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A Primer on Caspase Mechanisms

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Abstract

Caspases belong to a diverse clan of proteolytic enzymes known as clan CD with highly disparate functions in cell signaling. The caspase members of this clan are only found in animals, and most of them orchestrate the demise of cells by the highly distinct regulated cell death phenotypes known as apoptosis and pyroptosis. This review looks at the mechanistic distinctions between the activity and activation mechanisms of mammalian caspases compared to other members of clan CD. We also compare and contrast the role of different caspase family members that program anti-inflammatory and pro-inflammatory cell death pathways.

Keywords

Apoptosis; pyroptosis; regulated cell death; protease; gasdermin

1. INTRODUCTION

1.1 General preamble on caspases

Caspases are proteases that elicit and propagate signaling events resulting in cellular death: apoptotsis implemented by apoptotic caspases and pyroptosis performed by inflammatory caspases (Fig 1). Their name derives from the common use of a Cys side chain acting as nucleophile during peptide bond hydrolysis, and a rare primary specificity for cleaving after Asp – they are cysteine-dependent aspartate-specific proteases. The ancestor of caspases is ancient, and its descendants (officially peptidase clan CD) are found widespread across all life kingdoms [1], but the distinctive Asp specificity is unique to metazoans [2]. In vertebrates the apoptotic caspases can be further subdivided into initiator (caspases-8, −9 and −10) and effector (caspases-3, −6 and −7) caspases [3]. The apoptotic and inflammatory caspases have moderately well defined biochemical and biological mechanisms, but confusion reigns regarding the role of caspase-2. Caspase-2 has been reported to serve many roles within and outside apoptotic networks, and recent data suggests its participation in cell cycle regulation [4]. Readers interested in the many (sometimes conflicting) conflicting functions of caspase-2 are directed to the following reviews [4–9]. Caspase-14, generally not

considered an apoptotic or inflammatory caspase, seems to have a highly specialized, though mechanistically obscure, role in keratinization of the skin [10].

With the exception of caspase-1 - enriched in monocytes/macrophages, and caspase-14 - restricted to keratinocytes [11], caspases are widely expressed. Caspases are obligate cytosolic/nucleoplasmic proteins. Their sequences encode no export or import signals and, although they have been occasionally reported to be associated with mitochondria, these appear to have been artifactual [12]. Caspases begin life as single chain zymogens (enzymes awaiting activation) consisting of an N-terminal domain followed by a catalytic domain. The N-terminal domain is variable, can encode recruitment and activation signals, and defines the type of mechanism that caspases use. The C-terminal protease catalytic unit is a single domain, but often split into two chains by proteolytic cleavage during maturation. The catalytic dyad residues Cys and His reside in the large chain while the substrate recognition groove is formed primarily through residues from the small chain (Fig 2).

2. ACTIVATION MECHANISMS OF APOPTOTIC CASPASES

Caspases are restrained as inactive zymogens awaiting appropriate activation signals. The zymogens of apoptotic effector caspases 3 and 7 are obligate dimers and their activity is held in check by a linker separating the large and small chains. Proteolytic processing of the linker allows assembly of the catalytic site through rearrangement of characteristic mobile loops. Auto-proteolysis resulting in removal of the N-terminal pro domain or cleavage of the inter-chain linker sometimes follows activation. Although this has no impact on the inherent proteolytic activity [13, 14] cleavage of the linker enhances dimer stability, and contributes to other downstream regulatory events [3, 15]. There is essentially no dispute about this activation mechanism.

