

Caspases: Keys in the Ignition of Cell Death

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Caspases: Keys in the Ignition of Cell Death

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I. Introduction

Cell death is obligatory for harmonious cell life in animals, from the cavitation of the early embryo to the removal of infected or cancerous cells in the adult. Although there is more than one way for any given cell to die, it is generally the apoptotic mechanism that is used by the organism because it is a tidy, organized, and inflammation-free process. At the center of this death process is a family of proteases named caspases (the “c” is intended to reflect a cysteine protease mechanism, and “aspase” refers to their ability to cleave after aspartic acid, the most distinctive catalytic feature of this protease family) (see Figure 1). Members of the family are found in worms to flies to humans and can be traced back to

distantly related proteases from *Porphyromonas gingivalis*. In humans, caspases are ubiquitously expressed cytosolic proteases synthesized as latent zymogens awaiting an appropriate activation stimulus. Whereas seven members (caspases 2, 3, and 6–10) are usually considered to be part of the apoptotic machinery, three others (caspases 1, 4, and 5) are employed by another innate defense mechanism, the activation of proinflammatory cytokines. Despite the distinction between both processes, cyto-

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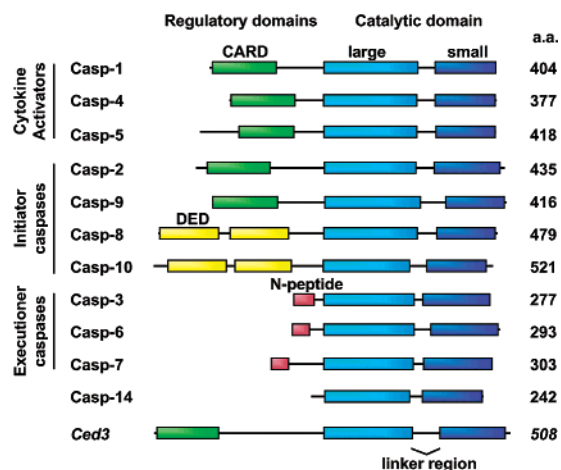


Figure 1. Schematic of primary structures of caspases. Human caspases are grouped according to their *in vivo* role: cytokine activators, initiator and executioner caspases. The five known domains of caspases are color coded: CARD (green) and DED (yellow) are involved in recruitment and activation; the N-peptide of executioner caspases (red) that is removed during apoptosis; the large subunit (cyan) and small subunit (blue) forming the catalytic domain are separated by a linker region. Caspases are drawn to scale and the number of amino acids of the longest known isoform of each human protein is shown on the right. Ced3 is from *C. elegans*, but is included since it is one of the founders of the family.

kine activators and apoptotic caspases have more similarities than differences. Finally, the odd one among this family, caspase 14, maybe involved in keratinocyte differentiation and is discussed below. Caspases are thus a very attractive field of research at the intersection of development, cancer, infection, and degenerative diseases.

A. Apoptosis

During the past decade, many proteins were shown to be cleaved during apoptosis in a caspase-dependent manner. Surveys using 2D-gel electrophoresis revealed that approximately 100 proteins are cleaved during apoptosis.^{1–3} An important question that has yet to be resolved is whether these are true and necessary substrates of caspases, or simple bystanders, indeed, only a few have yet been demonstrated to be important for the proper process of apoptosis. Hallmark phenotypes of apoptosis are generally a result of a single protein being cleaved. Much of the time it is an inactivation event, but there are few examples of gain of function caused by caspase proteolysis. Loss of function cleavages include proteolysis of poly(ADP-ribose) polymerase (PARP) implicated in DNA repair,⁴ inhibitor of caspase activated DNAase (ICAD/DFF45) that causes DNA fragmentation,^{5–7} 70 kDa U1 snRNP implicated in RNA splicing⁸ and GRASP65, a Golgi reassembly and stacking protein.⁹ Cleavage may also cause a gain of function by the removal of a negative regulatory domain: protein kinase C δ activation,¹⁰ and ROCK I activation implicated in membrane blebbing and apoptotic body formation.^{11,12} All those cleavage events fit caspase 3/7 substrate preferences. On the other hand, caspase 6 is effective in lamin B₁ pro-

teolysis¹³ and keratin 18 cleavage¹⁴ that result in nuclear architecture dismantling and cytoskeletal alterations. Cleavage of Bid, a pro-apoptotic Bcl-2 family member, by the initiator caspase 8 allows the extrinsic pathway to tie into the intrinsic pathway.¹⁵ These cleavage events represent some of the core events of apoptosis whose combined effect is ultimately to allow the proper disposal of the doomed cell.

B. Initiation of Apoptosis

Initiation of apoptosis is a tightly regulated mechanism. The constantly growing list of regulators may be confusing, but a simplified picture is shown in Figure 2. At the starting point of the extrinsic pathway is the ligation of a death receptor by its cognate ligand. Several pairs of receptor–ligand interactions have been shown to activate initiator caspases. On the cytoplasmic face of the membrane, a protein complex assembles forming the death inducing signaling complex (DISC). An adapter protein with a death domain (DD) to interact with the receptor and a CARD (caspase recruitment domain) or DED (death effector domain) to bind the initiator caspase (caspases 2, 8, 10) links the receptor to caspases. Because the DISC is a scaffold of signaling proteins, many pro-caspase molecules are gathered together, and the increased local concentration of zymogens is hypothesized to be the grounds for caspase activation.¹⁶ This hypothesis relies on the low zymogenicity of initiator caspases, a small amount of activity displayed by the zymogen. For example, pro-caspase 8 is 1% as active as the mature enzyme in contrast to the executioner caspase 3 that is >10 000 more active when processed.¹⁶ This property could account for the activation of the first caspase zymogen at the DISC, and the signal is now amplified and transmitted to the executioner caspase 3.

