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An efficient method to express and refold a truncated human procaspase-9: A caspase with activity toward Glu-X bonds

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Abstract

A truncated form of human procaspase-9 missing the first 111 amino acids, and a variety of mutants derived therefrom, have been expressed in *Escherichia coli* inclusion bodies. Upon refolding to active enzymes, Δ(1–111) procaspase-9 and mutants were recovered at purity greater than 95% and with a final yield of 20-35 mg/L cell culture. Our active procaspase-9 retains its pro-segment, while undergoing major auto processing at Asp^{315} and a minor (20%) cleavage at Glu^{306}. This unusual cleavage at a Glu-X bond also took place in the D315E mutant, and we describe herein the inhibitor Z-VAE-fmk that shows enhanced inactivation of procaspase-9 over caspases-3. The bond at Asp^{330}, not processed by procaspase-9, is cleaved by caspase-3 and the resulting procaspase-9 variant, missing the 316–330 bridge, is six times as active as the non-mutated Δ(1–111) proenzyme. A deletion mutant lacking residues 316–330 underwent auto activation by cleavage at Asp^{315}-Ala^{331} bond. Moreover, substitution of Glu^{306} by an Asp residue in this mutant led to rapid removal of the peptide spanning Ser^{307} to Asp^{330}, and resulted in an enzyme that was 7.6 times as active as the non-mutated Δ(1–111) procaspase-9. Finally, replacing both Asp315 and Glu306 with Ala generated a procaspase-9 mutant incapable of auto processing. This single chain procaspase-9 was fully as active as the non-mutated Δ(1–111) enzyme processed at Asp^{315} or Glu^{306}. Our demonstration that unprocessed procaspase-9 mutants are active as proteases with caspase-type specificity suggests that the role of procaspase-9 in cascade activation of executioner caspases might, in some circumstances, be carried out alone and without association of the apoptosome.

Keywords: Apoptosis; Apoptosome; Caspase 9; Caspase inhibitors; Mitochondria; Procaspase-9

Cysteine-dependent aspartate-specific proteases (Caspases)[2] are a family of cysteinyl endopeptidases that are involved in apoptosis, or programmed cell death [1–5]. These enzymes cleave their substrates at Asp-X bonds and show some degree of substrate specificity for amino acids in positions P_4–P_1 [6]. The 14 mammalian caspases identified to date are thought to exist in cells as inactive zymogens, referred to as procaspases, and much effort has been made to understand their modes of activation and regulation. All procaspases are organized into three regions: an N-terminal pro-segment; a large N-terminal subunit region; and a small C-terminal subunit region. In some procaspases, the large and small subunits are bridged by a short stretch of amino acids. Activation is brought about by autoproteolysis, or cleavage by another caspase or granzyme, at specific aspartic acids that define these regional boundaries. The result of procaspase processing is the shedding of the

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[2] *Abbreviations used:* Caspases, cysteine-dependent aspartate-specific proteases; IMS, intermembrane space; IB, inclusion body.

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pro-segment and the bridge with formation of a large and small subunit that assemble into a heterotetramer composed of two small and two large subunits. The heterotetramer is the active form of caspases. With respect to apoptosis, caspases have been classified as initiator and effector enzymes. Initiator, or upstream caspases, such as, -2, -4, -5, -8, -9, and -10 are believed to be activated as a result of signals coming from outside or inside the cell and they, in turn, activate downstream effector caspases such as -3, -6, and -7 [4,5,7,8]. The effector caspases are the executioners of apoptosis, being involved directly in breakdown of a variety of important cellular proteins, other caspases, and some Bcl-2 family members [9–11]. This results in an irreversible commitment to cell death. Two distinct pathways that lead to caspase activation in apoptosis have been characterized: the cell surface death receptor pathway and the mitochondria initiated pathway [12,13]. Both lead to activation of caspases that cleave proteins critical to cell survival [7,8].

