The structure of procaspase 6 is similar to that of active mature caspase 6¹

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To investigate the structural characteristics and activation mechanism of the precursor caspase, genes encoding the inactive pro-form and the active mature form of caspase 6 were expressed in *Escherichia coli* and the proteins of both forms were purified to homogeneity. The structure of each protein was characterized by chemical cross-linking, size-exclusion chromatography, CD and fluorescence spectroscopies. The pro-form caspase 6 exhibits a dimeric structure and its overall secondary structure was found to be similar to that of the mature caspase 6. Upon the maturation of procaspase 6, the maximum fluorescence wavelength $\lambda_{\max}$ was red-shifted from 330 to 337 nm and the fluorescence intensity of $\lambda_{\max}$ was increased. This fluorescence spectral change indicates

that the environment of a tryptophan residue in the substrate-binding site can be changed to a more polar one when the procaspase 6 is processed. Taken together, our results strongly demonstrate that precursor caspase 6 exists as a dimer and its overall structure is similar to that of the active caspase 6. Our results also suggest that the local conformational change at the substrate-binding site, with no drastic change in the overall structure, seems to enable precursor caspase 6 to become the active mature enzyme.

Key words: activation mechanism, apoptosis, protease, zymogen.

INTRODUCTION

Apoptosis, programmed cell death, is an essential biological process that plays a critical role in the normal development of multicellular organisms and the maintenance of tissue homeostasis [1,2]. Although the exact details need clarification, the coordinated activation of a family of caspases known as cysteine proteases appears to be crucial for apoptosis to occur. Caspases involved in apoptosis can be divided into two classes, initiators and executioners, based on their sequence homology, function and substrate specificities [3–6]. All mature initiator caspases are derived from procaspases with a long N-terminal prodomain. Three initiator caspases, 2, 8 and 10, have been implicated in the apoptotic pathway triggered by the death receptor [3,7,8]. Caspase 9, another initiator caspase with a long prodomain, is activated by forming a complex with the apoptotic protease-activating factor-1 upon the release of cytochrome *c* from mitochondria [5,6,9]. Caspases 3, 6 and 7, with short N-terminal propeptide, are involved in the limited proteolysis of various cellular substrates during apoptosis, and the activation of these executioner caspases depends on the proteolytic activation by the upstream initiator caspases [4–6].

Crystal structures of mature forms of caspases 1, 3, 7 and 8 have been reported previously [10–16]. The overall architectures of these caspases were found to be similar to each other and showed that all the enzymes are composed of a pair of large subunits surrounding two adjacent small subunits with a dimeric interface. Based on either the three-dimensional structure of mature caspases as observed by X-ray crystallography [10–12] or the complementation experiment [17], several models of association and activation mechanisms of caspase precursors have been proposed. It has been speculated that the procaspase exists as an inactive monomer, because the configuration of the region between the large and small subunits interferes with dimerization and the proteolytic cleavage allows subsequent association of subunits for the heterotetrameric mature structure [10,18].

Alternatively, each precursor molecule may already be folded to form a dimer with two large and small subunits before procaspase is processed [11,12,17]. For initiator caspase 8, it was shown that procaspase 8 could be activated directly by oligomerization, and this was thought to be sufficient to initiate the processing of procaspase [19,20]. Therefore it was argued that caspase precursors oligomerized while interacting with other molecules before autoproteolytic activation. The activation of caspase 9 did not require the proteolytic processing, but the activity was dependent on the cytosolic factors such as apoptotic protease-activating factor-1, cytochrome *c* and deoxyadenosine triphosphate [21,22]. Recently, the crystal structure of caspase 7 was published [23,24]. It showed that procaspase 7 is a dimer and the core structural elements of procaspase 7 are identical with those of the mature form of caspase 7, whereas the region surrounding the catalytic cleft exhibits a significant structural change upon maturation.