The zymogens of apoptotic initiator caspases are inert monomers, and there is little dispute about their activation mechanism (Fig 3). The most parsimonious model for apical caspase activation, sometimes known as the induced proximity model [16, 17], has been widely tested and holds that apical caspases require dimerization for activation, and that dimerization is the fundamental activating event. Initiator caspases are recruited by adaptor molecules to oligomeric activation platforms following an apoptotic signal. The induced proximity model postulates that local increase in concentration drive proximity-induced dimerization and therefore activation [15]. Much of the biochemical and structural work on activation of apical caspases has focused on caspases-8 and 9, but the same concept is thought to hold true for the activation of pro-inflammatory caspases, as we describe below. In cultured cells treated to undergo apoptosis, apoptotic caspases drive a characteristic morphology that includes membrane blebbing, chromosomal DNA fragmentation, packaging of cell constituents into “apoptotic bodies” and eventually cell death. *In vivo* few of these morphologies can be observed because apoptotic cell fragments are rapidly cleared by macrophages [18, 19], but in the nematode *C. elegans* the entire process of cell death and disposal may be visualized. Apoptosis is an immunologically silent cell demise, indeed it may be anti-inflammatory, and therefore complex signaling networks activated by apoptotic caspases are required to dismantle and package cells for removal [19–21].

3. PLATFORMS FOR APICAL CASPASE ACTIVATION

The role of the apical apoptotic caspases is to activate downstream effector caspases, particularly caspases-3 and 7. The apical caspases are the point at which a cellular death signal is converted to proteolytic activity, and this is achieved by remarkably coordinated clustering mechanisms that lead to apical caspase dimerization. Although apical apoptotic caspases respond to very different signals, and are activated in distinct activation platforms, the nature of such activation has striking similarities.

3.1 The DISC

The DISC (Death Inducing Signaling Complex) is a transmembrane assembly that acts as a conduit for extracellular death signals emanating from engagement of certain death receptors of the TNF family. The cytosolic face of the complex contains the adapter protein FADD, which acts as a bridge between the apical caspase and the C-terminal of the transmembrane death receptor. The key components of the recruitment are the DEDs of FADD and caspases-8, 10 and/or FLIP. Current models suggest that the caspase-8 and FLIP form elongated chains connected by their DEDs [22]. Importantly, only the long transcript of FLIP ($FLIP_L$) can encode a caspase activator because the short transcript ($FLIP_S$) has a stop codon following the DEDs, and encodes no catalytic domain homology unit. Thus, $FLIP_L$ activates caspase-8 by heterodimerization, on the platform of DISC chains, to generate a single active site. Indeed, in models of DISC formation, $FLIP_L$ is a better activator of caspase-8 than is caspase-8 itself – heterodimerization is favored over homodimerization [23, 24]. Because FLIP is an NFkB-regulated gene its concentration in cells is variable and this, accompanied by transcript splicing events to generate differential amounts of $FLIP_L$ or $FLIP_S$, would have a major impact on the role of FLIP in activating apical caspases. Some rodents (mice and rats) express only one apical caspase of the extrinsic pathway – caspase-8, but other rodents (guinea pig and squirrel), and primates possess genes for both caspase-8 and caspase-10. Although there has always been debate about the function of caspase-10, current thinking is that it participates in NFkB activation by re-wiring the DISC towards cell survival outcomes [25], and in apoptosis in cell lines deprived caspase-8 [26].

3.2 The Apoptosome

Apaf-1 (Apoptotic Protease Activating Factor-1) is a AAA-ATPase protein that, upon release of cytochrome-*C* from mitochondria, undergoes polymerization in an ATP or dATP dependent mechanism [15, 27]. Unlike the DISC, and as we will see later the Inflammasome, the Apaf-1 polymers seem to be self-limiting and form a donut-shaped ring of 7 or 8 monomers [28]. At the center of the donut are the CARDs of Apaf-1 which recruit caspase-9 via its CARD to form the apoptosome. Induced proximity of caspase-9 monomers results in their dimerization and generation of a proteolytic signal [29]. Upon activation caspase-9 undergoes auto-cleavage between the large and small subunits of the catalytic domain with two important results. Cleavage unmasks a neo-epitope on the small subunit that permits binding of its cognate inhibitor XIAP to terminate activity, while at the same time promotes dissociation from the apoptosome with subsequent monomerization. Thus the apoptosome acts as a proteolytic-based molecular timer to generate a pulse of apoptotic

signal [30]. The utility of the timer, while mechanistically-intriguing, awaits explanation from a cell survival/cell death perspective.