The intrinsic pathway uses a different mechanism to activate caspase 9. It can be triggered by a plethora of chemicals, especially DNA damaging agents, which are integrated at the protein level by members of the Bcl-2 family.^{17,18} The next key event seems to be the interaction of Bcl-2 family members with mitochondria, followed by the release of cytochrome *c*. In the presence of cytochrome *c* and ATP, the caspase 9 activator/cofactor Apaf 1 forms a ~700 kDa heptamer rosette.¹⁹ CARDs of the Apaf 1 molecule are gathered on the top of the structure and form a rendezvous point for pro-caspase 9. Pro-caspase 9 is a monomer at cytosolic concentration and it is the dimerization, not the proteolytic cleavage of the linker region that activates this particular initiator caspase.^{20,21}

C. The Apoptotic Cascade

Biochemical studies (reviewed in refs 22–26) have made it possible to place the apoptotic caspases in a pathway, such that some are activated by others: initiator caspases → caspase 3 → caspase 7. This is supported by the inherent substrate preference of caspases;²⁷ initiator caspases prefer sequences that are found in the linker domain of executioner cas-

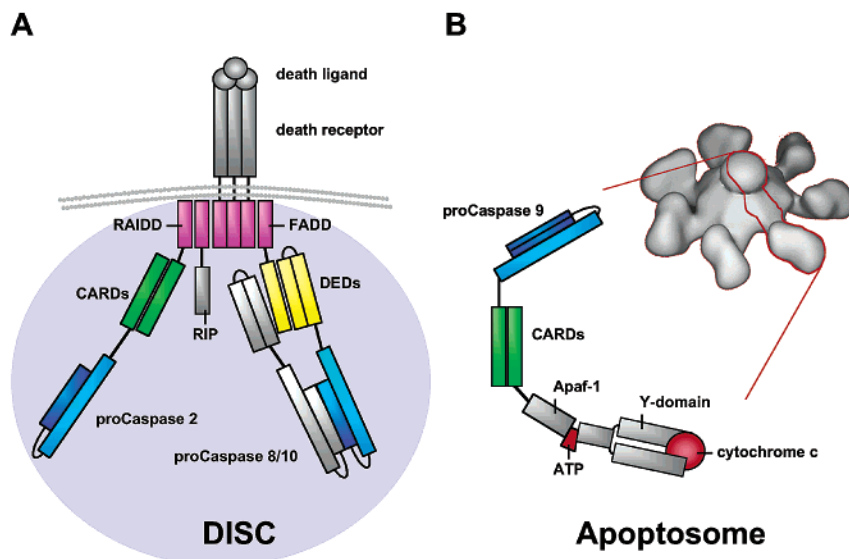


Figure 2. The extrinsic and intrinsic pathways. The extrinsic pathway starts with ligation of death receptors such as Fas/CD95. This leads to the formation of a huge complex of proteins including adaptors, regulators and pro-caspases. The DISC depicted here models how a receptor can lead to the activation of CARD or DED containing caspases. With proteins having both DD (pink) to bind the activated receptors and a CARD (e.g., RAIDD) or DED (e.g., FADD) domain to bind the pro-caspase, the receptor can indirectly bind both types of initiator caspases. Gathering of many caspase molecules at the DISC is thought to increase the local concentration of pro-caspases and leads to caspase activation according to the induced proximity model.¹⁶ Still not understood in this model is the stoichiometry of the DISC, which DED of the pro-caspases interacts with the adaptor, and if the bound pro-caspase is monomeric or dimeric. The intrinsic pathway achieves the same goal but in a very different manner. Release of cytochrome *c* by the mitochondria is the initial step of this pathway. Apaf 1 is activated in the presence of ATP and oligomerizes forming the apoptosome, shown here as a 3-D reconstruction (reprinted from ref 19, Copyright 2002, with permission from Elsevier Science). This structure binds several molecule of pro-caspase 9 on its top via homotypic CARD/CARD interactions, thereby significantly increasing the local pro-caspase 9 concentration. The driving force of caspase 9 activation is dimerization catalyzed by the apoptosome.

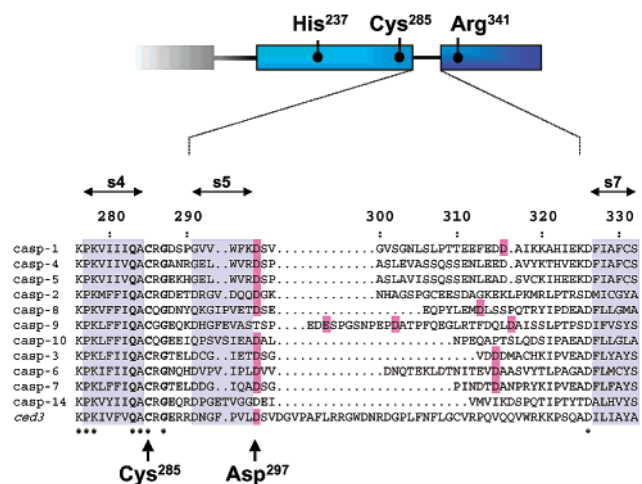


Figure 3. Alignment of the linker domains of human caspases. The numbering convention of caspase 1 is used. The schematic representation of a caspase is presented above the alignment and shows the relative position of the catalytic dyad and the specificity determinant R341. With the known exception of caspase 9, processing of the linker region is thought to be the general mechanism of caspase activation. It occurs most often at D297 (highlighted in red). Other sites may also be processed, generally by autolytic cleavage. The significance of the second sites is yet to be confirmed, though it has been suggested that cleavage at the DQLD/A site is important in removing the ability of IAPs to inhibit caspase 9.

pases (see Figure 3). This enables the initiators to specifically process and activate the executioner zymogens. Initial findings suggest that the N-termi-

nal peptides of caspases 3 and 7 have no effect on activity or "activability" in vitro. However, in vivo both caspases seem to require the removal of the N-peptides for efficient activation. Indeed, in some cells caspase 3 is required to remove the N-peptide of caspase 7 before the latter can be activated by an initiator caspase.²⁸ An additional degree of complexity in understanding the role of presumptive apoptotic caspases is revealed by the preference of caspase 2 for executioner type substrate motifs and caspase 6 for initiator type substrate motifs.²⁷ This is in contrast to the expected position of caspase 2 as an initiator caspase²⁹ and caspase 6 as an executioner caspase.^{30,31} So, although much has been learned of the inherent properties of caspases, several of their roles are in no way certain. This review focuses on the caspase family from the perspective of their structure, activity, and regulation.

