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Molecular insight into the role of the leucine residue on the L2 loop in the catalytic activity of caspases 3 and 7

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Synopsis

Various apoptotic signals can activate caspases 3 and 7 by triggering the L2 loop cleavage of their proenzymes. These two enzymes have highly similar structures and functions, and serve as apoptotic executioners. The structures of caspase 7 and procaspase 7 differ significantly in the conformation of the loops constituting the active site, indicating that the enzyme undergoes a large structural change during activation. To define the role of the leucine residue on the L2 loop, which shows the largest movement during enzyme activation but has not yet been studied, Leu^{168} of caspase 3 and Leu^{191} of caspase 7 were mutated. Kinetic analysis indicated that the mutation of the leucine residues sometimes improved the K_m but also greatly decreased the k_{cat}, resulting in an overall decrease in enzyme activity. The tryptophan fluorescence change at excitation/emission = 280/350 nm upon L2–L2′ loop cleavage was found to be higher in catalytically active mutants, including the corresponding wild-type caspase, than in the inactive mutants. The crystal structures of the caspase 3 mutants were solved and compared with that of wild-type. Significant alterations in the conformations of the L1 and L4 loops were found. These results indicate that the leucine residue on the L2 loop has an important role in maintaining the catalytic activity of caspases 3 and 7.

Key words: apoptosis, caspase, crystal structure, hydrophobic interaction, proenzyme activation, tryptophan fluorescence

INTRODUCTION

Apoptosis is a controlled cell-death process that is crucial for maintaining homoeostasis in multicellular organisms. It also has central roles in cell turnover, immune system function, embryonic development, metamorphosis and chemical-dependent cell death [1]. An imbalance in apoptosis underlies the aetiology of many human diseases [2]. Whereas insufficient apoptotic death causes cancers, excessively premature apoptosis may result in Alzheimer's, Parkinson's and Huntington's disease, ALS (amyotropic lateral sclerosis), multiple sclerosis or spinal muscular atrophy [3,4]. As caspases 3 and 7 are the final executioners of apoptosis, both the inhibition and activation of their catalytic activity are of significant interest for the development of therapeutic strategies for neurodegenerative diseases and cancers [4–7].

Mature caspase 3 is a dimer of heterodimers arranged in an $\alpha\beta\beta\alpha$ configuration, where α and β represent the large and small subunits respectively. The active site of mature caspase 3 comprises four loops from one heterodimer: L1 (residues 52–66), L2 (residues 163–175), L3 (residues 198–213) and L4 (residues 247–263). Caspase 7 also consists of four loops in a similar arrangement. L2 and L4 appear to be further stabilized by direct contact with L2′ (residues 176′–192′ in caspase 3) from the other heterodimer [7,8]. Clark and co-workers reported on the crucial roles of the loop bundle hydrogen bonds formed by Glu^{167} and Asp^{173} in the maturation of procaspase 3 and the activity of mature caspase 3 [8]. Although L2 and L2′ are covalently linked in the same heterodimer of the procaspase, the maturation process separates the two loops by cleavage between Asp^{175} and Ser^{176}. This process releases the constraint imposed on the loop L2–L2′ of the proenzyme, leading to a probable change in the enzyme conformation.

Abbreviations used: Ac-DEVD-CHO, *N*-acetyl-Asp-Glu-Val-Asp aldehyde; Ac-DEVD-*p*NA, *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide; DTT, dithiothreitol; IPTG, isopropyl-*β*-D-thiogalactoside; rmsd, root mean square deviation.

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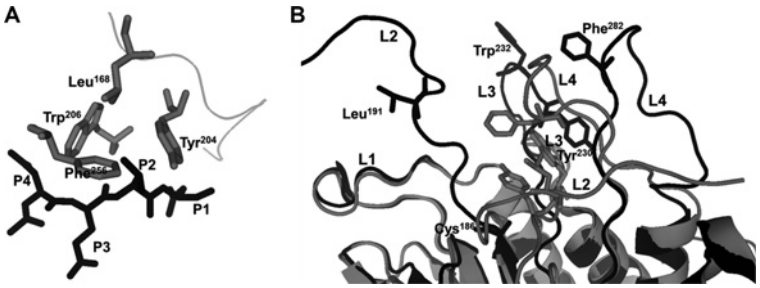

Figure 1 S2 subsite of caspases 3 and 7

(**A**) Recognition of the P2 residue of the inhibitor by the caspase 3 S2 subsite. The caspase 3 S2 subsite (grey), consisting of four hydrophobic amino acids (Leu^{168}, Tyr^{204}, Trp^{206} and Phe^{256}), accommodates the P2, Val, of the inhibitor, Ac-DEVD-CHO (black). Leu^{168} on the L2 loop from the large subunit participates in S2 subsite formation through a hydrophobic interaction with the other three aromatic amino acids from the small subunit. This Figure was created from PDB (ID: 2J33). (**B**) Structural comparison of procaspase 7 (black) created from PDB (ID: 1GQF) and mature caspase 7 (grey) created from PDB (ID: 1K86). The four hydrophobic amino acids (Leu^{191}, Tyr^{230}, Trp^{232} and Phe^{282}) are represented as black sticks (procaspase 7) and grey sticks (mature caspase 7). Leu^{191}, Tyr^{230}, Trp^{232} and Phe^{282} are expected to move from the aqueous environment to form the hydrophobic S2 subsite. The catalytic Cys^{186} showed only a small change in location, but its orientation was significantly changed.

