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Engineered Hybrid Dimers: Tracking the Activation Pathway of Caspase-7

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Summary

Caspase-7 is an obligate dimer of catalytic domains, with generation of activity requiring limited proteolysis within a region that separates the large and small chains of each domain. Using hybrid dimers we distinguish the relative contribution of each domain to catalysis by the whole molecule. We demonstrate that the zymogen arises from direct dimerization and not domain swapping. In contrast to previous conclusions, we show that only one of the catalytic domains must be proteolyzed to enable activation. The processed domain of this singly cleaved zymogen has the same catalytic activity as a domain of fully active caspase-7. A transient intermediate of singly cleaved dimeric caspase-7 can be found in a cell-free model of apoptosis induction. However, we see no evidence for an analogous intermediate of the related executioner caspase-3. Our study demonstrates the efficiency by which the executioner caspases are activated in vivo.

Introduction

Caspases constitute a family of cytosolic proteases with a stringent specificity for cleaving target proteins after aspartate residue. They are synthesized as zymogens awaiting activation by one of the routes available within a cell. During apoptosis in humans, initiator caspase-8, -9 and/or -10, which integrate molecular signals into proteolytic activity, are activated by dimerization at the death-inducing signaling complex (DISC, caspase-8 and -10) or the apoptosome (caspase-9). Once activated, the initiators activate the executioner zymogens by direct limited proteolysis, thus amplifying the apoptotic signal (Boatright and Salvesen, 2003). Activated executioner caspases cleave a limited set of cellular substrates (Fischer et al., 2003), thereby causing the demise of the cell through a set of hallmarks (Green and Evan, 2002).

Cleavage in a region between the large and small subunits (or chains), called the interchain connector, is the only requirement of executioner caspases to gain full activity. Whereas the site of cleavage within the connector may not directly impact the activity of the caspase (Zhou and Salvesen, 1997), it has important repercussions for its regulation. Cleavage of caspases reveals a new N terminus that often facilitates caspase inhibition by endogenous regulators (Riedl and Shi, 2004; Scott et al., 2005).

The zymogens of the executioner caspase-3 and -7 are homodimers, and therefore, they contain two potential active sites and two interchain connectors per molecule. A molecule of procaspase-3 originates from the association of two monomeric folding intermediates, and the domains are tightly associated in the procaspase-3 dimer (Bose and Clark, 2001). It is likely that this holds for caspase-7, which from a structural and mechanistic point of view provides a better paradigm, because atomic resolution structures are available in the uncleaved zymogen form, the cleaved, unliganded form, and the cleaved, ligand bound form (Wei et al., 2000; Chai et al., 2001; Riedl et al., 2001). Together, these structures reveal that activation requires the movement of three surface loops that contain crucial elements of the catalytic apparatus and specificity sites. It is considered that cleavage in the interchain connectors has two essential functions: (1) removal of blocking segments that prevent translocation of one of these loops in the zymogen form and (2) allowing interactions between the newly formed terminals of the small and large chains from neighboring domains to stabilize the active conformation by forming a loop bundle with each other.

The prevailing view is that both interchain connectors must be cleaved to allow formation of an active enzyme, because both connectors must be removed from the central cavity at the dimer interface, with subsequent formation of the loop bundle (Chai et al., 2001; Riedl et al., 2001). Moreover, the active initiator caspases-8 and -10 are dimeric enzymes with active sites that can be docked, at least in silico, onto the general space occupied by the interchain connector region of procaspase-7, leading to the hypothesis that a concerted and cooperative mechanism may account for simultaneous cleavage of both connectors of the zymogen. The available crystal structures cannot answer these fundamental hypotheses, primarily because the interchain connector is an intrinsically mobile loop. Therefore, we tested the importance of the hypothesis that both interchain connectors in a procaspase-7 molecule must be cleaved to become active by a combination of kinetics, mutagenesis, and selecting engineered caspase-7 hybrids. We also tested the two competing models for the formation of executioner caspase zymogens: a domain swapping model (Pop et al., 2001; Roy et al., 2001; Wei et al., 2000; Wilson et al., 1994) and a direct dimerization model (Bose and Clark, 2001; Denault and Salvesen, 2002; Pop et al., 2001).

Results

Activation of Recombinant Caspase-7 by Initiator Caspases

Upon expression in *E. coli*, single chain procaspase-7 is converted to a large and a small chain by cleavage in the interchain connector, with a $>10^4$-fold increase in catalysis (Zhou and Salvesen, 1997). The connector contains