The first level of caspase regulation is by conversion of zymogens to their active forms in response to inflammatory or apoptotic stimuli. The second level of regulation is organized by the specific inhibition of active caspases by natural inhibitors. Members of three protein families have been found capable of ablating caspase activity in vitro and in vivo. One of these, the inhibitors of apoptosis protein (IAP) family regulates cellular apoptosis by direct caspase inhibition in animals from flies to humans,³² and this process will be touched on briefly at the end of the review. The two other types of inhibitors include the cowpoxvirus protein CrmA, a serpin, and the baculovirus protein p35.

II. Structure and Function

A. Family Members

1. Cytokine Activators

Caspase 1 is a cytokine activator. This was first shown by the necessity of an aspartic-specific cysteine protease for the maturation of pro-IL- β .^{33,34} In agreement with this proposed role of caspase 1,^{35,36} gene ablation experiments result in mice that cannot process pro-IL-1 β and pro-IL-18.³⁷ These mice are highly resistant to septic shock³⁸ and have lower production of a variety of other cytokines (IL-1 α , IL-6, TNF α , and interferon γ) in response to lipopolysaccharides.³⁷ However, no overt apoptotic phenotype or developmental effect was observed demonstrating that caspase 1 is not related to apoptosis, except in a paracrine manner by sensitizing cells to apoptosis. Even though caspases 4 and 5 are poorly studied enzymes, they are most likely cytokine activators because of the high protein identity (see Table 1), domain organization (see Figure 1) and comparable substrate specificity to caspase 1. Presumably, caspases 4 and 5, which seem to be orthologs of mouse caspases 11 and/or 12, process cytokines in response to different inflammatory stimuli or pathological situations than caspase 1, or cooperate with caspase 1 to produce an adequate inflammatory response.

2. Initiator Caspases

Initiator caspases represent the transmission arms in the apoptotic engine; they convert apoptotic signaling to proteolytic activity. They integrate signals in response to death receptor ligation (extrinsic pathway with caspases 8 and 10, and possibly caspase 2) or in response to developmental cues or cellular stress (intrinsic pathway with caspase 9). Recruitment and activation of the initiator caspases is achieved by adapter molecules that bridge to death

receptors via homophilic DEDs (for caspases 8 and 10), or via CARD, between caspase 9 and the cofactor Apaf 1 (reviewed in ref 39). In many cultured cellular models in which caspase 8 and 10 are expressed, they seem redundant⁴⁰ probably owing to the high identity (48%), similar substrate specificity and domain organization. Nevertheless, in mice the caspase 8^{-/-} phenotype is lethal suggesting that either caspase 8 and 10 complement each other during development, or that they are differentially expressed, or that mice lack caspase 10. The need for initiator caspases may be 3-fold. First, they permit sensing and integration of different inputs, transmitting to a common execution phase. Second, they enforce amplification of the apoptotic system by generating substantial amounts of active executioner caspases. Third, they allow for a point of regulation before the final commitment to death. The prominence of initiator caspases in development is underscored by the phenotype of gene ablated mice, which demonstrate lethality for caspases 8 (embryonic lethality)⁴¹ and 9 (perinatal lethality).^{42,43} Caspase 2^{-/-} mice have a much less severe phenotype of excess oocytes and are resistant to specific pathologic stresses.^{44,45}

3. Executioner Caspases

In contrast to the initiators, caspases 3, 6, and 7 each possess a short distinct N-terminal peptide (23–28 residues). The reason for having three executioner caspases is not clear, and indeed the evidence for the importance of caspase 6 is lacking. Whereas caspase 3 is essential for normal embryonic development,⁴⁶ the phenotype of mice ablated in caspase 6 appear to be normal.⁴⁷ The phenotype of mice ablated in caspase 7 has yet to be reported, yet it is evident that caspases 3 and 7 are almost synonymous in their substrate and inhibitor specificity. Nevertheless, biochemical experiments place the activation of caspases 6 and 7 downstream of caspase 3,³⁰ and so we will consider them as executioner caspases.

4. Other Caspases

Reportedly expressed mainly in keratinocytes (at least on the protein level), caspase 14 is the odd one among this family. It was shown to be expressed and processed in epidermis and in an artificial skin model although no role has been proposed yet for the protein.^{48,49} It is interesting to note that caspase 14 is a minimal caspase with no particular module in front of its catalytic domain. Early reports⁵⁰ suggested that a 12th member of this family (caspase 13) existed, but it seems that there is no caspase 13 in humans and that the caspase family is probably complete with its 11 members.

In humans, a distant homologue to caspases, termed paracaspase/MALT1, has been described.⁵¹ Currently, no enzymatic activity has been ascribed to paracaspase although a recent study suggests its possible involvement in NF- κ B signaling pathway induced by Bcl10 protein.⁵² Other even more distant members of the superfamily include the proteases separase (required for sister chromatid separation

Table 1. Homology Table between Catalytic Domains of Caspases and *C. elegans* Ced3 Protease^a

	Caspases										
%	4	5	2	8	9	10	3	6	7	14	Ced3
1	61	60	29	18	24	20	28	21	22	23	26
4		83	29	16	24	22	27	19	23	23	25
5			30	16	24	22	27	22	23	24	28
2				26	30	26	31	27	30	31	32
8					34	48	38	24	39	29	32
9						32	37	29	34	24	31
10							34	30	38	27	30
3								42	57	30	34
6									39	25	31
7										29	34
14											28

^a Data are expressed in percentage identity on a pairwise comparison basis of human proteins. Values above 40% are shaded.

during anaphase)⁵³ and legumain (involved in lysosomal/endosomal protein processing).⁵⁴ Further details on caspase phylogeny can be found in ref 55.