4. INFLAMMATORY CASPASES AND PYROPTOSIS

The innate immune response of macrophages to bacteria and viruses is initiated by a highly conserved group of receptors known as pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) derived from pathogens. There are several PRR families, such as Toll-like receptors (TLRs), c-type lectin receptors and scavenging receptors localized on the outer cell surface or endosomal membrane, retinoic acid inducible gene-I-like receptors, absent in melanoma 2 (AIM2)-like receptors (ALRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) located in cytoplasm [31]. Some of these receptors signal to NFkB to induce cytokine expression, and some signal to inflammasomes – activators of inflammatory caspases [32].

Activation of inflammatory caspases results in a lytic cell death known as pyroptosis. The term ‘pyroptosis’, in analogy to apoptosis but substituting the first part of the word with *pyro*, relating to fire or fever, was first proposed to describe a pro-inflammatory programed cell death and distinguish it from apoptosis [33, 34]. While apoptosis is generally considered to be non-lytic and quietly removes unwanted cells, pyroptosis is a lytic and plasma membrane disrupting form of cell death, removes pathogen infected cells and prompts the recruitment of monocytes to site of injury through the release of inflammatory cytokines [35]. Uncontrolled or excessive pyroptosis can lead to widespread cell death, resulting in tissue damage, organ failure and lethal septic shock [36]. The secretion of inflammatory cytokines has been studied for several years, but not until recently was Gasdermin D (GSDMD) discovered to be a substrate of inflammatory caspases that is responsible for membrane pore formation [37, 38]. Cleavage of GSDMD produces an N-terminal fragment that forms pores in the plasma membrane and leads to the rapid lysis of cells – reviewed in [39], thus explaining the old question of how cytokines of the IL1 family – devoid of secretion signals – could exit a cell following an inflammatory stimulus. The exit of these cytokines is coupled to cell lysis. Caspase-1 also cleaves and activates the precursors of cytokines proIL-1b and proIL18, primary pro-inflammatory cytokines, thus the processing of these cytokines is intimately linked to cell lysis driven by GSDMD cleavage. However, there exists somewhat of a controversy in that cytokine exit has been reported to be dissociated from cell death [40, 41], although no mechanisms have been proposed. Perhaps the controversy results from the definition of cell death versus membrane permeabilization, and this seems important to rationalize.

4.1 Inflammatory Caspases-1, −4, −5, −12

Interleukin-1b (IL-1b) converting enzyme (ICE), now known as caspase-1, was discovered as the enzyme responsible for processing inactive proIL-1b to active IL-1b in macrophages [42–44] and confirmed by caspase-1 knock-out mice resistant to the lethal effect of endotoxins [45, 46]. ProIL-18, also known as interferon-γ-inducing factor, is also processed and activated by caspase-1. Caspase-1 sponsors inflammation by effecting the maturation of pro-interleukins. The caspase-1 subfamily, generally referred to as inflammatory caspases,

includes human caspase-4, −5 and −12 showing the closest sequence identity to caspase-1, the first caspase to be discovered [3, 42]. Genes of inflammatory caspases are tightly linked forming an inflammatory cluster [34, 47, 48]. Sequence analysis of human caspase-4 and −5 suggest these enzymes originated from gene duplication of mouse caspase-11. Therefore, caspase-11 is the murine ortholog of human caspases-4 and-5 [49].

The current consensus is that caspase-12 is an inactive homolog, based on conflicting results in the literature [50–52] and the substitution of critical residues that surround the active site in mouse, rat and human caspase-12. In humans, a single nucleotide polymorphism at amino-acid position 125 in caspase-12 results in the synthesis of either a truncated or a full-length enzyme. The full-length allele was identified only in populations of African descent (about 20% of them) [53]. However, some species caspase-12, including African elephant, cat and dog, seems to contain sequences compatible with activity. Thus, it is possible that caspase-12 may serve as an active caspase in its own right, or an activator of caspases-1 or 11, depending on the species.