In the present study, we prepared pro- and mature forms of caspase 6 to compare their structural characteristics with each other. The structures of both forms were investigated utilizing chemical cross-linking, size-exclusion chromatography, fluorescence and CD spectroscopies. Our results revealed that most of pro-form caspase 6 exists as a dimer with an overall secondary structure similar to that of active mature caspase 6. However, a tryptophan residue in the binding site of the substrate can become more accessible to aqueous environment upon processing of procaspase 6, suggesting that the configuration of the substrate-binding site needs to be changed for the activation.

MATERIALS AND METHODS

Construction of expression plasmid and site-directed mutagenesis

Caspase 6 gene was amplified by PCR with two oligonucleotide primers, 5'-AAGTC GACGC CTTCT ATAAA AGAGA AATG-3' and 5'-AACTG CAGTT AATTA GATT TGGAA

Abbreviations used: D316A, Asp³¹⁶ → Ala; DTT, dithiothreitol; W175F, Trp¹⁷⁵ → Phe; rCaspase 6, recombinant wild-type caspase 6.

¹ This paper is dedicated to Professor Sang Chul Shim on the occasion of his retirement.

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Figure 1 Schematic representation of the different forms of rCaspase 6

'Caspase 6' represents the structure of the precursor form of caspase 6 *in vivo*. Three cleavage sites (↓) were denoted by dotted lines and arrows with the flanking amino acid sequences. 'rCaspase 6' is the recombinant caspase 6 whose propeptide was replaced with the histidine tag to facilitate the purification by Ni^{2+} -resin, and 'D316A' is a mutant rCaspase 6 with mutation at the cleavage site. The locations of two tryptophan residues at positions 175 and 348 are indicated as follows: P, propeptide; LS, large subunit; I, linker region; SS, small subunit; H, histidine tag. The caspase 1 numbering convention was used as described previously [32].

AGAA-3'; underlined sequences represent restriction-enzyme sites for *SaII* and *PstI* respectively. The amplified gene was digested with *SaII* and *PstI*, and subcloned into the same restriction sites of pQE-31 plasmid vector (Qiagen) to construct pQE6M. The recombinant plasmid pQE6M was used to transform *Escherichia coli* M15 [pREP4] (Qiagen) for the expression of the wild-type caspase 6 with the propeptide-truncated recombinant wild-type caspase 6 (rCaspase 6) (Figure 1), and was also utilized as a template for site-directed mutagenesis. The mutagenesis was performed by PCR using the following primers with *PstI* sites (underlined sequences); 5'-TGGCTGCAGCCTCCGTTTACACGCTG-3' and 5'-AGGCTGCAGCACCTCAGTTATGTTGGTGT-3'. The amplified gene was digested with *PstI* and then self-ligated to generate pQED316A for the expression of the Asp³¹⁶ → Ala-mutant (D316A) caspase 6. Similarly, pQEW175F was constructed for the expression of the Trp¹⁷⁵ → Phe-mutant (W175F) caspase 6, using the following primers containing *Eco47III* sites (underlined sequences); 5'-AGAAGCGCTCATGATGGAAGATTAAAGCAA TTCC-3' and 5'-AAGA GCGCT TCTTT TTCCA CTAA CACTG CCAGA AAGGC-3'. All the mutations were confirmed by determining the nucleotide sequence of the entire caspase 6 gene using the Genetic Analyzer (ABI PRISM 3100, Applied Biosystems).

Expression and purification of recombinant caspases 6 and 3

E. coli M15 [pREP4], transformed with pQE6M, pQED316A and pQEW175F, were grown. Induction of the expression for the respective protein and the subsequent purification of the protein were performed as described previously [25], but with minor modifications. When the absorbance of the culture media reached 0.6–0.8 at 600 nm, isopropyl 1-thio- β -D-galactopyranoside was added to a final concentration of 0.02 mM to induce the expression of the caspase 6 for 18 h at 25 °C. Bacterial cells were harvested by centrifugation and then lysed by sonication in a buffer (100 mM Tris/HCl, pH 8.0, 100 mM NaCl, 10 mM imidazole). Cell debris and insoluble materials were removed by centrifugation at 12000 *g* for 30 min. The soluble fraction was mixed with nickel-nitrilotriacetic acid agarose resin (Qiagen) and the mixture was poured into an empty column. Proteins bound to the resin were eluted with a washing buffer (100 mM Tris/HCl,

