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Caspase-15 is autoprocessed at two sites that contain an aspartate residue in the P_1' position

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Abstract

Our recent characterization of porcine caspase-15 suggested that the amino terminus of the small catalytic subunit is formed by proteolytic processing between the consecutive aspartate residues D277 and D278. Since a charged residue (D278) is highly unusual in the P_1' position of a caspase cleavage site, we further characterized the mechanism of caspase-15 autoproteolysis. Amino acid sequence alignments showed that D277 and D278 as well as another pair of aspartates, D270 and D271, were evolutionarily conserved among species of the mammalian clade Laurasiatheria. Site-directed mutations of these four residues and analysis of recombinant proteins showed that D270 was crucial for autoproteolysis whereas the three other aspartates were dispensable for separation of the catalytic subunits. Mutation of D270 prevented catalytic activation and abolished subsequent processing at D277. Together with previous reports, our results show that caspase-15, unlike all other caspases, efficiently cleaves sites with an aspartate in the P_1' position.

Keywords: Caspase; Apoptosis; Autoprocessing; Enzyme activation; P_1' residue; Specificity of cleavage

Caspases are aspartate-specific cysteine proteases that cleave distinct substrate proteins critical for apoptosis, inflammatory signaling or other processes [1]. So far, 15 mammalian caspases have been reported, of which caspase-11 and 13 are actually orthologs of human caspase-4 [2–4]. Caspases are expressed as enzymatically inactive proenzymes. Distinct intracellular or extracellular stimuli lead to the formation of so-called caspase activation platforms, i.e., protein complexes to which initiator caspases bind via their prodomains. Thereby, structural changes in the caspase proenzyme and/or the proximity of two proenzymes induce activation which involves autoproteolytic separation of the two catalytic subunits, p20 and p10 [5–7]. Caspases with prodomains of less than 30 amino acid residues, i.e., caspase-3, 6, and 7, are activated by proteolytic processing by other caspases or granzyme B [1]. Short prodomain caspases absolutely require processing for activation whereas caspase-9 and other caspases with long prodomains can have significant activity even in the unprocessed state [8]. The proteolytic separation of the catalytic caspase subunits is generally mediated by cleavage of the proenzyme at two aspartate residues in the intersubunit linker region, with only caspase-10 and 14 being cleaved at a single site in this region [7]. After additional proteolytic cleavage between the prodomain and the large subunit, the mature catalytic caspase subunits form a heterotetrameric complex $(p20, p10)_2$ which cleaves substrates at amino acid motifs that are specific for individual caspases or groups of closely related caspases [9].

Caspase-15 is the member of the caspase family identified most recently [4]. The caspase-15 gene is expressed in diverse species of mammals such as pig, cow, dog (belonging to the superordinal clade Laurasistheria), and opossum (Marsupialia) but has been deleted in the superordinal clade of Euarchontoglires which includes both rodents and primates [10]. Caspase-15 contains a prodomain with

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a predicted pyrin domain structure and a catalytic domain that shows the highest sequence similarity to caspase-14. Recombinant porcine caspase-15 cleaves the Bcl-2 family protein Bid and induces apoptosis upon overexpression in mammalian cells [4]. Expression of the caspase-15 catalytic domain in *Escherichia coli* is associated with autoproteolytic processing into two subunits. D278 has been determined as the amino-terminus of the mature small subunit [4], showing that the cleavage occurs on the carboxy-terminal side of D277. The presence of an aspartate residue in the position immediately after the scissile bond, the P_1' position, makes this site unusual among caspase cleavage sites. Caspases-1, 3, 4, 6, 7, and 8 have been shown to prefer small amino acid residues such as alanine, glycine, and serine in the P_1' position whereas substrates with a P_1' aspartate are cleaved with low efficiency or not at all [11–13]. Accordingly, cleavage sites with an aspartate residue in the P_1' position are extremely rare among caspase substrates with Hsp90β being the only, yet unconfirmed, substrate of this category reported so far [14] and all reported caspase autoprocessing sites, except for caspase-15 D277 [4], having P_1' residues other than aspartate [7].

The present study demonstrates that auto-processing of caspase-15 at D277 requires prior cleavage at another site characterized by two consecutive aspartate residues. Our results show that the ability of caspase-15 to cleave substrate motifs with an aspartate in the P_1' position is utilized for a specific mode of autoactivation.

Materials and methods

Sequence analysis. Nucleotide sequences of caspase-2, 9, and 15 were either retrieved from the GenBank database or identified in the National Center for Biotechnology Information (NCBI) trace archive by discontiguous Mega BLAST (http://www.ncbi.nlm.nih.gov/blast/tracemb.shtml). Canine caspase-2 (GenBank Accession No. XM_843508) and 9 (XM_847070) and porcine caspase-15 (NM_001037147) were used as query sequences in the respective discontiguous Mega BLAST searches. The deduced amino acid sequences were aligned with the BLAST software package on the website of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/) and with the ClustalW algorithm at the website of the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw/).