5. ACTIVATION MECHANISM OF INFLAMMATORY CASPASES

In principle, inflammatory caspases are synthesized as inactive zymogens, just like initiator apoptotic caspases. Structurally, the inflammatory caspases have an N-terminal CARD followed by a catalytic domain with an architecture very similar to the apoptotic initiator caspase-9 (Figure 2). In innate immune cells they are considered as monomers awaiting recruitment to their cognate activation platforms, known as inflammasomes, to be dimerized and become active enzymes, and thus initially it was thought that inflammasomes may have the same architecture as the apoptosome [54]. However, more recent studies have provided for alternative models, spurred on by the finding that macrophages seem to possess a single caspase activation focus per cell [55].

5.1 Canonical Inflammasomes

Pyroptosis relies on proteolytic activity of inflammatory to undergo canonical (caspase-1-mediated) or non-canonical (caspase-4/11 or caspase-5-mediated) pathways [56]. The canonical inflammasome activation is dependent on the adapter protein ASC (apoptosis associated speck-like protein containing a CARD) and caspase-1. The formation of canonical inflammasomes varies in degree to the insult or threat the cell faces, ultimately all induce inflammatory responses by processing caspase-1. Canonical inflammasomes are protein complexes of either NOD-like receptors (NLRs) or pyrin domain (PYD)- containing non-NLRs assembling apoptosis-associated speck-like protein containing a C-terminal CARD (ASC) and pro-caspase-1 [31, 57]. Some of the most well studied canonical inflammasomes include the NLR family, which includes NLRC4, NLRP1, NLRP3 and AIM2, all differentially stimulated by different ligands. The activation mechanism has been proposed to result in the ACS-dependent polymerization of either chains [58] or large spherical assemblies [55] of inflammasome components, forming a much larger activation platform than is seen for caspase-9. The hypothesis has been put forward that this single focus results from rounds of polymerization that, unlike the caspase-9 apoptosome where polymerization is self-limiting, results in huge macromolecular assemblies of filamentous

inflammasome components [59]. The subsequent activation of caspase-1 remains unknown, but is probably analogous to apoptotic apical caspases, with the inflammasome assemblies acting as platforms for proximity-induced dimerization.

5.2 Non-Canonical Inflammasomes

Recent studies suggest that caspase-4, −5 and −11 may participate in a non-canonical activation process that does not require inflammasome components [32, 56]. Mouse caspase-11 and human orthologous caspases-4 and −5 are components of the non-canonical inflammasome, which do not require adaptor molecule, ASC. Caspase-11 was the first enzyme discovered to participate in a non-canonical or non-caspase-1 mediated inflammasome [56] as a response to gram-negative bacteria. This then led to the discovery of caspase-11's proposed role as direct sensor of intracellular LPS from gram-negative bacteria during macrophage-mediated inflammatory responses [60, 61]. Recombinant studies using caspase-4, −5 and −11 propose the formation of a multimer in the presence of LPS and monomers in the absence of LPS [60].

6. ACTIVATION MECHANISMS OF OTHER CLAN CD PROTEASES

Clan CD is an ancient group of proteases with representatives in every biotic kingdom. MALT1, also known as paracaspase [62], is the closest relative to the caspases in terms of its activation mechanism, though its biological function and primary specificity are totally different. Like caspases it is an obligate dimer, and like apical caspases it is activated by dimerization [63, 64]. Other clan CD members are distinct in their activation mechanisms, sampling most of the possible activation mechanism of proteases [1]. Legumain is a secreted and/or lysosomal protease involved in peptide degradation and maturation, and is activated by a pH switch that results in autolytic removal of a pro-domain that blocks access of substrates to the active site [65]. Separase is required for sister chromatid separation during anaphase, and is activated by proteasomal degradation of its cognate inhibitor, securin [66, 67]. PIGK (Glycosylphosphatidylinositol:protein transamidase) removes a C-terminal peptide from target proteins before they attach glycosylphosphatidylinositol anchors in the endoplasmic reticulum, but the mechanism of PIGK activation remains unclear, although it may be constitutively active because of its mechanism of action in the endoplasmic reticulum.

