DEVD and the reaction rate of DEVDase activity in vitro (Fig. S3), regions I, II and III may determine additional substrate specificity, which has not been clarified. These results are well consistent with the observation that the preferred peptide sequence of caspase-3 is not identical to the peptide sequences in preferred cellular substrates of caspase-3 (3,26).

Regions A, B, C, D, I, II and III in caspase-3 and 7 could be classified into three groups by position in the tertiary structure; structural region ABC, structural region I II III, and region D. Structural regions ABC and I II III each straddle the dimer’s interface by interacting with the same structural region of the partner. These two structural regions lie on the opposite sides of caspases separated by a curtain of β-sheets. Meanwhile, region D, which is situated away from the dimer’s interface in the 3D structure, is not engaged in the homo-dimer’s formation. Because region D and structural region ABC had a synergistic effect on DEVDase activity, they would function differently to regulate the DEVDase activity. Region D may influence the DEVDase activity by directly affecting the binding of the substrate, DEVD-pNA, and/or its reaction intermediate.

For both the selection of a partner for dimerization and DEVDase activity in vitro, the most important part of region B was region b. When cleaved executioner caspases bind to their substrates, the small subunit side of region B containing region b reportedly assembles the so-called “loop-bundle” together with loops L2 and L4 of the partner. Because the “loop-bundle” has been considered important to the protease activity of executioner caspases, structural region ABC may affect DEVDase activity by regulating its formation. In addition, part of region B containing region b was reported to bury a central cavity located at the center of structural region ABC in both caspase-3 and 7 (10). The central cavity was reported to be opened when the catalytic groove is occupied by a substrate, but closed when the groove is empty (9,27). The burying of the central cavity in structural region ABC may be involved in the regulation of in vitro DEVDase activity.

Structural regions ABC and I II III have several structural and functional characteristics in common. It has been unclear how the structural region I II III, which does not contribute to DEVDase activity, contributes to the cellular substrate-cleaving activity within cells. A cavity other than the central cavity was reported to be located at the center of structural region I II III in caspase-7 but not in caspase-3 (28), whereas the central cavity is observed in both caspase-3 and 7. The caspase-7-specific cavity in structural region I II III of caspase-7 might be involved in the impaired cellular substrate-cleaving activity within cells during apoptosis. Recently, a non-redundant function of caspase-7 was reported in LPS-induced apoptosis (29), suggesting that caspase-7 and caspase-3 infrequently show independent apoptosis-inducing functions in vivo. Structural region I II III of caspase-7 containing the caspase-7-specific cavity may be involved in the specific function of caspase-7 in apoptosis.

In this study, the biological functions and structural characteristics of executioner caspases, caspase-3 and 7, during apoptosis were examined by generating and analyzing chimera. The structural analysis of our chimeric caspases will lead to further clarification of the apoptotic cascade by resolving the relation between the structure and functions of executioner caspases, caspase-3 and 7.
REFERENCES


-7-

**FOOTNOTES**

The abbreviations used are: 3D, 3-dimensinal; aa, amino acid; CHX, cycloheximide; DEVD-pNA, Asp-Glu-Val-Asp-p-nitroanilide; DKD cells, double knockdown cells in which expression of both caspase-3 and 7 is down-regulated; shRNAs, short hairpin RNAs.
FIGURE LEGENDS

Fig. 1. Inhibition of Cell death by expression of shRNAs specific to caspase-3 and 7. A. HeLa and Jurkat cells were simultaneously infected with lentiviral vectors encoding shRNAs to caspase-3 and -7 (sh-casp3/7) and total cell extracts were analyzed by immunoblotting with anti-caspase-3 (anti-casp3) and anti-caspase-7 (anti-casp7) antibodies. Actin was also analyzed as a control. B. HeLa cells, expressing sh-lacZ or sh-casp3/7, were treated with 250 ng/ml of anti-Fas mAb CH-11 together with 1 µg/ml of CHX for 4 h and observed under a phase contrast microscope. C. Whole extracts of the HeLa cells described in (B) were prepared and analyzed for activity to cleave the colorimetric caspase substrate DEVD-pNA in vitro. Relative cleaving activity was calculated by comparing the DEVDase activity of each sample to that of sh-lacZ-expressing control cells. The results represent the mean ± SD for at least three independent experiments. The zero point represents the DEVDase activity of control cells treated with neither CH-11 nor CHX. D. Extracts of the HeLa cells described in (B) were prepared and analyzed by immunoblotting with antibodies specific for targets of executioner caspases, SETβ and lamin A. The bands of cleaved products are indicated. E. Jurkat cells were left untreated or treated with 250 ng/ml of CH-11 together with 1 µg/ml of CHX for 2 h and DNA prepared from the cells was analyzed by 1.8 % agarose gel electrophoresis to visualize any DNA-ladder.

