Caspases are central to apoptosis, and the principal executioner caspases, caspase-3 and -7, were reported to be similar in activity, primary structure, and three-dimensional structure. Here, we identified different activity in caspase-3 and -7 within cells and examined the relationship between their structure and function using human cells expressing almost equal amounts of exogenous caspase-3, caspase-7, and/or chimeric constructs after down-regulation of endogenous caspase-3 and -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, an 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 three-dimensional structures that were located at the interface of the homodimer of procaspase-7 on opposite sides. In addition, procaspase-3 and -7 revealed specific homodimer-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 homodimer-forming activity, and three-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 disorders, 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 C-terminal side of an aspartate residue in substrates. In mammals, 14 kinds of caspases have been identified, at least seven 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 include 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 homodimer both before and after activation by intrachain 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 caspase-7 is DEXD, whereas that of caspase-8, -9, and -10 is (I/L)EXD (2).

Among executioner caspases, caspase-3 and caspase-7 share 54% identity in amino acid (aa)3 sequence (7) and exhibit a highly similar three-dimensional 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 three-dimensional 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 caspase-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 caspase-7 double knock-out mouse embryonic fibroblast cells, caspase-3 and caspase-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 in caspase-3 and caspase-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 units/ml penicillin, and 100

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3 The abbreviations used are: aa, amino acid; CHX, cycloheximide; DKD, double knockdown; pNA, p-nitroanilide.
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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 mixture (Roche Diagnostics). For immunoprecipitation, the cell lysate was incubated with anti-FLAG antibody M2 for 1 h and subsequently with protein G-Sepharose (Amersham Biosciences) for 1 h at 4 °C. The precipitates were extensively washed with lysis buffer and eluted with 0.3 µg/ml FLAG-peptide for 1 h at 4 °C. Cell lysates and immunoprecipitates were resolved by SDS-PAGE and analyzed by Western blotting as described previously (16). The antibodies used in this study were those for caspase-3 and lamin A/C (Cell Signaling), caspase-7 (4G2) and cleaved SETβ (MBL Co.), FLAG tag (M2) and HA tag (12CA5) (Sigma), and actin (C4) (Chemicon).

cDNAs—For the expression of exogenous caspase-3, caspase-7, and chimeric caspases in double knockdown (DKD) cells, we utilized cDNAs with silent mutations in the target nucleotides of the shRNAs used in the generation of DKD cells. All of the cDNAs with silent mutations were generated with the QuikChange site-directed mutagenesis kit (Stratagene). cDNAs for chimeric caspases were generated using a PCR-driven overlap extension method as described (17) and/or the QuikChange site-directed mutagenesis kit. Primer sequences are listed in supplemental Tables 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 GTACGGTCCTCCTTCAGTA for caspase-7 cDNA (accession number NM_001227). The shRNA-encoding DNA oligonucleotide inserts were generated using the Insert Design Tool for pSilencer vectors (Applied Biosystems).

Transient Transfection of Expression Vectors—For transient transfection, HeLa cells were seeded at 1 × 10^5 cells/well in 6-well plates, cultured for 1 day, and then transfected with various expression vectors (0.7 µg each well) using Lipofectamine Plus (Invitrogen) according to the manufacturer’s protocol.