7. CONTEMPORARY AND FUTURE DIRECTIONS

The requirement of two inflammatory caspases for the different inflammatory pathways is still not understood, however it has been proposed that the non-canonical pathway abrogates the need for the priming step of the canonical pathway. Thus, canonical inflammasome activation is dependent on two sequential signals in macrophages- 1) priming and 2) triggering. Priming is the process in which PAMPs bind to PRR on macrophage cell surfaces recognize stimulants such as LPS, resulting in upregulation of NF-kB pathway and production of inflammasome components. Triggering is the signal necessary for full activation of inflammasomes. The direct recognition of LPS rapidly oligomerizes caspases-4, −5 and −11 resulting in generation of inflammatory proteolytic signals without the need for NFkB-mediated gene transcription. Although inflammatory caspases fall neatly

into the apoptotic initiator caspase description, the activation mechanism of these enzymes has not been well explored. Biochemical studies targeting the dimerization, auto-processing and catalytic sites should be able to shed light on their activation mechanism. Another important aspect to further probe is the involvement of LPS in caspase activation. Are there other proteins or complexes that aid caspase-4 −5 and −11 in the direct sensing of LPS that so far have remain hidden? Most studies seem to suggest that caspases 1, 4 and 5 are biochemically synonymous. Is this correct, or are there differences in the substrate repertoire of inflammatory caspases?

The significance of caspase-12 as a potential inhibitor to inflammatory caspases is a very intriguing field. $FLIP_L$ is a non-catalytic enzyme that heterodimerizes with apoptotic caspase-8. This heterodimer is thought to alter the targets of caspase-8 activity leading it away from apoptosis and towards blocking necroptosis. Could it be possible that caspase-12 acts as a modifier to inflammatory caspases in order to alter their specificity in an analogous manner? From this perspective, could caspase-10 also function as a modulator of caspase-8 activity by heterodimerization, given the findings that caspase-10 can apparently drive NFkB activation [25]?

The pyroptotic mediator GSDMD is not only cleaved by inflammatory caspases, but also by caspase-3. However, the caspase-3 cleavage inactivates the pyroptotic potential of the protein, with the consequence that apoptosis seems to prevent pyroptosis [68]. This may be a mechanism to ensure that infected macrophages that evaded pyroptosis are eliminated, and so an interesting question becomes what subtype of macrophages allow pathogens and viruses to utilize them without eliciting pyroptosis or apoptosis. Are there alterations in expression or mutations in GSDMD in pathologies associated with inflammatory diseases? Links between the different regulated cell death pathways may exist. Thus DFNA5 (deafness, autosomal dominant 5 – a Gasdermin family member) has been suggested to switch apoptosis to pyroptosis via cleavage by caspase-3 [69]. At first glance this may seem to be a strange outcome, since one would expect an inflammatory-silent form of cell death (apoptosis) to absolutely reject an inflammatory one. The pyroptosis-inducing outcome of caspase-3 activating DFNA5 seems at odds with the pyroptosis-defeating outcome of caspase-3 inactivating GSDMD, and it seems that highly cell-specific events must be invoked to explain these observations.

