Chapter 2
Caspases – Key Players in Apoptosis

Christine E. Cade and A. Clay Clark

Abstract Caspases are the terminal proteases involved in apoptosis, as well as being involved in inflammation. The apoptotic caspases can be classified as either initiator or effector caspases based on both their position in the caspase cascade and their activation mechanism. Initiator caspases require dimerization to be activated, and cleavage of a loop called the intersubunit linker stabilizes the active enzyme. Effector caspases, on the other hand, are found as dimers in the cell and cleavage of the intersubunit linker is the key step in their activation.

The name caspase is short for cysteinyl aspartate-specific protease. As their name suggests, these enzymes hydrolyze peptide bonds after certain aspartate residues using a catalytic cysteine (with the aid of an active-site histidine residue). Caspases can be inhibited by endogenous inhibitors such as XIAP, by synthetic inhibitors which target either the active site or an allosteric site, or by post-translational modification. Further research is needed to find novel activators and inhibitors of caspases to treat diseases which involve misregulation of apoptosis.

Keywords Caspase • Apoptosis • Protease • Intersubunit linker • Prodomain • Dimerization • Allostery

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Abbreviations

ASC	Apoptosis-associated speck-like protein containing a CARD
CARD	Caspase activation and recruitment domain
Caspase	Cysteinal aspartate-specific protease
DAMPs	Danger-associated molecular patterns
DD	Death domain
DISC	Death inducing signaling complex
FADD	Fas-associated death domain
FasL	Fas ligand
FLICE	FADD-like interleukin 1β-converting enzyme
FLIP	FLICE-like inhibitory protein
$FLIP_L$	Long splice variant of FLIP which forms a heterodimer with caspase-8
$FLIP_S$	Short splice variant of FLIP which blocks caspase-8 from binding death receptor
ICE	Interleukin 1β-converting enzyme
IL-1β	Interleukin 1β
IL-18	Interleukin 18
PS	Phosphatidylserine
Smac	Second mitochondrial activator of caspases
TNFR	Tumor necrosis factor receptor
XIAP	X-linked inhibitor of apoptosis protein

Caspases

Caspases (**c**ysteinal**asp**artate-specific prote**ases**) [1] are enzymes which utilize a catalytic cysteine to cleave their peptide substrates after specific aspartate residues. The first caspase was discovered in 1992 and because of its function was named interleukin-1-β converting enzyme (ICE) [2, 3] but was later renamed to caspase-1. In 1993, Ced-3 from *C. elegans* was found to be homologous to ICE [4] and the corresponding human protein CPP32 (later named caspase-3) was found in 1994 [5]. The official caspase nomenclature was decided on in 1996 to alleviate the confusion that went along with discovery of ten different caspases, some with multiple names [1].

1 Structure

Caspases are expressed as proenzymes (zymogens) called procaspases, which then become activated to the mature caspase form. Procaspase structure can be divided into three domains: an N-terminal prodomain, a large subunit, and a small subunit. The first step in maturation is dimerization. Then, proteolytic processing removes the prodomain and cleaves a loop called the intersubunit linker between the large and small subunits.

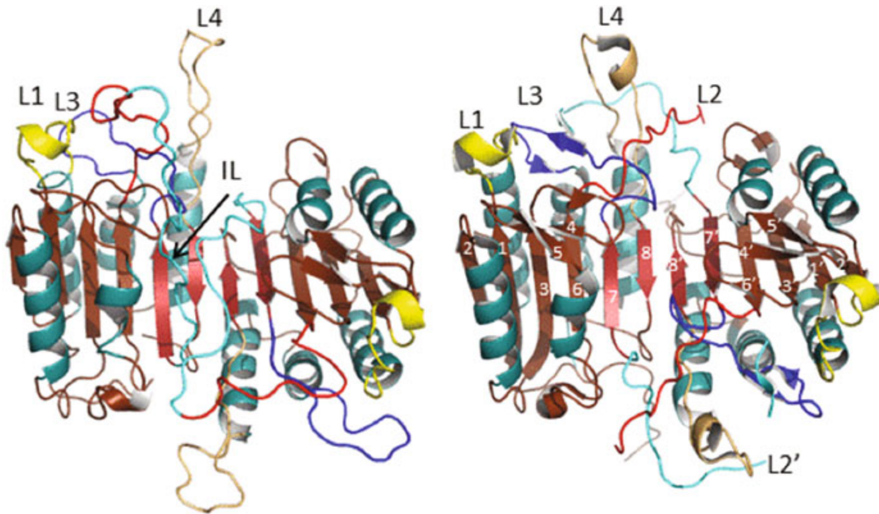


Fig. 2.1 Procaspase-3 model and crystal structure of caspase-3. Active site loop coloring: *yellow* = L1, *red* = L2, *cyan* = L2′, *blue* = L3, *tan* = L4

The secondary structure of mature caspases consists of six core β-strands in a slightly twisted sheet in each monomer, with two main helices on one face (the "front") of the protein and three helices on the other face (the "back") of the protein (Fig. 2.1). The first four core β-strands and helices 1–3 form the large subunit, whereas the last two core β-strands and helices 4–5 form the small subunit.