B. Domain Structure

1. Regulatory Domains

Regulatory domains of caspases consist of a CARD, DEDs, or a short N-peptide. Whereas the latter have no clearly defined function, the first two enable recruitment of the initiator caspases to their respective activation complexes, and their respective structures have been determined. Both the CARD and DED are members of the Death Domain family of adapters, found in many biologic instances where proteins are recruited by homophilic interactions. The basic form of the protein fold has six antiparallel helices tightly wrapping a hydrophobic core (reviewed in ref 39). The activation of pro-caspase 9 necessitates the union of several zymogen molecules at the apoptosome via the interaction of caspase 9's CARD and the activation-competent CARD of Apaf 1. It is the complementary charge distribution between these two CARDS that provides the specificity of the interaction, as evidenced by mutation of selected basic residues of the caspase 9 CARD.⁵⁶ The opposite is also true, changing acidic residues on Apaf 1 prevents binding to caspase 9.

Two DEDs comprise the N-terminal domain of caspases 8 and 10. In the case of caspase 8, and presumably also caspase 10, DED domains are necessary for recruitment to the death receptor Fas/CD95 via the adapter FADD. In this case, the primary recognition surfaces are hydrophobic, and point mutations in the hydrophobic interaction surface to nonaromatic residues diminish caspase 8 binding.⁵⁷

Finally, the short N-peptides found in the three executioner caspases have no clearly defined function, and no structure is currently available. However, there may be no specific binding of these N-peptides to the main catalytic domain, since crystal structures of pro-caspase 7 failed to reveal any density for the segment.^{58,59} It is noteworthy that N-peptides are highly charged sequences; for example, the caspase 7 N-peptide is composed of 1/3 acidic residues. The N-peptides of caspases 3 and 7 have no influence on the inherent activation or activity of the proteins in vitro,⁶⁰ though they may have an influence in vivo.²⁸ Among the potential roles are the silencing of caspase 3 activity⁶¹ and the subcellular localization for caspase 7,⁶² but further studies will be required to detail the mechanistic significance of those findings. Because N-peptides are removed during caspase activation, further investigation on this domain would probably reveal interesting findings.

2. Catalytic Domain

Primary sequence analysis of the catalytic domain of caspases reveals several major characteristics (see Table 1). Cytokine activators present higher identity (60–83%) to each other than any other caspase subgroup. This reinforces the current hypothesis that this subgroup as a whole is not involved in apoptosis.

High protein identity is also found in subsets of caspases though to play similar roles and have similar substrate preferences [cytokine activators, (W/Y)EHD↓; executioner caspases 3 and 7, DEXD↓; apical caspases 8 and 10, (I/L/V)EHD↓].^{27,63} Finally, all caspases share identity in a similar range (25–34%) to the Ced3 protease from *Caenorhabditis elegans*, clearly showing the evolutionary relatedness of caspases and their specialization in higher organisms.

Many groups have published crystallographic structures of the catalytic domain. Structures of caspases 1,^{64,65} 3,^{66–68} 7,^{58,69,70} 8,^{71,72} and 9²¹ alone or complexed with various types of inhibitors have been solved and revealed a closely related common fold (see Figure 4A–C). A catalytic unit, as defined by a large and a small subunit duo, is folded into a compact cylinder with a six-stranded β -sheet in the center surrounded by five helices positioned on opposing sides of the plane formed by the β -sheets. Even though some structures present extra strands, the overall form is conserved. Active caspases exist as a dimer of catalytic units related by 2-fold rotational symmetry. Both active sites are thus on the same side of the dimer; the small subunits provide the interface of the dimer. The key residues C285 (caspase 1 residue numbering convention)^{64,65} and H237 necessary for catalysis are found on the large subunit and form the catalytic dyad. It has been proposed that the backbone carbonyl of residue 177 may form a third catalytic component (see Figure 4D).^{64,65} This proposal is based on the highly conserved H-bonding distance between τ nitrogen of H237 and the carbonyl of residue 177, which may serve to position the catalytic His, even though the side chain of residue 177 is not conserved. Unfortunately, this implication of a third residue in the catalytic process, common to most other cysteine and serine proteases families, cannot readily be tested in caspases because of the difficulty to assess the role of the peptide backbone by mutagenesis. In the case of papain, a clan CA cysteine protease, the third residue on the triad (Asn175) was shown to help catalysis (high k_{cat}) but is not essential suggesting that a third residue is not mandatory for caspases.⁷³ The proteolytic mechanism of caspases is reminiscent of other cysteine proteases, with caveats. Once a substrate is bound to the catalytic site, the NH backbone of G238 and C285 forming the oxyanion hole donate hydrogen bonds to the carbonyl oxygen, thus polarizing the carbonyl group of the scissile bond. The nucleophilic C285 can now attack the electrophilic carbon (first tetrahedral intermediate). However, because of the greater than normal distance between the catalytic His and Cys (5–6 Å), it is unlikely that the proton of the thiol group can be passed to the neighboring His to act as a catalytic acid. More likely, the catalytic H237 acts by protonating the amine group of the leaving peptide product, and abstracting a proton from a water molecule to complete the deacylation step. Added complexity to the catalytic mechanism has recently been suggested by the observation of the crystal structure of unbound caspases 7 and 9. Whereas most structures have peptide inhibitors bound, the un-