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Abbreviations

PRRs	pattern recognition receptors
PAMPs	pathogen-associated molecular patterns
TLRs	Toll-like receptors
AIM2	absent in melanoma 2

ALRs	AIM2-like receptors
NOD	nucleotide-binding oligomerization domain
NLRs	NOD-like receptors
PYD	pyrin domain
CARD	caspase recruit domain
DED	death effector domain
ASC	apoptosis-associated speck like protein containing a C-terminal CARD
GSDMD	gasderminD

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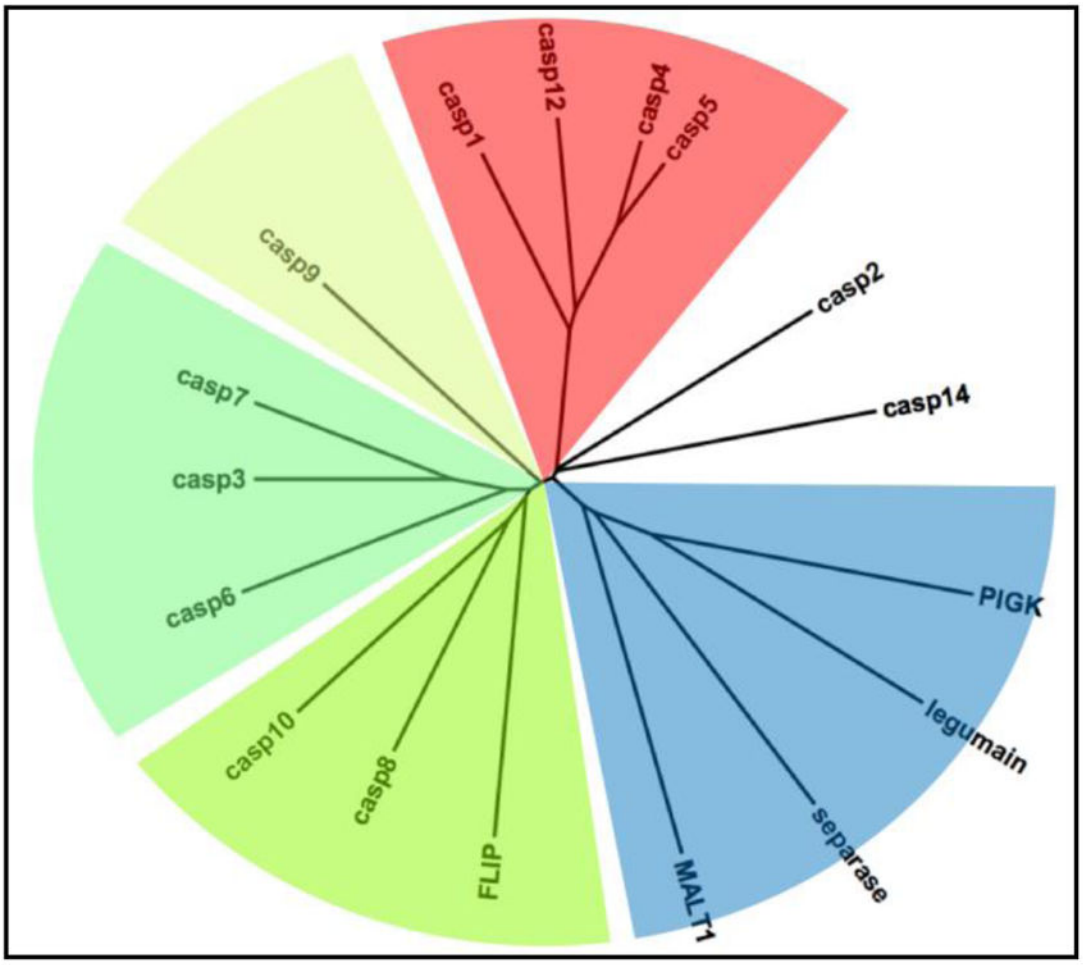

Figure 1. Peptidase clan CD dendrogram illustrating evolutionary relationships of human caspases and their homologs

Apoptotic caspases are featured in green hues, inflammatory caspases in red, and the remaining four human members of peptidase clan CD are in blue. Caspases-2 and −14 are uncolored and unassigned, as they have primary roles unassociated with apoptosis or inflammation. Pseudoproteases, FLIP and caspase-12, are proteins with the characteristic fold but mutations in their catalytic machinery render them proteolytically incompetent.