pH 8.0, 500 mM NaCl, 50 mM imidazole) and then with an elution buffer (100 mM Tris/HCl, pH 8.0, 100 mM NaCl, 200 mM imidazole). After the buffer of eluted fractions was exchanged utilizing the Fast Desalting column HR 10/10 (Pharmacia) with an equilibration buffer [20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM EDTA], the protein was concentrated with Centricon 10 (Amicon). Protein concentration was determined by the Bradford method using the Coomassie Blue Plus Protein Assay Reagent (Pierce, Rockford, IL, U.S.A.) and BSA as a standard. Wild-type caspase 6 matured by autoproteolysis when the protein was incubated in the equilibration buffer overnight at 12 °C. A recombinant plasmid containing a caspase 3 gene in the pRSET B vector (Invitrogen) pR3M was kindly provided by Dr Byung-Ha Oh (POSTECH, South Korea), and the expression and subsequent purification of caspase 3 were performed as described previously [26].

Proteolytic activity assay

The catalytic activity of caspase 6 was determined by spectrophotometry utilizing a substrate Ac-VEID-pNA (Calbiochem). Caspase 6 (0.2 μM) was pre-incubated for 10 min at 30 °C in 495 μl of a pre-incubation buffer (100 mM Hepes, pH 7.5, 10 mM DTT, 10 % sucrose, 0.1 % CHAPS) and then mixed with 5 μl of 10 mM substrate dissolved in DMSO. The amount of *p*-nitroaniline released from the hydrolysis of the peptide substrate was determined by measuring the absorbance at 405 nm.

Chemical cross-linking and size-exclusion chromatography

D316A caspase 6 (6 μM) was incubated in glutaraldehyde with the final concentrations of 0.005, 0.02 and 0.1 % for 3 min at room temperature and then subjected to SDS/PAGE (12 % gel). Proteins on the gel were stained with Coomassie Brilliant Blue R-250. The molecular size of the D316A caspase 6 was estimated in non-denaturing condition by size-exclusion chromatography. D316A (130 μg) was applied to a Superdex 200 HR 10/30 column (Pharmacia) and then eluted with the equilibration buffer at a flow rate of 0.5 ml/min while the absorbance at 280 nm was monitored. BSA, ovalbumin and chymotrypsinogen A were used as molecular-mass standards (Pharmacia).

CD and fluorescence spectroscopies

CD spectra of the proteins were obtained by using a spectropolarimeter (J-715, Jasco). Far-UV CD spectra were obtained in a cuvette with a 2 mm path length, and near-UV CD spectra in a cuvette with a 5 mm path length. The concentrations of recombinant caspases were adjusted to 5 μ M for far-UV spectra and 10 μ M for near-UV spectra. The difference in α -helical content ($\text{Diff}_{\alpha\text{-hel}}$) was estimated approximately from the mean residue ellipticity $[\theta]$ at 222 nm, as described previously [27]:

$$\text{Diff}_{\alpha\text{-hel}} = \frac{[\theta] + 3000}{33000}$$

The fluorescence spectra were obtained by using a spectrofluorimeter (RF-5301PC, Shimadzu). Fluorescence intensities were measured from 300 to 400 nm with an excitation wavelength of 295 nm. To monitor time-course changes of CD and fluorescence spectra during the processing of the precursor caspase 6, 1 ml of 10 μ M D316A was mixed with 10 μ l of 10 μ M active caspase 3 at 25 °C and spectral changes were monitored at various time points. At each time point, CD measurements were averaged for 1 min with a response time of 1 s at 208 and 222 nm, and fluorescence emission spectra were obtained with the excitation wavelength at 295 nm.