Caspases 3 and 7 have a high sequence identity, with the conservation of key residues, including the catalytic cysteine and other active site residues [9,10]. The active site of caspases 3 and 7 consists of four subsites, S1–S4, to accommodate substrate binding. Of these, the S2 subsite is unique because of its hydrophobic nature, which comes from the four highly conserved hydrophobic amino acids: leucine residue on the L2 loop, tyrosine and tryptophan residues on the L3 loop and phenylalanine residue on the L4 loop [8] (Figure 1A). Accordingly, the S2 subsite accommodates hydrophobic amino acids such as valine, leucine or methionine as a P2 residue on substrates or inhibitors, whereas the other subsites recognize hydrophilic residues, such as aspartate or glutamate. Many other reports in the literature have demonstrated the importance of the three aromatic amino acids at the S2 subsite for enzyme activity [11–14]. Although the mutation of Leu^{168} to Ala^{168} on the L2 loop of caspase 3 impairs enzyme activity, the molecular mechanism remains unknown [15].

The activation of caspases 3 and 7 separate the L2–L2′ loops, leading to a large rearrangement of the four loops. As L2 belongs to the large subunit and the other loops to the small subunit, the L2 loop rearrangement is critical for stabilizing the newly generated interaction between the small and large subunits during the activation of caspases 3 and 7. As shown in Figure 1(B), a comparison of the crystal structures of caspase 7 and its precursor shows that the caspase activation results in a large movement (10–16 Å) of the four hydrophobic amino acids, accompanied by a structural re-organization of the loop bundle where the amino acids belong [16,17]. As Leu^{168} is accompanied by the catalytic Cys^{163} on the same loop in caspase 3, its movement should change the orientation or location of Cys^{163}, thereby directly affecting the enzyme activity. Feeney et al. [8] showed that the loop bundle hydrogen bonds are important in stabilizing the structure of the active caspase 3. Considering that effective hydrogen bonding requires a short distance (<3.5 Å) and correct directionality, however, the L2 loop rearrangement at the beginning is likely driven by another type of weak bonding interaction, such as hydrophobic interactions, which are less strongly affected by distance and directionality. The leucine residue on the L2 loop is the only hydrophobic amino acid that has no interaction with the other residues in procaspase 7 but forms hydrophobic interactions after loop rearrangement [16,17]. Thus, this leucine was anticipated to be involved in the L2 loop rearrangement and stabilization of mature caspases 3 and 7 by participating in S2 subsite formation. To further investigate the role of the leucine residue on the L2 loop at the molecular level, the corresponding leucine residues on caspases 3 and 7 were mutated to alanine, aspartic acid, phenylalanine, tyrosine, tryptophan and valine, and the resultant mutant enzymes were analysed with kinetic analysis, fluorescence spectroscopy and X-ray crystallography.

EXPERIMENTAL

Materials

Primer synthesis and DNA sequencing were performed by Bioneer and Genotech respectively. The primers used for plasmid construction are listed in Table 1. All reagents were purchased from Sigma–Aldrich unless stated otherwise. IPTG (isopropyl-β-D-thiogalactoside) was purchased from Duchefa. The caspase substrate, Ac-DEVD-*p*NA (*N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide) was purchased from Anaspec. Protein expression and cleavage were monitored by SDS/PAGE (15 % gel). Caspase activity was measured by monitoring the absorption changes at 405 nm, based on the release of *p*-nitroaniline from substrate hydrolysis, on a DU 800 UV-VIS spectrophotometer (Beckman Coulter). Fluorescence spectra were monitored using an LS 50B luminescence spectrometer coupled with FL WinLab® Software (PerkinElmer). The amino acid sequences of the engineered

Table 1 Constructs and corresponding primers for caspase preparation
Italicized bases represent restriction sites, and underlined bases were changed by mutagenesis.