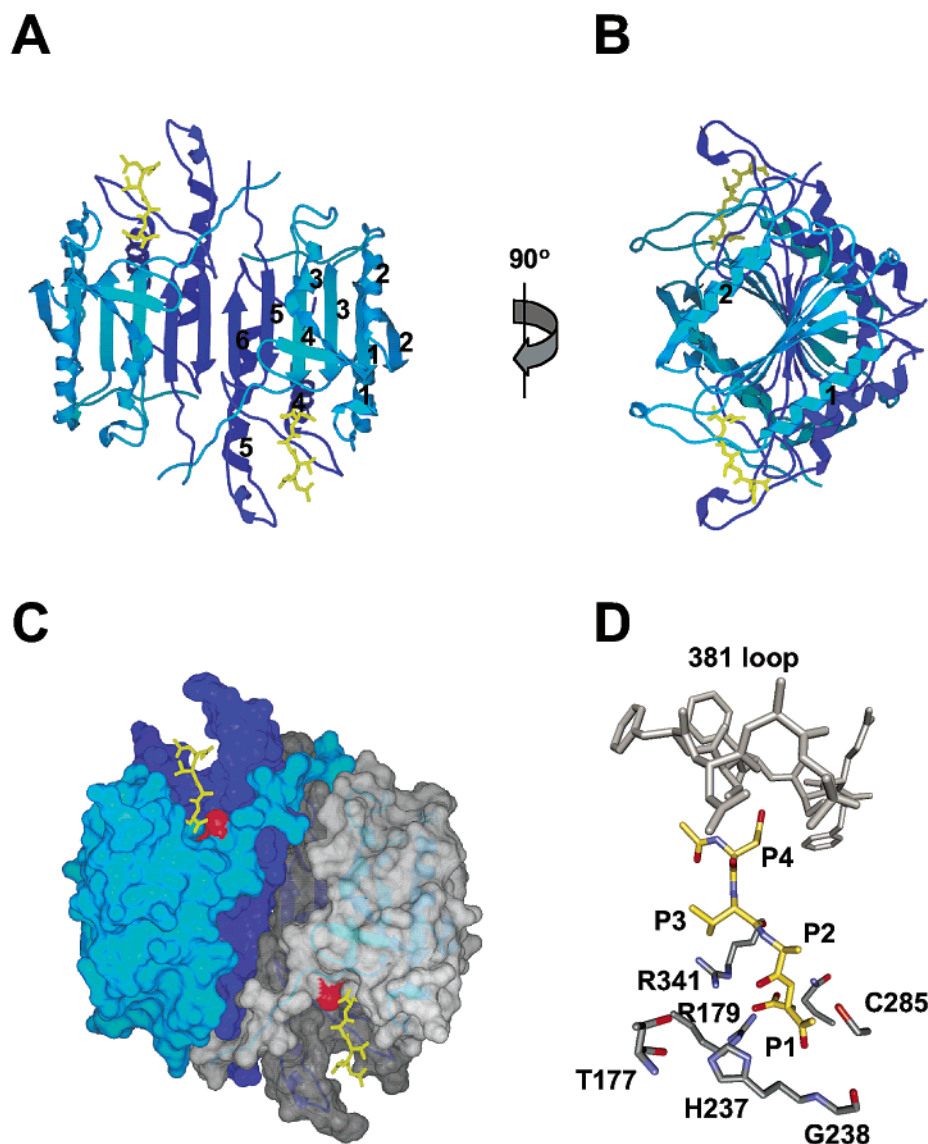


Figure 4. Structure of a caspase dimer. The ribbon (A, B) and surface model (C) of caspase-3 (PDB ID code 1CP3) is presented as a catalytic unit dimer (large subunit, cyan; small subunit, blue). A tetrapeptide aldehyde occupying the S_1 – S_4 subsites is shown in yellow sticks. A rotation of 90° (B) of the standard view allows visualization of the $\alpha\beta\alpha$ sandwich configuration and the orientation of both catalytic sites of the dimer facing the same side. In the surface model (C) showing the globular aspect of the dimer, with the catalytic C285 in red, the second catalytic unit of the dimer is transparent to show the underlying ribbon model. It also clearly shows that the dimer interface is provided mainly by the small subunits (strand $\alpha 8$). (D) Stick model of the substrate binding pockets and catalytic site of caspase 3 with the irreversible inhibitor AcDEVD-fmk (yellow backbone). The side-chains of key residues mentioned in the text are presented. The 381 loop in gray provides selectivity for the P4 residue.

bound form of caspase 7 seems to show a conformation in which the catalytic site is not yet fully formed, with the catalytic cysteine rotated away from its normal position.⁵⁹ This means that caspase 7, at least, may undergo substrate-induced activation. Interestingly, the unbound catalytic subunit of caspase 9 shows a more conventional conformation,²¹ essentially the same as the peptide bound form, and so there must presently be some uncertainty about the first steps in the catalytic cycle.

C. Activity Determinants

1. Specificity Determinants

The first step in proteolysis is to position the scissile peptide bond across the catalytic apparatus,

and different protease families have developed distinct surface pockets for substrate binding to properly align the substrate with the catalytic apparatus. Thus, the primary specificity of matrix metalloproteases is dominated by a hydrophobic S'_1 pocket,⁷⁴ members of the papain family have a dominant S_2 pocket,⁷⁵ and serine proteases from both the subtilisin and chymotrypsin families, have a dominant S_1 pocket.⁷⁶ Perhaps the closest similarity to the caspase solution for substrate binding is found in the subtilisins, which utilize a combination of S_1 and S_4 pockets to control specificity.⁷⁷ The high selectivity observed of the caspases in both S_1 and S_4 are, however, unparalleled. The caspases are among the most specific of selective proteases described so far, and they have never been seen to cut more than just

a few bonds in their target protein.²⁴ What are the molecular determinants of this extreme specificity?