RESULTS

Expression and purification of pro- and mature forms of caspase 6

Recombinant wild-type caspase 6, rCaspase 6, was overexpressed and purified to homogeneity. Most of the protein was not processed, and only a small portion of caspase 6 was obtained in the mature form as ascertained by SDS/PAGE analysis (Figure 2A, lane 1). After incubating the purified protein overnight with 1 mM DTT at 12 °C, the pro-form of caspase 6 was completely processed to the mature form (Figure 2A, lane 2), and this also exhibited a drastic increase in catalytic activity. Compared with the cleavage sites of caspase 6 reported previously [28], the processed products of rCaspase 6 indicated that the precursor was supposed to be cleaved at the peptide bond after Asp³¹⁶ when analysed by SDS/PAGE (Figure 1). To prevent the autocatalytic processing of the specific site, Asp³¹⁶ of rCaspase 6 was replaced with Ala to make the D316A mutant. D316A showed no autocatalytic cleavage at any potential cleavage site and no increase in the catalytic activity was observed even after incubation for a week at 12 °C. The processed mature form of D316A was obtained by cleaving the peptide bond after Asp²⁹⁷ with active caspase 3. This processing event was expected to occur during the apoptotic proteolytic cascade [28,29].

Dimeric structure of pro-form caspase 6

Chemical cross-linking and size-exclusion chromatography were utilized to investigate the multimeric state of pro-form D316A caspase 6. When D316A was incubated in glutaraldehyde with the final concentrations of 0.005, 0.02 and 0.1% at room temperature for 3 min, the protein band of D316A was shifted gradually from 33.5 kDa to approx. 67 kDa, with an increase in the concentration of glutaraldehyde as ascertained by SDS/PAGE analysis (Figure 2B), indicating that D316A could form a dimer. To investigate further the dimeric state of the protein in non-denaturing condition, size-exclusion chromatography was performed with 10 μ M D316A while the absorbances at 280 nm were monitored. BSA (67 kDa), ovalbumin (45 kDa) and chymotrypsinogen A (25 kDa) were used as calibrating standards to

Figure 2 SDS/PAGE and size-exclusion chromatography of caspase 6

(A) SDS/PAGE of rCaspase 6. Proteins were separated by SDS/PAGE (12% gel) and stained with Coomassie Brilliant Blue. Most of the protein was not processed immediately after purification (lane 1). After treatment with 1 mM DTT, the unprocessed form (P) was completely processed into large (L) and small (S) subunits after an overnight incubation at 12 °C (lane 2). (B) SDS/PAGE of the pro-form cross-linked by glutaraldehyde. D316A caspase 6 (lane 1) was treated with 0.005% (lane 2), 0.02% (lane 3) and 0.1% glutaraldehyde (lane 4). Cross-linked proteins were separated by SDS/PAGE (12% gel) and stained with Coomassie Brilliant Blue. (C) Size-exclusion chromatography of D316A. Size-exclusion chromatography was performed with 10 μ M D316A, while absorbances at 280 nm were monitored. BSA (67 kDa), ovalbumin (45 kDa) and chymotrypsinogen A (25 kDa) were loaded as standards to assess the molecular mass of D316A. Molecular mass (kDa) of the proteins is indicated on the right-hand side as follows. P, unprocessed form (33.5 kDa); L, large subunit; S, small subunit; D, expected dimer.

assess the size of the protein. The retention volume of D316A was found to be nearly the same as that of BSA (67 kDa), as shown by the size-exclusion chromatography (Figure 2C). Results of both chemical cross-linking and size-exclusion chromatography for D316A suggest that procaspase 6 is a dimer.