The dimer interface consists of the final β-strand from each monomer, side-by-side in an antiparallel manner. The two monomers are related through a C2 axis of symmetry such that one monomer is "upside-down" compared to the other monomer.

Five loops are important for the formation of the active site. Once the intersubunit linker is cleaved, the two halves of the cleaved linker are called L2 and L2′. Active site loops L1, L2, L3, and L4 come from one monomer, and loop L2′ comes from the other. The catalytic cysteine is part of loop L2, and the catalytic histidine is part of a loop extending from the C terminal end of β3.

2 Classification

Caspases are divided into two main categories based on their function: apoptotic caspases and inflammatory caspases. The apoptotic caspases are further divided into two categories based on time of entry into the apoptotic cascade: initiator caspases and effector caspases.

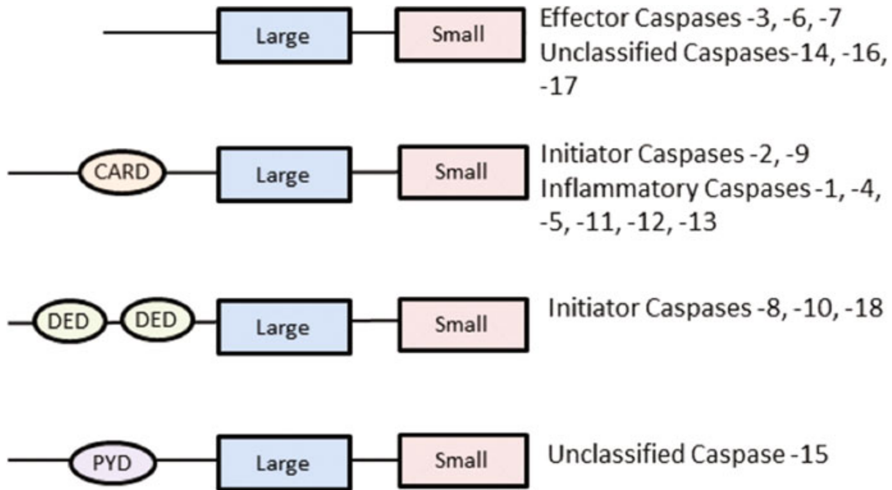


Fig. 2.2 Domain arrangement of mammalian caspases

2.1 Apoptotic Caspases

2.1.1 Initiator Caspases

Initiator caspases are stable monomers in the cell until they are activated by dimerization. Once dimerized, initiator caspases have sufficient activity to autoprocess, cleaving their prodomain and intersubunit linker. An induced proximity model for dimerization was first invoked for caspases-8 and -10 but now seems to be generalizable to initiator caspases as a whole. This model says that activation complexes increase the local concentration of the initiator caspases, enabling them to dimerize [6]. The prodomains of initiator caspases contain either a CARD (caspase activation and recruitment domain) or DED (death effector domain), which allow initiator caspases to bind to activation complexes (Fig. 2.2).

The initiator caspases-2 and -9 are involved in the intrinsic pathway, which is activated by mitochondrial damage, cytotoxic stress, chemotherapeutic drugs or certain developmental cues [7]. Activation of caspase-2 leads to release of cytochrome *c* from the mitochondria, which then binds to Apaf-1 and forms the heptameric apoptosome. The apoptosome binds procaspase-9 to dimerize and therefore activate it. Once active, caspase-9 activates downstream effector caspases.

The initiator caspases-8 and -10 are activated by the extrinsic pathway: in order to eliminate excess cells created during development or remove cells with tumorigenic properties, a molecule binds to a death receptor at the membrane which is part of the tumor necrosis factor receptor (TNFR) superfamily [8, 9]. One such ligand/receptor pair is FasL (Fas ligand) and CD95(APO-1/Fas). The cytosolic death domains (DD) of the receptor recruit an adaptor molecule such as FADD (Fas-associated death domain), allowing the complex to bind initiator procaspases-8 or -10 to forma death-

inducing signaling complex (DISC). Once the procaspases are part of the DISC, they are able to dimerize and therefore become active. The active caspase-8 or -10 then activates downstream effector caspases such as caspase-3.

2.1.2 Effector Caspases

The effector caspases-3, -6, and -7, are found as inactive dimers in the cell. They are activated once an initiator caspase cleaves their intersubunit linkers. Because they do not require death scaffolds for dimer formation [10, 11], their prodomains are short and lack the CARD and DED domains typical of initiator caspases. Their prodomains are, however, likely to be involved in targeting within the cell [12–15].