Caspase	Plasmid	Forward primer	Reverse primer
Caspase 3 (1)	P1	5′-GGGAATTC*CATATG*GAGAACACTGAAAACTCAG-3′	5′-CCG*CTCGAG*GTGATAAAAATAGAGTTCTTTTGT-3′
L168A caspase 3 (2)	P2	5′-TGCCGTGGTACAGAAGCGGACTGTGGCATTGAG-3′	5′-CTCAATGCCACAGTCCGCTTCTGTACCACGGCA-3′
L168D caspase 3 (3)	P3	5′-TGCCGTGGTACAGAAGATGACTGTGGCATTGAG-3′	5′-CTCAATGCCACAGTCATCTTCTGTACCACGGCA-3′
L168F caspase 3 (4)	P4	5′-TGCCGTGGTACAGAATTCGACTGTGGCATTGAG-3′	5′-CTCAATGCCACAGTCGAATTCTGTACCACGGCA-3′
L168Y caspase 3 (5)	P5	5′-TGCCGTGGTACAGAATATGACTGTGGCATTGAG-3′	5′-CTCAATGCCACAGTCATATTCTGTACCACGGCA-3′
L168W caspase 3 (6)	P6	5′-TGCCGTGGTACAGAATGGGACTGTGGCATTGAG-3′	5′-CTCAATGCCACAGTCCCATTCTGTACCACGGCA-3′
L168V caspase 3 (7)	P7	5′-TGCCGTGGTACAGAAGTGGACTGTGGCATTGAG-3′	5′-CTCAATGCCACAGTCCACTTCTGTACCACGGCA-3′
C163S caspase 3 (8)	P8	5′-CTTTTCATTATTCAGGCCAGCCGTGGTACAGAA-3′	5′-TTCTGTACCACGGCTGGCCTGAATAATGAAAAG-3′
Caspase 7 (9)	P9	5′-GGGAATTC*CATATG*GCAGATGAGCAGGGCTGTATTGAAG-3′	5′-CCG*CTCGAG*TTGACTGAAGTAGAGTTCCTTGGT-3′
L191A caspase 7 (10)	P10	5′-TGCCGAGGGACCGAGGCGGATGATGGCATCCAGGCC-3′	5′-CTGGATGCCATCATCCGCCTCGGTCCCTCGGCAAGC-3′
L191D caspase 7 (11)	P11	5′-TGCCGAGGGACCGAGGATGATGATGGCATCCAGGCC-3′	5′-CTGGATGCCATCATCATCCTCGGTCCCTCGGCAAGC-3′
L191F caspase 7 (12)	P12	5′-TGCCGAGGGACCGAGTTTGATGATGGCATCCAGGCC-3′	5′-CTGGATGCCATCATCAAACTCGGTCCCTCGGCAAGC-3′
L191Y caspase 7 (13)	P13	5′-TGCCGAGGGACCGAGTACGATGATGGCATCCAGGCC-3′	5′-CTGGATGCCATCATCGTACTCGGTCCCTCGGCAAGC-3′
L191W caspase 7 (14)	P14	5′-TGCCGAGGGACCGAGTGGGATGATGGCATCCAGGCC-3′	5′-CTGGATGCCATCATCCCACTCGGTCCCTCGGCAAGC-3′
L191V caspase 7 (15)	P15	5′-TGCCGAGGGACCGAGGTGGATGATGGCATCCAGGCC-3′	5′-CTGGATGCCATCATCCACCTCGGTCCCTCGGCAAGC-3′
C186S caspase 7 (16)	P16	5′-TTCTTCATTCAGGCTTCTCGAGGG ACCGAG-3′	5′-CTCGGTCCCTCGAGAAGCCTGAATGAAGAA-3′

proteins were numbered based on the wild-type procaspases 3 and 7.

Site-directed mutagenesis of the leucine residue on the L2 loop of caspases 3 and 7

The wild-type caspase 3 gene was amplified using the forward primer (C1F) 5′-GGGAATTC**CATATG**GAGAACACTGAAAACTCAG-3′ with an NdeI restriction site (marked in boldface) and the reverse primer (C1R) 5′-CCG**CTCGAG**GTGATAAAAATAGAGTTCTTTTGT-3′ with a XhoI restriction site (marked in boldface). The product was digested with the corresponding restriction enzymes and inserted into the corresponding sites of the pET21a plasmid (Novagen) digested with the same enzymes. The resultant construct was designated plasmid P1. Plasmid P9 for the expression of wild-type caspase 7 was prepared by the same method, using the forward primer (C9F) 5′-GGGAATTC**CATATG**GCAGATGAGCAGGGCTGTATTGAAG-3′ with an NdeI restriction site (marked in boldface) and the reverse primer (C9R) 5′-CCG**CTCGAG**TTGACTGAAGTAGAGTTCCTTGGT-3′ with a XhoI restriction site (marked in boldface). Constructs for the catalytic mutants, C163S caspase 3 and C186S caspase 7, were prepared using the QuikChange® site-directed mutagenesis kit (Stratagene) and the supplied protocol. Briefly, 1 μl of pfu Turbo polymerase (2.5 units/μl) was added to a mixture of the template plasmid (1 ng), primers (200 pM each, Table 1), dNTPs (250 μM each) and reaction buffer (supplied with the kit). After 17 cycles of PCRs on a thermocycler (95°C for 1 min, 55°C for 30 s and 68°C for 12 min), the reaction was cooled to 4°C, and DpnI (1 μl at 10 units/μl, New England Biolabs) was added to digest the parental DNA and hemimethylated plasmid. PCR products were transformed into XL-10 Gold (Stratagene). Individual colonies were picked and grown for DNA amplification, and the resultant DNAs were sequenced. Subsequent site-directed mutagenesis at Leu^{168} of caspase 3 and Leu^{191} at caspase 7 to alanine, aspartic acid, phenylalanine, tyrosine, tryptophan and valine were also performed on the background of plasmids P1 and P9 using the same method. The constructed plasmids and the corresponding primers are listed in Table 1.

Preparation of wild-type and mutant caspases

Escherichia coli Rosetta DE3 (Novagen) carrying the respective expression plasmid was grown at 37°C in LB [Luria–Bertani (broth)] medium until D_{600} reached 0.6–0.8. Caspase expression was induced by adding 1 mM IPTG at 18°C for 18 h. Cells were harvested, washed with buffer A (50 mM Tris pH 7.5, 250 mM NaCl, 5 % glycerol and 1 mM 2-mercaptoethanol) and lysed using ultrasonication. After centrifugation (29 820 ***g*** for 30 min), the supernatant was incubated with a cobalt affinity resin (TALON®, Clontech) on a rocker for 1 h at 4°C and washed with buffer A supplemented with 10 mM imidazole. The protein was eluted from the metal affinity resin by buffer A supplemented with 100 mM imidazole. After dialysis against buffer B [20 mM Tris/HCl, pH 8.0, 10 mM NaCl, 2.5 % glycerol and 1 mM DTT (dithiothreitol)], the protein was purified using Mono Q ion-exchange column chromatography (Amersham Pharmacia), further purified on a Sephacryl S-100 size-exclusion column (Amersham Pharmacia) with buffer C (20 mM Hepes, pH 7.5, 150 mM NaCl and 1 mM DTT) and concentrated to 4–6 mg/ml for crystallization.