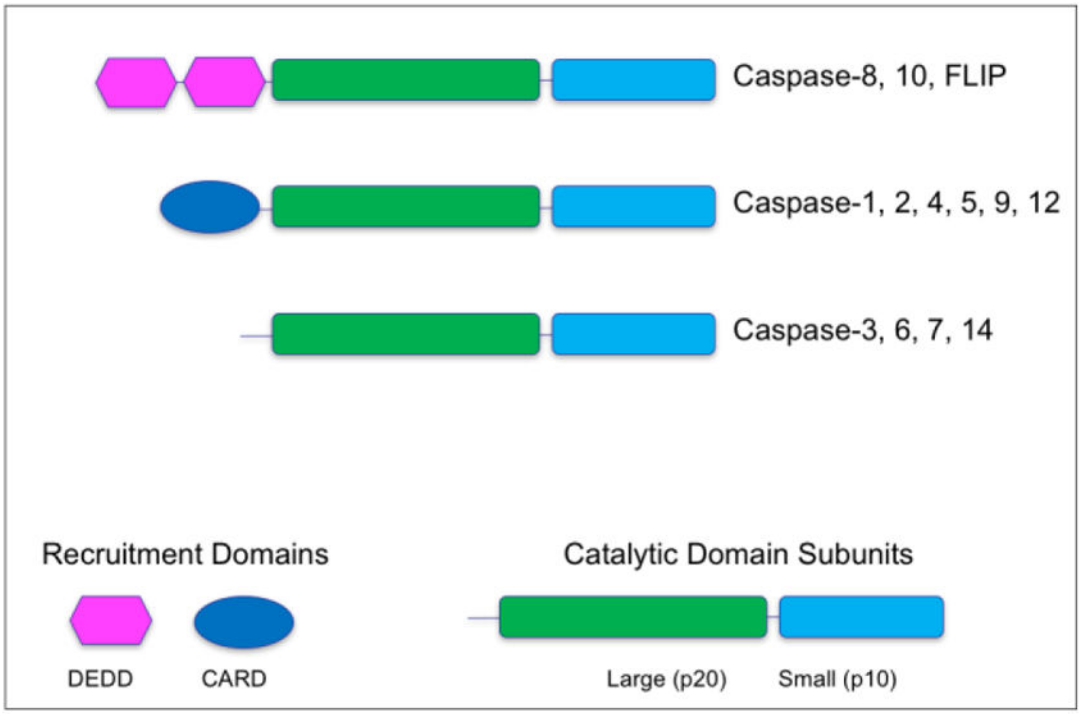

Figure 2. Domain structures of human caspases

Apical caspases contain domains that serve to recruit the zymogens to activation complexes through homotypic protein interactions – DED (Death Effector Domain) and CARD (Caspase Recruitment Domain). Effector caspases lack these recruitment domains, and are activated by cleavage within the catalytic domain by apical caspases. Cleavage within the catalytic domain is obligatory for effector caspase activation, but dispensable for apical caspase activation.