Fig. 2. Analysis of exogenously expressed caspase-3 and 7 in DKD cells. A. DKD HeLa cells and DKD Jurkat cells were infected with an empty lentiviral vector (control) or a lentiviral vector encoding FLAG-tagged caspase-3 (flag-casp3) or caspase-7 (flag-casp7). Total cell extracts were analyzed by immunoblotting with anti-caspase-3 and anti-caspase-7 antibodies. B. DKD HeLa cells expressing flag-caspase-3 or 7 were treated with 250 ng/ml of CH-11 and 1 µg/ml of CHX for 4 h. Whole cell extracts were prepared and analyzed for DEVDase activity, as described in Fig. 1B. The zero point represents the DEVDase activity of DKD HeLa cells not expressing exogenous executioner caspases treated with 250 ng/ml of CH-11 and 1 µg/ml of CHX for 4 h. C. The HeLa cells described in (A) were treated with CH-11 as in (B) and then cell extracts were analyzed for the proteolytic cleavage of SETβ and lamin A as described in Fig. 1C. D. Jurkat cells expressing flag-caspase-3 or 7 as in (A) were left untreated or treated with 250 ng/ml of CH-11 and 1 µg/ml of CHX for 2 h and analyzed for a DNA-ladder as described in Fig. 1D.
Fig. 3. Dimer-forming activity of caspase-3, caspase-7 and the chimeric constructs. A. DKD HeLa cells were transfected with HA-tagged caspase-3 (c3) or 7 (c7) together with flag-tagged caspase-3, caspase-7 or chimera as indicated in the figure. Cell lysates were immunoprecipitated (IP) with anti-flag antibody and the immunoprecipitates were analyzed by Western blotting (WB) with anti-flag or anti-HA antibody. Total cell lysates were also analyzed by WB. B. Primary amino acid (aa) sequences of human caspase-3 and 7 were aligned. Caspase-3 and 7 were similarly fractionated into 21 blocks, which are separated by vertical lines in the figure. Regions A, B and C; D; I and II; and III are indicated by pink, purple, cyan and blue lines, respectively. Region b and ii are the divided parts of region B and II, respectively. The positions of previously reported loops, L1, L2, L3 and L4, are also indicated by lines on top of the aa sequences. C. Tertiary structural views of procaspase-7 (PDB ID: 1GQF) are depicted from the side with regions A, B, C and D (left), and from the opposite side with regions I, II and III (right). Regions ABC, D, I II, and III are highlighted by pink, purple, cyan and blue lines, respectively, as in (B). E. DKD HeLa cells were co-transfected with flag- and HA-tagged-caspases, and a co-immunoprecipitation analysis was carried out as in a. F. Tertiary structural views of procaspase-7 (PDB ID: 1GQF) are depicted from the side with region b (left), and from the opposite side with regions I and ii (right). Regions b and I ii are highlighted by pink and cyan lines, respectively.