Kinetic analysis of wild-type and mutant caspases

The catalytic activities of the caspases were measured by a colorimetric assay using a known caspase substrate, Ac-DEVD-*p*NA. The enzyme (50 μl, final concentration of 10–80 nM) was added

Table 2 Statistics for data collection and refinement

	L168F caspase 3	L168D caspase 3	L168Y caspase 3
Resolution (Å)	1.7	1.7	1.7
Space group	$P2_1$	$P2_1$	$P2_1$
Unit cell			
a (Å)	50.182	49.834	50.192
b (Å)	68.245	68.553	68.751
c (Å)	94.048	93.811	93.666
α, β, γ (°)	90, 101.843,90	90, 101.875, 90	90, 101.778, 90
Number of reflections	65693	68014	68429
Completeness (%)	95.9	99.8	99.8
I/σ	19.8	24.0	21.5
R_{merge} (%)*	6.3	6.0	5.8
Redundancy	3.3	3.7	3.5
R_{cryst} (%)	19.7	18.8	19.5
R_{free} (%)	21.2	21.1	21.3
Rmsd for bond length (Å)	0.005	0.005	0.005
Rmsd for bond angles (°)	1.34	1.31	1.32
Rmsd for dihedral angles (°)	23.6	24.0	23.6
Rmsd for improper angles (°)	0.67	0.67	0.66
Average *B* factor (Å^2)	24.9	19.7	22.0
Number of protein atoms	3901	3890	3901
Number of water molecules	369	390	369
PDB ID	4DCP	4DCJ	4DCO

*$R_{merge} = \Sigma_{hkl}\ \Sigma_j |I_{hkl,\,j} - \langle I_{hkl}\rangle| / \Sigma_{hkl}\ \Sigma_j\ I_{hkl,\,j}|$, where *I* is the intensity for the *j*th measurement of an equivalent reflection with the indices *h*, *k*, *l*.

to 950 μl of the substrate solution at various concentrations (0, 15, 30, 60, 120 and 240 μM) in buffer D (50 mM Hepes, pH 7.4, 50 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA and 5 mM DTT) at room temperature (25°C). The *p*-nitroaniline released by the caspase reaction was monitored with an absorption change at 405 nm on a DU 800 UV-VIS spectrophotometer (Beckman Coulter). K_m and V_{max} were determined using Hyper32 version 1.0.0 (http://homepage.ntlworld.com/john.easterby/hyper32.html), and k_{cat} was calculated by dividing V_{max} (M/min) by the enzyme concentration (M).

Fluorescence measurement of wild-type and mutant caspases

Fluorescence spectra were recorded on a PerkinElmer LS50B Luminescence spectrometer coupled with FL WinLab® Software (PerkinElmer) using a cuvette with a 1-cm path length. Each caspase was incubated in buffer D at room temperature for at least 2 h before obtaining fluorescence spectra. Samples were excited at 280 nm, and fluorescence emission was scanned between 280 and 500 nm. All measurements were corrected for background signal.

Crystallization and data collection

Protein stock solutions were prepared by mixing the protein (4–6 mg/ml) in buffer C with 10 equivalents of Ac-DEVD-CHO (*N*-acetyl-Asp-Glu-Val-Asp-aldehyde). The resultant mixture was incubated at room temperature for 2 h. Each protein stock solution was mixed with the same volume of a reservoir solution (100 mM sodium citrate, pH 6.5 and 10 mM DTT) supplemented with 16–20 % (w/v) PEG 6000. Crystals of caspase 3 and its mutants grew within 3 days at room temperature using the hanging-drop vapour-diffusion method. X-ray diffraction data were collected on beamline 4A at Pohang Accelerator Laboratory using an ADSC Quantum 4 detector. The diffraction data were processed with DENZO and scaled with SCALEPACK [18]. The statistics for the data collection and refinement are shown in Table 2.

RESULTS AND DISCUSSION

Design and preparation of mutant caspases

Leu^{191} on the L2 loop of caspase 7 (active conformer) is located at the hydrophobic S2 subsite, but it is exposed to the aqueous environment in procaspase 7 (inactive conformer) (Figure 1B). The proenzyme exists exclusively as the inactive conformer owing to the strain induced by the covalently linked L2–L2′; however, the cleavage of L2–L2′ during activation may lead to an equilibrium shift between the active and the inactive conformers. This equilibrium shift should be controlled by the nature of the L2

Table 3 Kinetic constants of Ac-DEVD-*p*NA hydrolysis by caspases
ND, not determined; WT, wild-type.