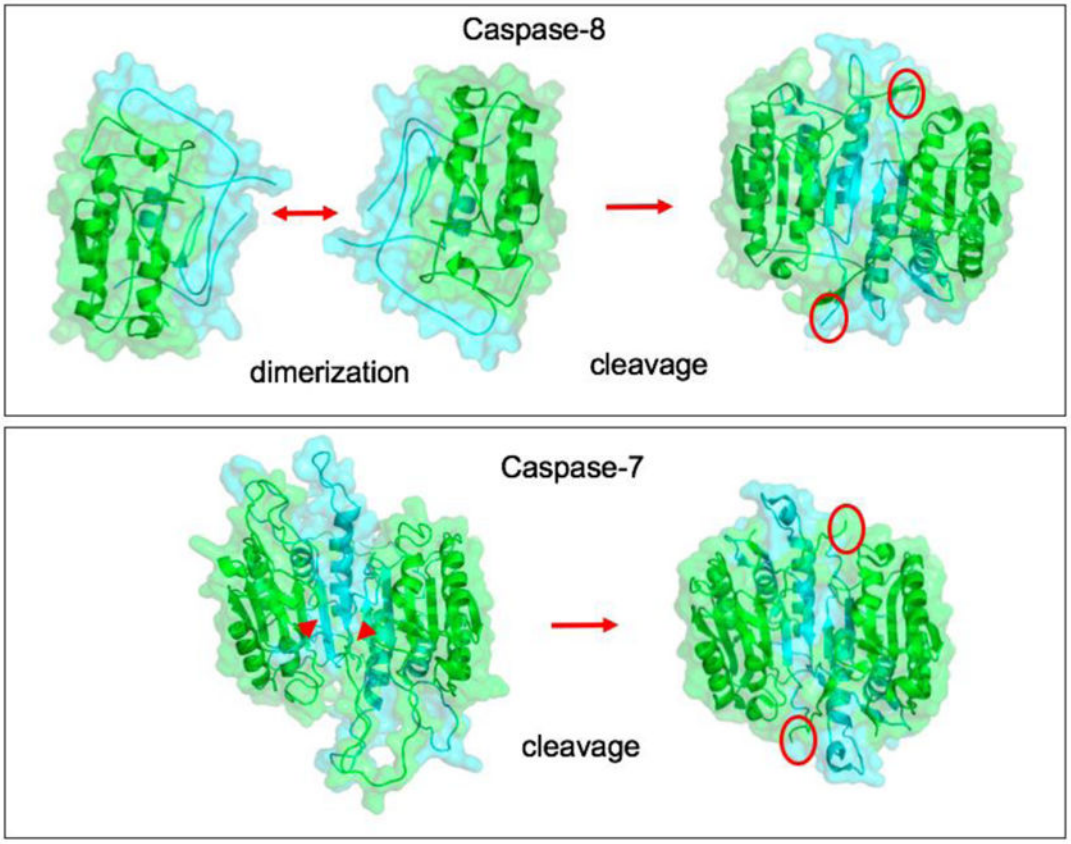

Figure 3. Distinct activation mechanisms of apical and effector caspases

Apical caspase-8 and effector caspase-7 shown as examples, based on the crystal structures reported in PDB codes 2K7Z, 3KJQ, 1GQF, 1F1J. Apical caspases undergo proximity-induced dimerization within activation complexes. Sometimes they also undergo cleavage within the catalytic domain to yield short segments of secondary structure that stabilize the resulting dimer (red circles). Cleavage of the pre-formed zymogen dimer of caspase-7 (red arrowheads) results in activation with an analogous further stabilization of the dimer (red circles).

Table 1

Human clan CD proteases, their biological functions, primary specificity, and activation mechanisms.

Clan CD member	Biological Role	Primary specificity	Activation mechanism
Caspase-1, 4, 5	Inflammatory caspases	Asp	No consensus – probably dimerization
Caspase-12	*Putative pseudocaspase - inflammation*	-	
Caspase-9	Intrinsic pathway apical caspase	Asp	Dimerization
Caspase-8, 10	Extrinsic pathway apical caspases	Asp	Dimerization
FLIP	*Pseudocaspase – extrinsic pathway*	-	
Caspase-3, 6, 7	Apoptotic effector (executioner) caspases	Asp	Intra-domain cleavage
Caspase-2	“Multifunctional” caspase	Asp	No consensus
Caspase-14	Keratinocyte differentiation	Asp	Intra-domain cleavage
MALT1	B, T-cell activation via NFkB	Arg	Dimerization
Legumain	Antigen presentation, lysosomal protein degradation	Asn	Removal of N-domain
Separase	Sister chromatid separation during anaphase	Arg	Removal of blocking inhibitor
PIGK	C-terminal processing of proteins destined for GPI anchor addition	Asn/Ser*	Unknown