Comparison of the secondary structural contents between pro- and mature forms of caspase 6

The CD spectrum of D316A was compared with that of the completely processed mature rCaspase 6. The concentration of the protein was adjusted to 5 μ M for far-UV CD spectra and to 10 μ M for near-UV spectra. Both CD spectral data indicated that the overall shapes and intensities of the spectra were strikingly similar between D316A and mature rCaspase 6. For quantitative comparison of the secondary-structural contents between precursor and mature forms, the α -helical content was estimated based on the $[\theta]$ values at 222 nm as described previously [27] and compared with each other. The difference in the α -helical content was only 5% of the total helical content, which corresponds to three amino acids out of approx. 60 amino acids constituting the α -helix based on the crystal structure of

Figure 3 Fluorescence spectra of pro- and mature forms of caspase 6

Fluorescence spectra of pro-form D316A caspase 6 (●) and mature rCaspase 6 (○) were measured. The maximum intensity of each spectrum was taken as 1. λ_{max} of these two spectra differ by 7 nm.

caspase 3 [13]. This means that the overall secondary structure of caspase 6 was almost unchanged even after the processing of the pro-form caspase 6 with the cleavage between large and small subunits in the primary structure.

To investigate further the structural changes involved in the processing of the cleavage site at Asp²⁹⁷, D316A was mixed with a trace amount of active caspase 3 as described in the Materials and methods section. The processing pattern and the change in $[\theta]$ at 208 and 222 nm were investigated in a single tube at various time points. In this time-course processing of D316A caspase 6 by caspase 3, which cleaves the peptide bond after Asp²⁹⁷ instead of Asp³¹⁶, the difference in the value of $[\theta]$ at 222 nm between the pro- and mature forms was only approx. 4%, which is almost comparable with the value of 5% for rCaspase 6, which can be activated by autocatalytic processing as described above. Comparison of the CD spectra of the pro- and mature forms consistently suggested that the secondary structure of procaspase 6 is already pre-organized similar to that of mature active caspase 6, which is generated by the cleavage of the peptide bond after either Asp³¹⁶ or Asp²⁹⁷.

Fluorescence spectroscopy

Fluorescence spectroscopy is a sensitive probe to monitor the change in the environment around tryptophan residues in the protein. The fluorescence spectra of D316A and the mature rCaspase 6 were obtained to assess the environmental change around tryptophan residues (Figure 3). The spectral data showed that the maximum fluorescence wavelength λ_{max} was red-shifted by 7 nm from 330 to 337 nm in active mature rCaspase 6. This result indicates that the tryptophan in the mature form of caspase 6 is exposed to the aqueous environment [30]. To investigate further the environment of the tryptophan residue in the substrate-binding site of caspase 6 in detail, we prepared W175F caspase 6. Based on the comparison of the sequence homology and crystal structures between caspases 3 and 7, W175F caspase 6 was expected to have a single tryptophan at position 348 in the substrate-binding site [14]. The W175F mutation reduced the autocatalytic processing activity. However,

Figure 4 Fluorescence spectral changes of W175F caspase 6 during maturation

(A) Time-course changes in fluorescence of W175F caspase 6 upon addition of active caspase 3. W175F (10 μM), which contains a single tryptophan located at the active site, was mixed with a small amount of active caspase 3. While the caspase 3 cleaved W175F, fluorescence spectral changes were monitored. T0, T2, T3, T4 and T6 represent the incubation times for 0, 2, 3, 4 and 6 h respectively. (B) SDS/PAGE of W175F during the maturation. At each time point, the sample was retrieved and analysed by SDS/PAGE (15% gel). The gel was stained with Coomassie Brilliant Blue. P and S represent the unprocessed form and the small subunit of W175F respectively. p22 and p20 indicate the large subunits of W175F obtained by the cleavage of the peptide bond after Asp³¹⁶ and Asp²⁹⁷ respectively. (C) Absorbance changes with time at 405 nm. The catalytic activity was measured as described in the Materials and methods section. Representation of absorbance changes: ●, pro-form; ○, completely processed mature form.

when the mutant protein was mixed with caspase 3, its enzymic activity was restored gradually while the processing of W175F by caspase 3 continued simultaneously. The changes in fluorescence spectra were correlated with the increase in activity during the processing of the pro-form caspase 6 (Figure 4). As the processing proceeded, the fluorescence intensities were increased significantly and the λ_{max} was red-shifted by 7 nm, similar to that

shown in the spectral differences between D316A caspase 6 and mature rCaspase 6.