2. Shared Specificity Determinants

The S_1 , S_3 , and S'_1 specificity pockets do not distinguish among most caspases, with Asp required in S_1 , Glu preferred in S_3 and small side-chains in S'_1 , as demonstrated by synthetic substrate scans^{27,63,78} and structural analysis. Both subunits participate in the formation of the S_1 substrate pocket: R179 and Q283 from the large subunit and R341 from the small subunit (see Figure 4D). This side-chain arrangement precludes any residue other than Asp from occupying the S_1 pocket, exemplified by the 20 000 ratio in k_{cat}/K_M of caspase 3 for a substrate having a P_1 Asp instead of a Glu residue.⁶³ This degree of discrimination between closely related residues appears to be the highest among known proteases, and even very specific prohormone convertases such as furin [preferred sequence RXXR(K/R)R↓ and minimal sequence RXXR↓] has a discrimination ratio of only 200 between Arg and Lys in S_1 .⁷⁹

The extended substrate specificity (S_2 – S_4 subsites) of each caspase is mainly provided by the small subunit. In addition to participating in formation of the S_1 pocket, the guanidinium group of R341 influences the substrate preference for Glu at the P_3 position although other side-chains are well tolerated here.²⁷ The specificity determinants of the S'_1 pocket are less easily recognizable in the structures, mainly because all of the standard inhibitors terminate before this position. Nevertheless, substrate scans reveal a common preference for small side-chains in P'_1 .⁶³ Although S'_1 occupancy does not increase catalysis, certain residues are undesirable due to unproductive interactions with this region of the enzymes.

3. Distinguishing Specificity Determinants

The seminal work of Thornberry and colleagues²⁷ demonstrated the importance of the S_4 pocket in distinguishing among substrates and this has formed the basis of most subsequent inhibitor and substrate design. The structure of caspase 1 reveals that the characteristic S_4 specificity for aromatic residues such as Trp/Tyr is formed by a hydrophobic groove partly bordered by residue 381. Interestingly, a loop of three additional residues in caspase 8 (S_4 preference for Leu) and nine additional in caspases 3 and 7 (S_4 preference for Asp) have been inserted at position 381, indicating a mechanism for selectivity in the S_4 subsite. However, this expansion in the executioner caspases may have evolved for another reason (see section on regulation by inhibitors below). On the basis of substrate specificity scanning, the caspases have been divided into three groups with the following P_4 – P_1 consensus: cytokine activators (1, 4, and 5) prefer (W/Y)EHD↓ peptides, initiator caspases (8, 9 and 10), and caspase 6 display activity preferentially on (I/L/V)EHD↓ substrates whereas executioner caspases 3 and 7 and caspase 2 are more active on DEXD↓ sequences.²⁷ For the moment, no substrate is known for caspase 14. These preferences enable characterization of caspases in purified systems, but

the use of supposedly selective substrates to discriminate between mixtures of caspases, for example, in extracts of apoptotic cells, is complicated by at least two factors. First, some caspases (such as caspase 3) have higher absolute k_{cat}/K_M values in general, presumably due to a higher intrinsic catalytic efficiency. Second, concentrations of caspases in a biological milieu are quite varied, with executioners such as caspases 3, 6, and 7 predominating over initiators such as caspases 8 and 9. Therefore, the activity of the most abundant and most active caspase will often determine the readout regardless of the substrate used. Consequently, most investigators will be measuring the activity of caspase 3, owing to its inherently high abundance and catalytic efficiency, regardless of which substrate they choose to use for the studies. The same statement can be made for inhibitors that, at least until now, have important cross-reactivity.

D. Molecular Mechanisms of Activation and Proteolytic Cascade

There are two fundamental ways to activate caspases. Executioner caspases are activated through limited proteolysis by initiator caspases. But because there are no proteases above the initiators, they must be activated in another manner. Indeed, this is a common thread through all proteolytic pathways, including coagulation and fibrinolysis. In the initiators, most is known of the caspase 9 activation mechanism, and for the executioners most is known for caspase 7.

1. Caspase 9 Model

At the cytosolic concentration (<50 nM), procaspase 9 is a monomer²¹ and requires oligomerization within the apoptosome to become active. Furthermore, procaspase 9 does not need to be cleaved in the linker region to become active.^{20,80} Not only is cleavage unnecessary, but also it is insufficient to produce an active enzyme. Instead, caspase 9 is activated by small scale rearrangements of surface loops that define the substrate cleft and catalytic residues (see Figure 5A,B). Structural evidence²¹ demonstrates an inactive conformation in which the catalytic dyad is slightly disoriented and the specificity determining residue R341 is completely dislocated (15.9 Å from C285 Cα compare to 8.8 Å in the active conformation); it faces the solvent instead of the catalytic cleft. During dimerization, a hydrophobic pocket of one monomer accepts F337 side chains of another monomer, allowing the substrate binding loop (W340–W348) to insert. This movement communicates the activation signal to residues in the vicinity of C285 aligning the catalytic apparatus. An interesting finding is that the dimer is made of an active and an inactive catalytic unit,²¹ and it has been proposed that the dimer disallows two active catalytic units. A major clash between F390 of both small subunits can best be reconciled if one of the catalytic units is inactive.

In vivo the dimerization of caspase 9 would occur following CARD-dependent recruitment to the multimeric apoptosome¹⁹ that allows a high local concentration. We hypothesize that this is sufficient for