	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\mu M \cdot min^{-1}$)
WT caspase 3	70.94 ± 3.70	1213.60 ± 35.98	17.12 ± 0.39
L168F caspase 3	43.47 ± 4.67	942.11 ± 53.34	21.73 ± 1.11
L168W caspase 3	ND	ND	ND
L168A caspase 3	326.55 ± 64.28	35.66 ± 7.63	0.11 ± 0.002
L168D caspase 3	ND	ND	ND
L168V caspase 3	81.15 ± 2.08	400.33 ± 21.86	4.93 ± 0.14
L168Y caspase 3	180.25 ± 6.72	902.83 ± 51.82	5.01 ± 0.10
WT caspase 7	131.8 ± 0.52	621.71 ± 4.65	4.71 ± 0.05
L191F caspase 7	76.09 ± 1.82	437.83 ± 8.84	5.75 ± 0.02
L191W caspase 7	388.86 ± 10.61	24.15 ± 0.09	0.06 ± 0.0002
L191A caspase 7	44.34 ± 3.66	26.84 ± 1.64	0.61 ± 0.09
L191D caspase 7	ND	ND	ND
L191V caspase 7	75.60 ± 2.07	124.57 ± 1.56	1.65 ± 0.06
L191Y caspase 7	202.78 ± 23.27	168.88 ± 4.47	0.83 ± 0.03

loop, including hydrogen bonding and hydrophobic or van der Waals interactions. In addition, the tryptophan residue on the L3 loop also moves from the aqueous environment to the hydrophobic environment of the S2 subsite; thus, a spectral shift or enhancement of tryptophan fluorescence is expected [19]. Owing to the sequence and structural similarities of caspases 3 and 7, Leu^{168} on the L2 loop of caspase 3 may undergo an almost identical movement. As the leucine residue moves from a hydrophilic to a hydrophobic environment, its hydrophobic nature and its size are likely critical for its movement to form the S2 subsite. To test the importance of the size and hydrophobicity of the side chain, Leu^{168} of caspase 3 and Leu^{191} of caspase 7 were each mutated to alanine, aspartic acid, phenylalanine, tyrosine, tryptophan and valine. As aspartic acid resembles leucine in its van der Waals radius but is hydrophilic because of the negative charge on the carboxylate at neutral pH, Leu-to-Asp mutants were prepared to test the importance of hydrophobicity. As alanine, valine, phenylalanine, tyrosine and tryptophan residues are all hydrophobic but vary in size, these mutants can be used to test the importance of size. In addition, the catalytic cysteines of wild-type enzymes and all leucine substitution mutants were mutated to serine residues to model the corresponding proenzymes. All prepared enzymes were analysed with enzyme kinetic assays and tryptophan fluorescence.

The expression of all catalytic mutants in *E. coli* resulted in the production of >10 mg of single chain proteins from 1 litre of culture (results not shown). Although they varied in catalytic activities, the leucine substitution mutants (proteins P2–P8 and P10–P16) could be expressed and purified as 2–3 mg of mature enzymes from 1 litre of culture. Interestingly, the Leu-to-Asp and Leu-to-Trp mutants on the L2 loop of caspases 3 and 7 showed negligible activity in the kinetic assays with the synthetic substrate but proceeded through maturation during expression, indicating that the catalytic cysteine is necessary for the maturation of caspases during their expression in *E. coli*. The primers, plasmids and proteins used are listed in Table 1.

Kinetic analysis of wild-type and mutant caspases 3 and 7 enzymes

The catalytic properties of the caspases prepared above were analysed using a synthetic substrate, Ac-DEVD-*p*NA (Table 3) [20]. Wild-type caspase 3 ($k_{cat}/K_m = 17.12\ \mu M^{-1} \cdot min^{-1}$) showed a 3-fold higher substrate specificity for Ac-DEVD-*p*NA than caspase 7 did ($k_{cat}/K_m = 4.93\ \mu M^{-1} \cdot min^{-1}$). As expected, the mutants presented huge differences in their catalytic activities, although the mutations at residue 168 of caspase 3 or residue 191 of caspase 7 were not expected to affect the loop bundle hydrogen bonds [8]. These results demonstrate that the leucine residue on the L2 loop is closely involved in the catalytic activity of the caspases through an additional interaction with the loop bundle hydrogen bonds. It is especially noteworthy that the L168D caspase 3 and L191D caspase 7 showed no significant catalytic activity. Considering that aspartic acid and leucine have similar van der Waals radii, the lack of catalytic activity in the Leu-to-Asp mutant enzyme must be due to the different polarities of the side chains. The importance of the hydrophobicity of leucine was also confirmed by comparing the phenylalanine and tyrosine mutants. Although phenylalanine and tyrosine are similar in size and structure, their substrate specificities (k_{cat}/K_m) vary 4-fold with caspase 3 and 7-fold with caspase 7. This result agrees well with an earlier report by Radzicka and Wolfenden, in which it was shown that phenylalanine is more hydrophobic than tyrosine and that their energy differences in hydrophobic interactions with octanol and cyclohexane are 0.46 and 3.12 kcal/mole respectively [21]. In the present study, Leu-to-Phe mutants were found to have a slightly better activity than the corresponding wild-type caspases, which can also be understood by analysing the structure, as discussed below.

A comparison of the kinetic constants of alanine, valine and tryptophan mutants with those of the wild-type caspases revealed the importance of the size of the amino acid side chain. The Leu-to-Val mutant was found to decrease the substrate specificity by

approximately 3-fold, and the Leu-to-Ala or Leu-to-Trp mutant resulted in negligible activities. These results suggest that tryptophan is too large to fit into the S2 subsite and alanine is too small. The dramatic decrease in catalytic activity with L168A caspase 3 has also been reported by Li and co-workers [15]. The k_{cat} and K_m of L168A is 34 and 4.6 times worse than those of the wild-type caspase 3. In all caspases 3 and 7 mutants, the k_{cat} values were much more impaired than the K_m values, indicating that the enzymes can bind substrate but cannot catalyse hydrolysis. Although L191F, L191A and L191V caspase 7 mutants showed an improvement in substrate binding (K_m), their substrate specificity was decreased owing to huge decreases in their catalytic ability (k_{cat}). This result suggests that the mutation of the leucine residue directly affects the reactivity of the catalytic cysteine as well as substrate binding. It may be that the S2 subsite adjusts the position and orientation of the catalytic cysteine on the L2 loop through binding of the leucine residue on the same L2 loop.