RESULTS

Evaluation of Physiological Activities of Caspase-3 and Caspase-7 within Human Cells—To examine the role of caspase-3 and caspase-7 within human cells, we generated double knockdown cells in which expression of both caspase-3 and caspase-7 was down-regulated (DKD cells). The expression of caspase-3 and caspase-7 in human HeLa and Jurkat cells was down-regulated by the expression of shRNAs using lentiviral vectors (Fig. 1A). In Fas-stimulated DKD HeLa cells, apoptotic morphological change was significantly inhibited (Fig. 1B). In addition, the activity cleaving a low molecular weight compound, DEVD-p-nitroanilide (pNA), in vitro (in vitro DEVDase activity) and the activity cleaving lamin A and SETβ within cells, observed during apoptosis, were also dramatically suppressed (Fig. 1, C and D). Lamin A was reported to be a direct target of caspase-6, which is a direct target of caspase-3, whereas SETβ is a direct target of caspase-3 and/or caspase-7 (20, 21). In DKD Jurkat cells, the formation of a DNA ladder was also remarkably inhibited (Fig. 1E), indicating that Fas-mediated apoptosis is inhibited in DKD Jurkat cells. Taken together,
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FIGURE 2. Analysis of exogenously expressed caspase-3 and caspase-7 in DKD cells. A, DKD HeLa and 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 FLAG-caspase-7 were treated with 250 ng/ml CHX for 2 h and analyzed for a DNA ladder. C, anti-FLAG, anti-lamin A, and anti-SETB were used for immunoprecipitation. D, DKD HeLa and Jurkat cells were infected with an empty lentiviral vector (control) or a lentiviral vector encoding FLAG-caspase-3 or FLAG-caspase-7. Total cell extracts were analyzed by immunoblotting with anti-caspase-3 and anti-caspase-7 antibodies.

We first analyzed the accuracy of the formation of the homodimer in procaspase-3 and procaspase-7. HA-procaspase-3 and HA-procaspase-7 were simultaneously expressed in DKD HeLa cells together with FLAG-caspase-3 or FLAG-caspase-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 procaspase-7 form only homodimers within cells.

We then analyzed the regions important for determining the specificity with which procaspase-3 and procaspase-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 with those of procaspase-3, designated procaspase-7(ABCII), could form a homodimer and a dimer with procaspase-3 but not with procaspase-7 (Fig. 3A). Interestingly, a procaspase-3-based chimera in which regions I and II of procaspase-3 were replaced with those of procaspase-7, designated procaspase-3(I), could form a dimer with both procaspase-3 and procaspase-7. In contrast, procaspase-7(ABC) could form a dimer with both procaspase-3 and procaspase-7, whereas procaspase-7(I) could form a dimer with either (Fig. 3A). We could not analyze either procaspase-7(II) or procaspase-3(ABCDII) because the expression levels of these molecules were too low to be...
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FIGURE 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 caspase-7 (c7) together with FLAG-tagged caspase-3, caspase-7, or the chimera as indicated. 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 (Ab). Total cell lysates were also analyzed by Western blotting. B, the primary aa sequences of human caspase-3 (casp3) and caspase-7 (casp7) are aligned. Caspase-3 and caspase-7 were similarly fractionated into 21 blocks, which are separated by vertical lines. Regions A, B, and C; region D; regions I and II; and region III are indicated by pink, purple, cyan, and blue lines, respectively. Regions b and ii are the divided parts of regions B and II, respectively. The positions of previously reported loops L1, L2, L3, and L4 are also indicated by on top of the aa sequences. C, tertiary structural views of procaspase-7 (Protein Data Bank code 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, D and E, DKD HeLa cells were cotransfected with FLAG- and HA-tagged caspases, and a co-immunoprecipitation analysis was carried out as described for A. F, tertiary structural views of procaspase-7 (code 1GQF) are depicted from the side with region b (left) and from the opposite side with regions I and ii (right). Regions b and ii are highlighted by pink and cyan lines, respectively.
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). We highlighted these regions in the tertiary structure of procaspase-7 (Protein Data Bank code 1GQF) (Fig. 3C) (10). Regions I and II, derived from both partners, are located near each other and occupy a position straddling the homodimer interface. Similarly, regions A, B, and C, derived from both partners, are also located close together, occupying a position straddling the homodimer interface on the opposite side of regions I and II. We designated the three-dimensional structural regions composed of regions I and II and of 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. Whereas procaspase-7(ABC I II) could interact with procaspase-3 but not with procaspase-7, procaspase-7(ABC) could bind to both procaspase-3 and procaspase-7. We then 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 the most important. Procaspase-3(B I II) could not bind procaspase-3 but could bind procaspase-7, whereas procaspase-3(I II) could interact with both procaspase-3 and procaspase-7, supporting the idea that region B is the most important in the formation of a homodimer 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 are located at the center of structural regions ABC and I II, respectively, in the three-dimensional structure of procaspase-7 (Fig. 3F). All of the results indicate that the center of structural regions ABC and I II exhibits the most important effect on the selection of a partner for dimerization.