Site-directed mutagenesis of caspase-15. A site-directed mutagenesis kit (Novagen) was used to mutate a series of aspartate residues in the intersubunit linker region of porcine caspase-15. D270, D271, D277, and D278 were mutated to asparagine in a pET-23a(+) (Novagen) expression construct of the catalytic domain of porcine caspase-15 fused to a carboxy-terminal His_6 tag [4]. D270 and D271 were also mutated in procaspase-15 that had been inserted into the mammalian expression vector pcDNA3.1 (Invitrogen) and thereby fused with a C-terminal V5-tag [4]. C258S caspase-15 constructs for expression in *E. coli* and mammalian cells have been described previously [4].

Production and characterization of recombinant caspase-15 mutants. The catalytic domain of wild-type caspase-15 and caspase-15 D270N, D271N, D277N, D278N as well as caspase-15 C258S (active-site mutant) [4] was expressed in the *E. coli* strain BL21 Star (DE3)pLysS (Invitrogen) and purified over a nickel-charged affinity resin (Invitrogen). Enrichment of recombinant protein was monitored by immunoblotting using anti-His-tag antibody (Invitrogen). Mouse antisera were raised against recombinant caspase-15 and used for Western blot analysis at a dilution of 1:2000 according to standard protocols [15]. The enzymatic activities of the recombinant caspase-15 variants were determined by incubation with IETD-pNA (Apotech) (100 μM) in a reaction buffer containing 10 mM EDTA, 50 mM NaCl, 10 mM DTT, 0.1% Chaps, 5% glycerol, and 50 mM Hepes (pH 7.2). Absorbance at 405 nm was measured at various time points during incubation at 37 °C. Activity units were defined arbitrarily as the increase in absorbance (mOD_{405}) within 1.5 h.

Overexpression of caspase-15 mutants in 293 cells. FreeStyle 293 cells (Invitrogen) were transfected with endotoxin-free plasmid preparations encoding full-length wild-type caspase-15, caspase-15 D270N, D271N, and C258S, respectively, using lipofectamine 2000 (Life Technologies). All open reading frames contained a C-terminal V5 tag. Twenty-four hours after transfection cells were lysed with a buffer containing 1% Nonidet P40 and complete protease inhibitor (Roche), and lysates were subjected to Western blot analysis with anti-V5-tag antibody (Invitrogen) as first step reagent.

Results

The amino acid sequence of the caspase-15 intersubunit linker region is highly conserved among Laurasiatherian mammals

Acting on the assumption that amino acid residues important for site-specific processing of the caspase subunits were evolutionarily conserved, we analyzed the amino acid sequences of caspase-15 and, for comparison, caspase-2 and 9 from 8 species of the mammalian superordinal clade Laurasiatheria: pig (*Sus scrofa*), cow (*Bos taurus*) (odd-toed ungulates), horse (*Equus caballus*) (even-toed ungulates), dog (*Canis familiaris*), cat (*Felis catus*) (carnivores), little brown bat (*Myotis lucifugus*) (bats), European hedgehog (*Erinaceus europaeus*), and common shrew (*Sorex araneus*) (eulipotyphlan insectivores) (Fig. 1A). These species were from 5 out of 6 orders of Laurasiatheria and, together, represented approximately 600 million years of independent evolution along different branches of the evolutionary tree [16].

Alignment of caspase-15 intersubunit linker sequences showed that, in addition to the aspartate residue, D277, previously shown to be the site of cleavage next to the mature small subunit [4], three other aspartate residues (D264, D270, and D271) were strictly conserved and a fourth one, D278, was conserved among Laurasiatherian mammals (Fig. 1A) but deleted in the marsupial, gray short-tailed opossum (*Monodelphis domestica*) [10]. The sequence around the D270 was identical in all Laurasiatherian species and contained the motif VETD which is an efficient autoactivation site motif in caspase-8 and highly similar to the preferred substrate of caspase-15, IETD [4].

Comparison of the intersubunit linkers of caspase-15 and other caspases showed that the caspase-15 linker was clearly shorter than those of most other mammalian caspases including caspase-2 and 9, which are otherwise structurally similar to caspase-15 [4] (Fig. 1B and C). Among 8 species of the Laurasiatherian clade, the sequence of the caspase-15 subunit linker was more conserved (70% identity) than that of the corresponding region of caspase-2 (47% identity) (Fig. 1B) and caspase-9 (31% identity) (Fig. 1C).