Tryptophan fluorescence analysis of wild-type and mutated caspases 3 and 7 enzymes

Caspases 3 and 7 have two tryptophan residues in their structures, but only one of them significantly changes its environment during activation. Owing to the movement of the tryptophan residue on the L3 loop from an aqueous to a hydrophobic environment, the activation of caspases 3 and 7 alters the tryptophan fluorescence. The corresponding catalytic Cys-to-Ser mutant was used as a model for the proenzyme of each leucine substitution mutant as it does not undergo cleavage during expression and purification [8].

While procaspase 7, procaspase 3 and caspase 3 have λ_{em} maxima at 357 nm, caspase 7 has an λ_{em} maximum at 371 nm (Figure 2A), corresponding to a 14 nm red-shift of the maximum fluorescence wavelength (λ_{max}) upon the activation of procaspase 7. This shift may be partially due to the polar aspartates near the tryptophan residue in the active form [19]. The activation of procaspases 3 and 7 to mature enzymes was found to increase their fluorescence intensity, which agrees well with the previous reports that tryptophan has a higher quantum yield in more hydrophobic environments and has higher fluorescence intensity in a folded protein than in a denatured protein [19]. A red-shift and increase in fluorescence intensity upon activation was also observed in caspase 6 [22].

In general, the trend in the fluorescence change between each leucine substitution mutant and the corresponding catalytic mutant correlated with the catalytic activity of each mutant (Figure 2). Specifically, the fluorescence difference between the proenzyme forms (mutated at the catalytic cysteine residue and leucine residue on the L2) and the mature forms (mutated at only the leucine residue on the L2) of the catalytically active mutants (phenylalanine, tyrosine and valine) was larger than that of the inactive mutants, such as Leu-to-Asp or -Trp mutants. The alanine mutant, however, showed a large change in the fluorescence intensity and a very low catalytic activity. In addition, the fluorescence intensity of the mature valine mutant was found to be higher than that of the active phenylalanine and tyrosine mutants (Figure 2G), suggesting that the substitution of the leucine residue on L2 with a smaller amino acid can place the tryptophan residue on L3 deeper into the hydrophobic S2 subsite. Indeed, the addition of the specific inhibitor Ac-DEVD-CHO, which is expected to push the tryptophan residue out of the S2 subsite, was found to decrease the fluorescence intensity of wild-type and L168A caspase 3 (Figure 2H). Accordingly, the tryptophan residue in the inhibitor-bound caspase 3 was more exposed to water than that in the free caspase 3 (Figure 3A). In addition, in general, caspase 3 mutants have a higher increase in fluorescence intensity upon activation than caspase 7 mutants. This result also correlates well with the observation that the tryptophan residue on L3 is more buried in the hydrophobic S2 subsite of caspase 3 than in caspase 7 (Figure 3B).

All observations suggest that the alanine mutant can form an active conformer in which the tryptophan residue has a tighter interaction with the tyrosine and phenylalanine at the S2 subsite than the other active mutants. Another interesting feature of the alanine mutants is that its k_{cat} was more impaired than its K_m (Table 3). The tryptophan mutant of caspase 7 showed the same trend. This observation suggests that the alanine mutants form the S2 subsite but their catalytic cysteines do not have the correct orientation to perform catalysis.

Interestingly, the mature L168D caspase 3 presented a lower fluorescence than the corresponding catalytic mutant, perhaps because the polar aspartate approaches the tryptophan residue [19]. This result indicates that Asp^{168} is located near the S2 subsite, which is consistent with the structural analysis described later in this paper. The slight increase in fluorescence upon L191D caspase 7 activation may suggest, however, that Asp^{191} is not close enough to the tryptophan residue at the S2 subsite. Again, the failure of both the Leu-to-Asp mutants of caspases 3 and 7 to perform catalysis may result from an inappropriate orientation or location of the catalytic cysteine, as found in the Leu-to-Ala caspase mutants.

The results of the fluorescence and kinetic studies indicate that the leucine residue on the L2 of caspases 3 and 7 adjusts the orientation of the catalytic cysteine for catalysis.

Crystallographic analysis of caspase 3 mutants

Crystallization of the mutants described above yielded high-quality crystals only for L168F, L168D and L168Y caspase 3 enzymes bound to Ac-DEVD-CHO. The others failed to provide sufficient crystal quality to obtain diffraction data.