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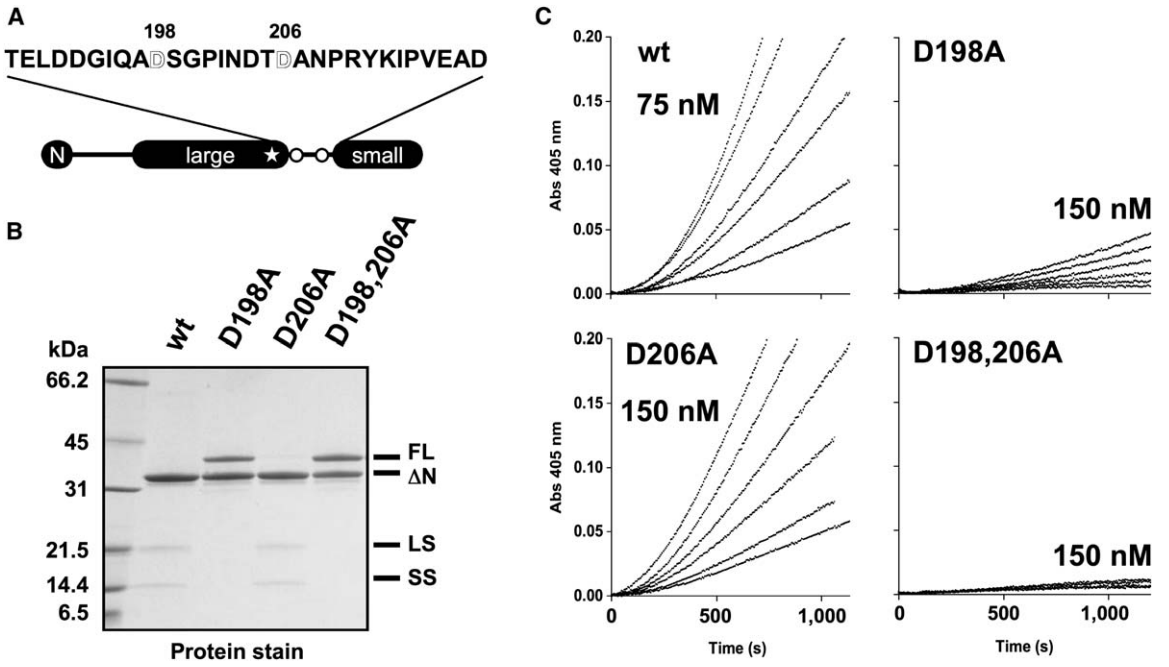

Figure 1. Kinetics of Procaspase-7 Activation by Initiator Caspases Show Strong Preference for Asp198

(A) Schematic representation of caspase-7 with the catalytic cysteine residue (star) and the two activation sites (open circles, outlined residues) found in its interchain connector. The "N" refers to the 23 amino acid N peptide removed during apoptosis and *E. coli* expression.

(B) The protein stain shows the integrity of the proteins used. Caspase-7 proteins carrying the D198A mutation are unable to activate efficiently and are less effective in processing the N peptide.

(C) Various concentrations of recombinant procaspase-7 activation site mutants were incubated with recombinant caspase-8 (25 nM) in the presence of 400 μM AcDEVD-pNA chromogenic substrate. A sample containing caspase-8 alone was used to determine its contribution to the hydrolysis of the substrate (<5%) and was subtracted to generate the datasets presented here. Similar experiments were carried out with caspase-2, -9, -10, and caspase-8:FLIP heterodimer. Activation rates were determined as described in the Experimental Procedures and are presented in Table 1. Procaspase-7 concentrations were as follows: wt: 75, 56, 42, 32, 24, and 18 nM; D198A: 150, 75, 56, 42, 32, and 24 nM; D206A: 150, 67, 44, 30, 20, and 13 nM; and D198A,D206A: 150, 75, 56, and 42 nM. The highest concentration of procaspase-7 is indicated for each panel. ΔN, full-length protein lacking the N peptide; FL, full-length; and LS or SS, large or small subunit.

two aspartate residues that could serve as proteolytic activation sites for apical caspases-2, -8, -9, and -10 (Figure 1A). It is unclear which of the sites are preferred by apical caspases, and to determine this, we mutated each site separately, purified recombinant proenzymes (Figure 1B), and determined the kinetics of activation (Figure 1C and Table 1). For comparison of apical caspase activation of procaspase-7, we employed high-salt buffer, where initiator caspase-8, -9, and -10 are maximally active (Boatright et al., 2004). Under this condition, procaspase-7 was activated with k_{cat}/K_M values from $1.6 - 3.4 \times 10^4$/M/s, and mutagenesis indicates that the preferred site is Asp198 (Figure 1C and Table 1). Interestingly, each of the apical caspases showed only minor use of Asp206 as an activation site. There is a formal possibility that cleavage at Asp198 enhances processing at Asp206. We thus tested this possibility by processing at 198 with the natural caspase activator granzyme B (GrB), followed by processing at 206 by caspase-8 (see Figure S1 in the Supplemental Data available with this article online). Because further processing at Asp206 cannot be measured enzymatically and could contribute to the readout, we used the catalytic mutant of procaspase-7 and analyzed the result on SDS-PAGE. Differences in the generation of the small subunit cleaved at 206 (see Figure S1) were less than 4-fold, suggesting that cleavage at Asp206 is relatively independent of the status of cleavage at Asp198 for its accessibility to proteolysis by an initiator caspase. In contrast to the apical caspase-8, -9, and -10, caspase-2 was unable to efficiently activate wild-type (wt) caspase-7 (Table 1) at either site.

The zymogen of caspase-7 is a dimer and contains connectors from both catalytic units juxtaposed in the general area of the dimer interface (Chai et al., 2001; Riedl et al., 2001). This raises the intriguing possibility that both catalytic units of the dimeric procaspase-7 zymogen may be simultaneously processed at their respective Asp198 sites in a concerted cooperative manner. If true, one would expect a caspase-8 homodimer (two active sites) to be more than twice as effective as a caspase-8 heterodimer (one active site). Caspase-8 heterodimers were obtained by incubation in the presence of the inactive caspase-8 paralog FLIP (Boatright et al., 2004). Homo- and heterodimers were used in activation assays in which the same concentration of active caspase-8 active sites was employed. Activation rates were similar for homodimer and heterodimer (Table 1), pointing to the absence of detectable cooperativity.

Analysis of the crystal structures of cleaved caspases reveals a critical role for residue 291 (caspase-1 numbering; residue 192 in caspase-7) in the formation of a loop bundle that stabilize the active site (Chai et al., 2001). To test the importance of residue 192 in