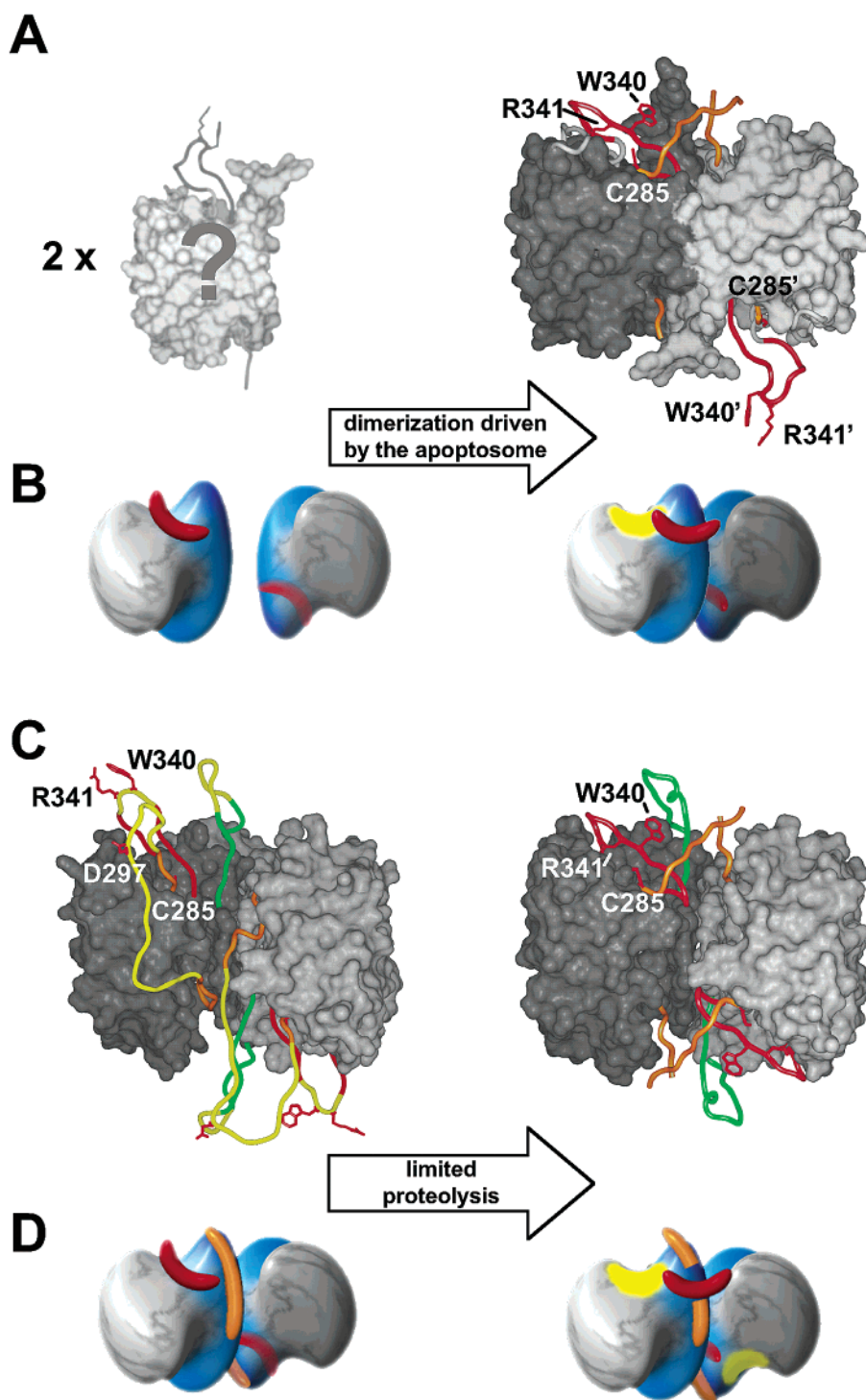


Figure 5. Two molecular mechanisms of pro-caspase activation. (A) Model of caspase 9 (PDB ID file of active dimer 1JXQ) activation loop and priming bulge. The model of the proposed inactive monomer of pro-caspase 9 (left) and the active dimer (right) are presented as surfaces with worm representation of the two principal loops involved in the activation mechanism. The interdomain linker (S330–S339, orange) and activation loop (W340–W348, red) are depicted and the key residues C285, W340, and R341 (red stick representation) are shown to illustrate the important displacement that occurs during activation. The conformation of pro-caspase 9 monomer (grayed) is probably similar to the inactive moiety of the active dimer. (B) Cartoon summarizing pro-caspase 9 activation mechanism. The red sausage depicts the 341 activation loop, and the yellow patch shows the location of the active site. (C) Model of caspase 7 (PDB ID files: zymogen dimer, 1GQF and active dimer, 1F1J) activation loop cleavage. Three loops around the active site undergo important conformation changes during pro-caspase 7 activation. The interdomain linker (orange) asymmetrically positioned in the inactive dimer, exposes D297 (red stick) to proteolysis by apical caspases. The activation loop (red) and the 381 loop (green) are also presented. Again, key residues C285, W340, and R341 are shown. Cleavage of D297 allows R341 to dive into the catalytic cleft and form the S1 pocket near the catalytic dyad. C285 and also H237 slightly reorient themselves to the optimal catalytic configuration during that process. (D) Cartoon representation of the activation mechanism of pro-caspase 7 with both active site depicted as yellow patches, the red sausages depicting the 341 activation loop, and the orange bananas depicting the interdomain connector that is cleaved during activation.

the caspase monomers to "find" the dimeric conformation and become active.

2. Caspase 7 Model

Executioner caspases are found as latent dimeric molecules and need cleavage in the linker region for their activation. The X-ray structure of pro-caspase 7^{58,59} revealed how such cleavage leads to formation of the catalytic site (see Figure 5C,D). Interestingly, the structure of pro-caspase 7 demonstrates that the zymogen is stabilized in almost exactly the same way as pro-caspase 9. The catalytic dyad position is disoriented and the S_1 pocket is missing because R341 is expelled from the catalytic cleft: R341 is 18.4 Å from C285 C α compared to 11.4 Å in the active species. This conformation is influenced by the linker segment that joins the large and small subunits of the catalytic domain, filling a central cavity in the zymogen dimer and preventing the enzyme from adopting its catalytic conformation. Cleavage of the linker region (at D297, see Figure 3) allows three loops surrounding the catalytic cleft to reorganize, promotes alignment of the catalytic Cys, and places R341 so the S_1 pocket is optimal for P₁ Asp recognition. Contrary to active caspase 9, both catalytic units are competent for catalysis in the caspase 7 dimer.