The overall structures of all of the mutants were essentially the same as that of wild-type caspase 3 (Figure 4A). When the wild-type structure was superimposed with those of the mutant forms, however, important changes were observed. All the mutations caused small but significant movements in the L1 loop (residues 55–63) and the L4 loop (residues 252–257), which surround the active site. The average rmsd (root mean square deviation) of the backbone atoms between the wild-type and mutant enzymes ranged from 1.3 to 1.7 Å for the L1 loop and from 1.0 to 1.5 Å for the L4 loop. Considering that the overall rmsd was 0.4–0.6 Å between the wild-type and mutants, the mutations on Leu^{168} have a remarkable effect on the conformation of the loops

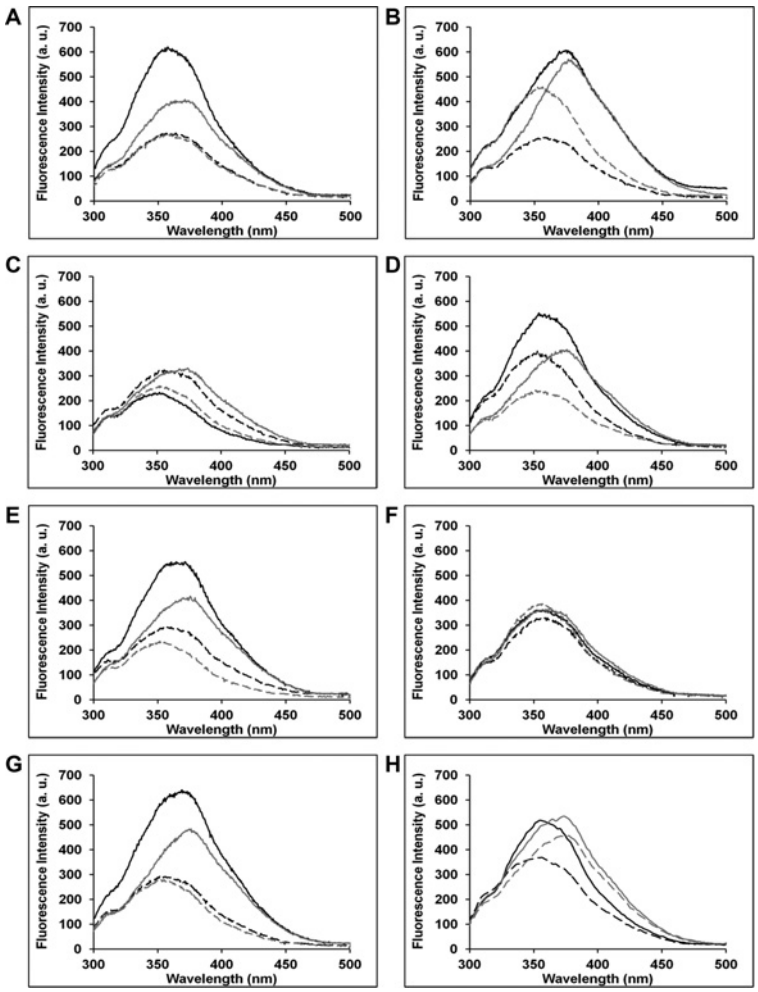

Figure 2 **Tryptophan fluorescence spectra of caspases and corresponding procaspases**

The fluorescence spectra of caspases 3 and 7 are represented by black and grey lines respectively and dotted and solid lines represent cysteine to serine catalytic mutants and L2–L2′ cleavage products respectively (**A**–**G**). (**A**) Fluorescence spectra of wild-type caspases 3 and 7 and their corresponding catalytic mutants. The maximum fluorescence wavelength (λ_{max}) for wild-type caspase 7 was red-shifted by 14 nm upon activation, with a moderate increase in fluorescence intensity. Mature caspase 3 exhibited a large increase in fluorescence intensity compared with its catalytic mutant. (**B**) Leu-to-Ala mutants. With red-shift, whereas caspase 3 exhibited a large increase in fluorescence intensity upon L2–L2′ cleavage, caspase 7 exhibited a small increase in fluorescence intensity. (**C**) Leu-to-Asp mutants. Caspase 7 showed a small fluorescence increase upon L2–L2′ cleavage, whereas caspase 3 exhibited a small fluorescence decrease. This small fluorescence increase resulted from the low absolute fluorescence intensity of mature enzymes. (**D**) Leu-to-Phe mutants. Caspase 3 showed a relatively small change in fluorescence intensity owing to the high fluorescence intensity of the catalytic mutant. In contrast, caspase 7 showed a very similar fluorescence change in the wild-type enzyme. (**E**) Leu-to-Tyr mutants. Both caspases 3 and 7 showed a similar fluorescence change in their wild-type enzymes. (**F**) Leu-to-Trp mutants. Both caspases 3 and 7 showed very small increases in fluorescence upon L2–L2′ cleavage. (**G**) Leu-to-Val mutants. Both caspases 3 and 7 showed similar fluorescence changes from their wild-type enzymes. (**H**) Fluorescence change upon addition of Ac-DEVD-CHO, a specific inhibitor, to caspase 3 (black) and L168A caspase 3 (grey), whereas the solid and dotted lines represent the absence and presence of the inhibitor respectively. Upon the addition of the inhibitor, caspase fluorescence intensity decreased in both wild-type and mutant enzymes. a.u., arbitrary units.

surrounding the active site. To identify the effect of the mutations, we investigated and compared the residues surrounding Leu^{168} in the wild-type and mutant forms (Figures 4B–4D). For L168D caspase 3, the side-chain of Asp^{168} pointed away from the catalytic core and is therefore not in noticeable contact with the hydrophobic residues at the S2 subsite. In contrast, in both the L168F and L168Y mutants, the side-chains of L168F and L168Y were located in near three hydrophobic residues and were engaging in extensive van der Waals contacts. We cannot exclude the possibility, however, that similar structures resulted from an induced-fit binding of the inhibitor to the active site of the mutants.