Fig. 4. Comparison of in vitro and in vivo protease activity of exogenously expressed caspase-3, caspase-7 and chimeric constructs. A. DKD HeLa cells were infected with a lentiviral vector encoding flag-tagged caspase-3, caspase-7, caspase-7 (D) or caspase-7 (ABC), and total cell extracts were analyzed by immunoblotting with anti-flag or anti-actin antibody. B. DKD HeLa cells in (A) were stimulated with anti-Fas mAb CH-11 and CHX and DEVDase activity in vitro was analyzed as described in Fig. 2B. C. DKD HeLa cells, infected with an empty lentiviral vector or a lentiviral vector described in (A), were treated with 250 ng/ml of CH-11 and 1 µg/ml of CHX for 4 h. Cell extracts were prepared and analyzed for the proteolytic cleavage of SETβ and lamin A as described in Fig. 1C. D. DKD HeLa cells were transiently transfected with an empty vector (control) or a vector encoding flag-tagged caspase-3, caspase-7 or caspase-7 (ABCD), and cultured for 16 h. Total cell extracts were analyzed by immunoblotting with anti-flag antibody. E. DEVDase activity of Fas-stimulated DKD HeLa cells in d was analyzed as in b. F. Proteolytic cleavage of SETβ and lamin A was analyzed in Fas-stimulated DKD cells in d.
Fig. 5. Caspase-7 (ABCD I II III) exhibits caspase-3-like activity both in vitro and in vivo. A. DKD HeLa and DKD Jurkat cells were infected with a lentiviral vector encoding flag-tagged caspase-3, caspase-7 or caspase-7 (ABCD I II III), and total cell extracts were analyzed by immunoblotting with anti-flag and anti-actin antibodies. B. The DKD HeLa cells in (A) were stimulated with anti-Fas mAb CH-11 and CHX and in vitro DEVDase activity was analyzed as described in Fig. 2B. C. The DKD HeLa cells in (A) were stimulated with anti-Fas mAb CH-11 and CHX and proteolytic cleavage of SETβ and lamin A was analyzed as described in Fig. 1C. D. The DKD Jurkat cells in (A) were stimulated with anti-Fas mAb CH-11 and CHX and the formation of a DNA ladder was analyzed as described in Fig. 1D.
Figure 1

A

<table>
<thead>
<tr>
<th></th>
<th>HeLa</th>
<th>Jurkat</th>
</tr>
</thead>
<tbody>
<tr>
<td>sh-lacZ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sh-casp3/7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-casp3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-casp7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-actin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

Fas stimulation 4h

sh-lacZ  sh-casp3/7

C

<table>
<thead>
<tr>
<th>relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>0.8</td>
</tr>
<tr>
<td>0.6</td>
</tr>
<tr>
<td>0.4</td>
</tr>
<tr>
<td>0.2</td>
</tr>
<tr>
<td>0</td>
</tr>
</tbody>
</table>

D

Fas stimulation 0h  4h

sh-lacZ  sh-casp3/7  sh-lacZ  sh-casp3/7

anti-lamin A  anti-SETβ  cleaved lamin A  cleaved SETβ

E

Fas stimulation 0h  2h

sh-lacZ  sh-casp3/7  sh-lacZ  sh-casp3/7


Figure 4

A

B

C

D

E

F

anti-flag

anti-actin

anti-lamin A

anti-SET?

cleaved lamin A

cleaved SET?

casps3

casps7 (A)

casps7 (ABC)

relative activity

0h

4h

control

casps3

casps7 (D)

casps7 (ABC)

relative activity

0h

4h

control

casps3

casps7

casps7 (ABCD)

relative activity

0h

4h

control

casps3

casps7

casps7 (ABCD)
Figure 5

A

DKD HeLa

DKD Jurkat

casp3
casp7

anti-flag

anti-actin

casp3

casp7

c7 (ABCD III)

C

Fas stimulation

0h

4h

control

casp3

casp7

c7 (ABCD III)

anti-lamin A

anti-SET8

cleaved lamin A

cleaved SET8

B

relative activity

0

0.2

0.4

0.6

0.8

1

1.2

casp3
casp7
c7 (ABCD I II III)

D

Fas stimulation

0h

2h

control
casp3
casp7

c7 (ABCD III)

Downloaded from http://www.jbc.org/ by guest on July 16, 2017
Identification of functional regions defining different activity between caspase-3 and caspase-7 within cells
Hirokazu Nakatsumi and Shin Yonehara

J. Biol. Chem. published online June 21, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M110.126573

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2010/06/21/M110.126573.DC1

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2010/06/21/jbc.M110.126573.full.html#ref-list-1