Despite the completely different activation mechanisms, the underlying similarities in the maintenance of the zymogens of caspases 7 and 9 are compelling. Pro-caspase 9 deviates structurally from the other pro-caspases. It possesses a seven-residue longer 240s surface loop, which in the caspase 9 dimer reaches over to the opposite monomer, thereby filling the central cavity. Therefore, in a pro-caspase 9 dimer, none of the intersubunit linkers can insert into the central cavity, and the blocking segments (seen in pro-caspase 7) are probably hindered from nestling into their respective docking sites in pro-caspase 9. Pro-caspase 9 possesses an exceptionally long linker, which could project much further out from the molecular surface than in pro-caspase 7. Consequently, the entry of the priming bulge from the opposite monomer is possible without proteolytic cleavage, in agreement with the significant proteolytic activity observed for the caspase 9 zymogen.²⁰ These observations allow for a pleasing explanation of the individual processes that generate the active forms of the executioner caspase 7 and the initiator caspase 9.

III. Regulation by Inhibitors

The endogenous inhibitors of caspases, those present in mammalian cells, are members of the IAP family. In addition to these endogenous regulators are the virally encoded cowpox virus CrmA and baculovirus p35 proteins that are produced early in infection to suppress caspase-mediated host responses. Each of the inhibitors has a characteristic specificity profile against human caspases, as determined *in vitro*, and these profiles agree with the biologic function of the inhibitors (reviewed in ref 81). Though XIAP and CrmA would be expected to regulate mammalian caspases *in vivo*, p35 would never be present nor-

mally in mammals because it is expressed naturally by baculoviruses.

The best-characterized endogenous caspase inhibitor is the X-linked inhibitor of apoptotic proteases (XIAP), a member of the IAP family. The IAPs are broadly distributed and, as their name indicates, the founding members are capable of selectively blocking apoptosis, having initially been identified in baculoviruses (reviewed in ref 82). Eight distinct IAPs have been identified in humans. XIAP (which is the human family paradigm) has been found by multiple research groups to be a potent but restricted inhibitor targeting caspases 3, 7, and 9 (reviewed in ref 32). Similarly, evidence implicates human cIAPs 1 and 2, ML-IAP, *Drosophila* DIAP-1 (reviewed in ref 82), as well as ILP2,⁸³ as caspase inhibitors. IAPs might have functions in addition to caspase inhibition because they have been found in organisms such as yeast, which neither contain caspases nor undergo apoptosis.⁸⁴

IAPs contain one, two, or three BIR domains, which represent the defining characteristic of the family. Currently, there is no known function for BIR1; however, domains closely related to the second BIR domain (BIR2) of XIAP specifically target caspases 3 and 7 ($K_i \approx 0.1$ –1 nM), and regions closely related to the third BIR domain (BIR3) specifically target caspase 9 ($K_i \approx 10$ nM, reviewed in refs 32 and 85). This led to the general assumption that the BIR domain itself was important for caspase inhibition. Surprisingly, the recent structures of BIR2 in complex with caspases 3 and 7 have revealed the BIR domain to have almost no direct role in the inhibitory mechanism. All the important inhibitory contacts are made by the flexible region preceding the BIR domain.^{68,70,86} Interestingly, the mechanism of inhibition of caspase 9 by the BIR3 domain requires cleavage in the intersubunit linker to generate the new sequence NH₂-ATPF.⁸⁰ In part, this explains the cleavage of caspase 9 during apoptosis, which as described above is not required for its activation. Paradoxically, it seems required for its inactivation by XIAP. This sequence is homologous to that of mature SMAC (NH₂-AVPI-) and HtrA2 (NH₂-AVPS-), proteins shown to antagonize the inhibitory function of XIAP toward caspase 9 (reviewed in ref 85). Since SMAC and HtrA2 are released from mitochondria during the early phase of apoptosis, it is hypothesized that they could constitute a positive feedback loop to amplify caspase activation by preventing BIR3 from inhibiting caspase 9.

Interestingly, neither CrmA-like nor p35-like inhibitors, which operate by mechanism-based inactivation⁸¹ have been chosen for endogenous caspase regulation; rather, IAPs have been adapted to regulate the executioner caspases. Although the reason for this is not certain, it seems likely that the IAP solution provides a degree of specificity that mechanism-based inhibitors cannot achieve. Thus, XIAP inhibition of caspases 3 and 7 requires a nonstandard interaction with the extended 381 loop that is specific to these two caspases. Possibly the 381 loop has evolved to achieve substrate specificity in the executioner caspases, as suggested above. But an equally

likely possibility is that the 381 loop has been generated to enable the IAP scaffold to provide a unique control level over the execution phase of apoptosis. Adding to this level of complexity, IAPs, but not CrmA nor p35-like proteins, are subject to negative regulation by mitochondrial pro-apoptotic proteins SMAC/Diablo and HtrA2.⁸⁷

IV. Conclusion

Our current knowledge of the biology of caspases clearly identifies them as prime targets for therapeutics. In principle, inhibition of caspases could deliver therapy in acute cellular degenerative diseases such as ischemic damage, or even in the longer term neurodegenerative diseases.⁸⁸ On the other hand, activation of caspases could be used as a way to therapeutically delete cells in proliferative diseases such as cancer. Chemotherapy is widely used as an anti-cancer treatment, and most chemotherapeutics act by initiating the intrinsic apoptosis cascade. Yet therapy design is challenging because apoptosis is the general mechanism of cell removal in a healthy organism, and so the problem must be approached cautiously. Knowledge of the biology and biochemistry of caspases is not completed yet but it has clearly set the basis for new opportunities in treatment of various diseases and pathological states. Now that the basic groundwork has been established, a clearer knowledge of the intricate regulation of caspase activation and upstream signaling events will undoubtedly shed light on new ways to control the death machinery to advantage.

V. Abbreviations

Apaf 1	apoptotic protease activating factor
BIR	baculovirus IAP repeats
CARD	caspase recruitment domain
CTL	cytotoxic T-lymphocyte
DD	death domain
DED	death effector domain
DISC	death inducing signal complex
FADD	Fas associated via DD
IAP	inhibitor of apoptotic proteases
SMAC	second mitochondrial activator of caspases
TNF α	tumor necrosis factor α
TNF-RI	TNF α receptor type I
XIAP	X-linked IAP

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