**Identification of Amino Acid 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 chimeras 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 that make direct contact with the low molecular weight substrate DEVD (13). The difference in region D between caspase-3 and caspase-7, which contains residues for direct binding to DEVD-pNA, would affect their distinct in vitro DEVDase activity.

Interestingly, region ABC is identical to those regions important for selecting a partner for dimerization. Region B contains residues for direct binding to the low molecular weight substrate DEVD near the active center (11, 12) and to contain aa residues that make direct contact with the low molecular weight substrate DEVD (13).
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loops L2 and L4 of the other subunit in the proform 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 were replaced with Ala and Ala and with Glu, respectively (supplemental Fig. S2A) (25). In these cells, expression of the caspase-3 mutant was expected to inhibit the formation of a homodimer of endogenous caspase-3 and to form a heterodimer between endogenous wild-type and mutant caspase-3. The heterodimer showed much weaker DEVDase activity in vitro than another heterodimer between wild-type and mutant caspase-3 in which the active center was inactivated but the intercleavage site was intact (supplemental 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 proform. Taken together, region b would be important for caspase-3 not only in selecting its partner but also in 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 chimeras, we analyzed their DEVD-pNA-cleaving activity in vitro using the Michaelis-Menten equation and calculated \(K_{\text{m}}\) and \(V_{\text{max}}\) parameters with Eadie-Hofstee plots (supplemental Fig. S3, A and B). \(V_{\text{max}}\) values differed significantly among caspase-3, -7, -7(D), and -7(ABC), consistent with their DEVDase activity, whereas \(K_{\text{m}}\) 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 (Fig. 4, C and 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 caspase-7 to depend on the difference in regions A, B, C, and D.

Amino Acid Regions Important for the Strong Activity of Caspase-3 in Cleaving Its Substrates within Cells—Next, we examined whether the chimeric constructs caspase-7(D), -7(ABC), and -7(ABCD) cleave cellular substrates in a similar manner to caspase-3 within cells. Surprisingly, all of the chimeras showed much lower activity levels than caspase-3 for the cleavage of SETβ and caspase-6-mediated cleavage of lamin A (Fig. 4F). These results suggest that in vitro DEVDase activity is not equivalent to intracellular cleaving activity and that 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 in cleaving cellular substrates between caspase-3 and caspase-7, we generated new chimeric constructs based on caspase-7(ABCD). Finally, we found that caspase-7(ABCD II III) expressed in DKD HeLa cells showed caspase-3-like activity in the cleavage of lamin A and SETβ within cells as well as the cleavage of DEVD-pNA in vitro (Fig. 5, A–C). In addition, caspase-7(ABCD 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 caspses 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 chimeras and found that the caspases differ in protease activity and that the difference depends on seven 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
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crucial to the difference in protease activity between caspase-3 and caspase-7.

Caspase-3 and caspase-7 differed in activity in cleaving 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. Whereas 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 (supplemental Fig. S3), regions I, II, and III may determine additional substrate specificity, which has not been clarified. These results are 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 caspase-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 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 B-sheets. Meanwhile, region D, which is situated away from the dimer interface in the three-dimensional structure, is not engaged in homodimer 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 to be important to the protease activity of executioner caspases, structural region ABC may affect DEVDase activity by regulating its formation. In addition, the 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 caspase-7 (10). The central cavity was reported to be open 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 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 caspase-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 caspase-3 and caspase-7.

In this study, the biological functions and structural characteristics of executioner caspases caspase-3 and caspase-7 during apoptosis were examined by generating and analyzing chimeras. 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 caspase-7.

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