Nearly identical CD spectra and the prominent difference in the fluorescence spectra indicated consistently that overall structures of both precursor and mature forms of caspase 6 should be almost indistinguishable. But the local structure and conformation, especially around the substrate-binding site, might be changed after the processing of precursor caspase 6.

DISCUSSION

Consistent results from both chemical cross-linking and size-exclusion chromatography suggest that D316A as the pro-form caspase 6 already exists as a dimer with an approximate size of 67 kDa (Figures 2B and 2C), and that two molecules of caspase 6 precursors interact before the processing between large and small subunits. CD spectra were essentially indistinguishable between pro- and mature forms of caspase 6. This suggests that both forms have approximately the same secondary structure and that procaspase 6 has a pre-organized dimeric structure, similar to that of the mature active caspase 6.

Although the overall structure of procaspase 6 is similar to that of the processed form, the pro-form does not exhibit the catalytic activity. To assess the structural change in the active site after maturation, we monitored the environmental change around a tryptophan residue at the substrate-binding site in caspase 6. There are only two tryptophan residues at positions 175 and 348 in rCaspase 6. Based on the sequence alignment and the crystal structure of caspases 3 and 7 complexed with the tetrapeptide-aldehyde inhibitor, one of the two tryptophans in rCaspase 6 Trp³⁴⁸ is supposed to be located at the substrate-binding site and conserved among the caspases [12,14]. The other tryptophan at position 175 is expected to be located on the loop so far from the substrate-binding site that it might not be directly involved in the binding of the substrate to caspase 6. In the W175F mutant containing only Trp³⁴⁸ at the substrate-binding site, the red-shift and the increase in the fluorescence intensity at $\lambda_{\max}$ were observed when the mutant was activated by caspase 3 (Figure 4A). Almost the same fluorescence spectral changes were observed as with rCaspase 6 which was processed autocatalytically (Figure 3). These observations indicate that the environment around Trp³⁴⁸ instead of Trp¹⁷⁵ was changed in the maturation of caspase 6 and that Trp³⁴⁸ makes a major contribution to the fluorescence spectral change. Because the fluorescence spectrum provides a good indication of the environmental change around the tryptophan residue in the protein, the red-shift of $\lambda_{\max}$ and the simultaneous increase in the fluorescence intensity at $\lambda_{\max}$ after the processing of pro-form caspase 6 could imply that the substrate-binding site in the enzyme could become more exposed to the aqueous environment [30].

Our results, combined with structural changes observed in the activation of caspase 7 [23,24] and caspase 9 [31], provide an insight into the general activation mechanism of caspases. The crystal structure of procaspase 7 showed that the core structural elements of procaspase 7, including the central six-stranded β -sheet and α -helices are very similar to those of mature caspase 7. However, three of the four loops in the active site, designated as L2, L3 and L4, have drastically different conformations [23]. Trp³⁴⁸ is well conserved among caspases 6, 7 and 9 [32]. Using hydrophobic interaction, Trp³⁴⁸, together with Val³³⁴, Val³⁷⁷ and Phe³⁸⁰ in procaspase 7 contributes to the stabilization of the zymogen conformation of the Val³³⁴-Pro-Gly³³⁶ segments. These segments are located approximately at the site occupied by the Ser³³⁹-Trp-Arg³⁴¹ segments in the active caspase 7 [24]. The crystal structure of caspase 9 also showed that structural

rearrangements are required for the formation of the competent active site, even though the overall caspase folds are not changed significantly [31]. Even if activation is induced by the dimerization of inactive caspase 9 monomers, unlike the case of caspase 7, then the activation loop forming the substrate-binding site in caspase 9 is re-orientated by interactions at the dimer interface. Therefore the drastic change in fluorescence upon the activation of caspase 6 is consistent with the structural change in the substrate-binding site in the activation of both caspases 7 and 9. The activation mechanisms of procaspase 6 seem to be very similar to those of procaspase 7 and caspase 9, since the proteolytic cleavage between the large and small subunits of procaspase 6 allows drastic conformational changes around the active site.

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