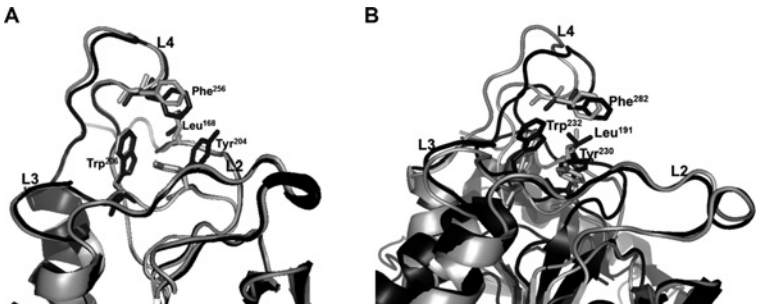

Figure 3 Comparison of tryptophan at S2 subsite

(**A**) Trp^{206} of caspase 3 in the presence (black cartoon created from PDB ID: 2J33) and absence (grey cartoon created from PDB ID: 1QX3) of the inhibitor. Because Tyr^{204} and Phe^{256} (black sticks) are pushed away from Trp^{206} in the presence of the inhibitor Ac-DEVD-CHO, Trp^{206} has a weak interaction with Tyr^{204} and Phe^{256} compared with caspase 3 without the inhibitor. (**B**) Trp^{232} (black stick) in caspase 7 (black cartoon created from PDB ID: 1K86) is located farther from the S2 subsite than is Trp^{206} (grey stick) in caspase 3 (grey cartoon created from PDB ID: 1QX3). Consequently, tryptophan is more exposed to water in caspase 7 than in caspase 3. The residue numbers given are based on caspase 7.

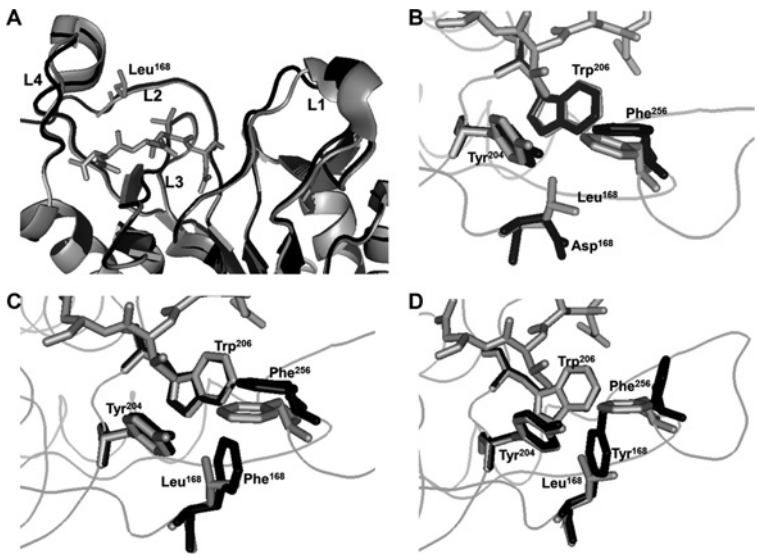

Figure 4 Structural comparison of wild-type caspase 3 with Leu^{168} mutants

(**A**) The structure of wild-type caspase 3 (grey) is superimposed on to that of the L168F mutant (black). The Ac-DEVD-CHO inhibitor is shown using a stick model. The secondary structural elements, important loops and location of the mutation are indicated. The active site of wild-type caspase 3 is superimposed with those of L168D (**B**), L168F (**C**) and L168Y (**D**) mutants. The wild-type caspase 3 is represented as grey cartoons and sticks. The residues participating in the hydrophobic interaction with Leu^{168} and bound inhibitors are shown as sticks. The corresponding residues in the mutant structures are shown only for clarity.

Consistent with the kinetic data, the hydrophobic interaction of the leucine residue on the L2 loop at the S2 subsite appears to be important for the proper catalysis of caspase 3.

Conclusions

To explore the role of the leucine residue on the L2 loop of caspases 3 and 7, this residue was mutated to various amino acids, and the resultant mutants were studied by kinetic analysis, fluorescence spectroscopic analysis and crystallographic analysis. The kinetic data revealed that the leucine on the L2 loop is critical for catalytic activity. The fluorescence data correlated well with the catalytic activity of the mutated caspases. The crystallographic analysis of caspase 3 mutated at Leu^{168} showed a significant difference in the interaction of the mutated residue with the hydrophobic residues at the S2 subsite. The kinetic data obtained with L191A caspase 7 showed a highly impaired k_{cat} but an improved K_m compared with wild-type caspase 7, indicating that the leucine residue is important for maintaining the catalytic cysteine for catalysis. In general, the mutation of the leucine residue on the L2 loop affected k_{cat} more strongly than K_m, indicating that the exact association of the leucine residue on the

L2 with the S2 subsite is required for caspases 3 and 7 to remain catalytically active. This study has demonstrated that the leucine residue on the L2 loop of caspases 3 and 7 plays a critical role in enzyme activity through its hydrophobicity and size.

AUTHOR CONTRIBUTION

Hyo Jin Kang and Young-mi Lee performed all of the experiments with caspases 3 and 7 respectively, with the exception of the protein crystallization. Myeong Seon Jeong prepared the protein crystals. Hyo Jin Kang also performed crystal diffraction experiments and prepared Figure 2 and the Tables. Seung Jun Kim performed structural refinement and prepared Figures 1, 3 and 4. Sang Chung conceived the study and wrote the paper. Moonil Kim and Kwang-Hee Bae contributed to the conception of the study and revision of the paper.

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