Caspase-9 initiates a mitochondrial pathway for propagation of apoptotic signals. When cells receive apoptotic stimuli, mitochondria release cytochrome *c*, which binds to cytoplasmic Apaf-1, the mammalian homolog of the *Caenorhabditis elegans* protein, CED-4. This complex, together with dATP, recruits procaspase-9, leading to its auto-activation by cleavage at Asp^{315} to produce a large and a small subunit [13–17]. Procaspases-3, and -7 are targets for cleavage and activation by caspase-9, and this sets the stage for destruction of cellular proteins leading to cell death [18]. In addition, studies in vitro and in intact cells have shown that caspase-3 is also in the apoptosome. Here, caspase-3 cleaves procaspase-9 at Asp^{330}, removing the bridge segment from 316 to 330, and generating p35 and p10 subunits with concomitant increase in apoptosome activity [14,19,20]. This significant increase in apoptosome activity (up to 8-fold) was observed by Zou et al. [21] in a system containing purified, recombinant Apaf-1, caspase-9, and caspase-3, again suggesting a synergistic effect of caspase-3 on caspase-9 activity in the cytosolic apoptosome.

Though procaspases reside mainly in the cell cytosol, they are also localized in subcelluar compartments. For example, procaspase-2 [22], procaspase-3 [23,24], procaspase-8 [25], and procaspase-9 [22,26–28] have been identified in the mitochondrial intermembrane space (IMS). Recent work [27] supports the belief that, upon induction of apoptosis, mitochondrial procaspase-9 and caspase-3 migrate to the cytosol where they become activated, and then return to the mitochondria. Yet another study [28] found that procaspase-9 processing takes place in purified mitochondria exposed to H_2O_2, supporting the notion that caspase-9 can work in the absence of the apoptosome.

This communication describes the cloning, expression and refolding of an N-terminally truncated form of recombinant human procaspase-9 missing residues 1–111. The Δ(1–111) procaspase-9 variant, recovered from *Escherichia coli* inclusion bodies, was refolded, activated, and characterized in terms of kinetic properties, and protein chemistry.

A series of Δ(1–111) procaspase-9 mutants was also generated in this study, including some described earlier by Zou et al. [21] in an investigation of procaspase-9 activation in the presence of components of the apoptosome. All of our work has been done with the purified procaspase-9 variants alone, excluding any of the apoptosome cofactors. In contrast to other caspases described thus far, our procaspase-9 variant and mutants showed remarkable activity in autoprocessing at Glu^{306} and at the Glu^{315} residue in the D315E mutant. This led us to test whether a conventional caspase inhibitor such as Z-VAD-fmk might be modified to Z-VAE-fmk to yield a compound specific for caspase-9. Indeed, Z-VAE-fmk showed enhanced inhibition of our procaspase-9 variant over caspases-3 and -8, in keeping with the broader specificity of procaspase-9 toward Glu-X bonds. In the course of this work, the greatest activities derived from mutants having small subunits beginning at Ala^{331}. Extensions of the large subunit had no effect on catalytic activity. Finally, an intact, single chain procaspase-9 mutant incapable of autoprocessing at Glu^{306} or Asp^{315}, nevertheless showed substantial activity toward model substrates in the absence of any of the usual components of the apoptosome.

Materials and methods

Reagents

Quick-clone human heart cDNA was purchased from Clontech (Palo Alto, CA). TA cloning vector, restriction enzymes, and media for cell culture were purchased from Invitrogen (Carlsbad, CA). pQE-30 vector, M15 [pREP4] cells, plasmid miniprep kit were from Qiagen (Chatsworth, CA). XL1 Blue cells, *Pfu* DNA polymerase, QuickChange site-directed mutagenesis kit, and T4 DNA ligase were purchased from Stratagene (La Jolla, CA). Oligonucleotide primers were synthesized by Sigma-Genosys (The Woodlands, TX). The substrates for enzyme assays, Ac-LEHD-α-(4-methyl-coumaryl-7-amide) and Ac-IETD-α-(4-methyl-coumaryl-7-amide) were purchased from Peptide Institute (Osaka, Japan). Z-VAD-fmk and Z-VAE-fmk were obtained from Peptide International (Louisville, KY). Recombinant caspase-3 and -8 were prepared as previously described [29,30], respectively. All other chemicals were purchased from Sigma unless specified otherwise.

Cloning and expression of procaspase-9 and mutant proteins

The cDNA containing the full length procaspase-9 plus 151 bp of 3′ non-translated region was amplified from human heart cDNA using forward primer: 5′-CGGCCTG GAGTCTTAGTTGG and reverse primer: 5′AAGAGCC TGTCTGTCACTGG by standard PCR. The PCR product was purified, sequenced to confirm the identity of the procaspase-9, and cloned into vector PCRII using the TA cloning kit. The procaspase-9 was amplified from the PCRII-procaspase-9 using forward primer: 5′ CGCGGAT