2.2 Inflammatory Caspases

Similarly to the initiator caspases, the inflammatory caspases-1, -4, -5, -11, -12, and -13 are activated by dimerization. Their prodomains contain a CARD which allows them to bind to activation complexes. Similarly to apoptosome formation, a multiprotein complex called the inflammasome consists of a NOD-like receptor such as NLRP1, an adaptor protein such as ASC (apoptosis-associated speck-like protein containing a CARD), and the inflammatory procaspase, particularly procaspase-1 [16]. In some cases, the procaspase can also be recruited to CARD domains in the receptor directly, without the aid of an adaptor molecule [17].

Once the inflammatory caspases become active, they are activators of cytokines through cleavage of their preforms. In monocytes and macrophages, caspase-1 activates interleukin-1β (IL-1β) [3] and interleukin-18 (IL-18). These cytokines mediate innate immunity and inflammation [18].

The mouse caspase-11 is a homolog of human caspase-4 [19]. In humans, caspase-12 is generally truncated due to a premature stop codon, but in some people of African descent, a read-through mutation causes expression of the full-length protein, causing increased risk of sepsis due to decreased inflammatory and immune response to endotoxins [20]. Caspase-13 is a bovine ortholog of human caspase-4 [21].

2.3 Other or Unclassified Caspases

Caspase-14 expression is restricted to epidermal keratinocytes and is involved in differentiation [22]. Like the effector caspases, it has a short prodomain with no adaptor regions. Several caspases are not yet classified: 15, 16, and 17 [23]. Caspase-15 is expressed in several mammalian species including pigs, dogs, and cattle [24]. It contains a pyrin-like region in its prodomain similar to that found in zebrafish

caspases caspy and caspy2 [25]. Caspase-16 is found in marsupials and placental mammals and contains a short prodomain with no adaptor regions [23]. Caspase-17 is found in vertebrates except for marsupials and placental mammals and also does not contain adaptor regions in its prodomain. Caspase-18 is found in opossums and chickens and, like caspases-8 and -10, contains two DED regions in its prodomain, so it is likely also an initiator apoptotic caspase [23].

3 Mechanisms

3.1 Activation

Activation of caspases generally requires two events: they must be a dimer and the intersubunit linker must be cleaved. Removal of the prodomain is not necessary for activation; in fact, the prodomain may serve to stabilize the active enzyme [26].

After dimerization, cleavage of the intersubunit linker occurs first, followed by cleavage of the prodomain. Prior to cleavage, the intersubunit linker from one monomer occupies the dimer interface. Upon cleavage of the intersubunit linker, the C-terminal portion of the linker, L2′, vacates the central cavity and rotates about 180 degrees toward the active site, forming contacts with L2, L3, and L4 from the opposite monomer. These loop bundle contacts stabilize the active site. The movement of L2′ out of the dimer interface allows L3 to slide in towards the interface and form the substrate binding pocket. Rotation of a key arginine on L2 from a solvent-exposed position into the interface allows its neighboring residue, the catalytic cysteine, to assume its proper position for catalysis.

For effector caspases, equilibrium favors the inactive dimer. For initiator caspases, however, dimerization is the main challenge to be overcome for activation. Addition of kosmotropes such as sodium citrate causes caspase-8 to dimerize and become activated [27]. At least partly because the initiator caspases have longer intersubunit linkers than effector caspases, cleavage of the intersubunit linker is not necessary for activation, but rather, stabilizes the active conformation.

Effector caspase mutants, particularly procaspase-3 V266E, can also be activated without cleavage of the intersubunit linker [28]. This mutant is even more effective at inducing apoptosis than the wild-type (WT) enzyme [29]. The enhancement of activation caused by the mutation is predicted to occur because the mutation keeps the intersubunit linker from binding to the dimer interface. In general, when the intersubunit linker is in the dimer interface, the protein is inactive, whereas when it is out of the interface it can become active.

The conformational ensemble of effector procaspases includes both active and inactive conformers. Although the inactive ensemble is favored, binding of allosteric activators could shift the equilibrium to the active ensemble. On the other hand, binding of allosteric inhibitors to the active caspase could inactivate it. Manipulating the position of the intersubunit linker could lead to allosteric activation or inhibition. A drug which binds at the dimer interface and holds the intersubunit linker in place

could inactivate the enzyme. Conversely, a drug which binds at the dimer interface and keeps the intersubunit linker from binding could activate the procaspase. In fact, a small molecule has been suggested to activate procaspase-3 by this mechanism [30].

Additionally, Wells and coworkers have found a small molecule termed 1541 which forms nanofibrils that act as a scaffold for (pro)caspase-3 binding and increase activation of the procaspase [31]. They suggest that the procaspase is activated through induced proximity, similar to the activation of initiator caspases. *In vitro*, amyloid-β (residues 1–40) fibrils were also shown to activate procaspase-3. The activation of caspases by fibrils may play a role in neurodegenerative diseases [32].

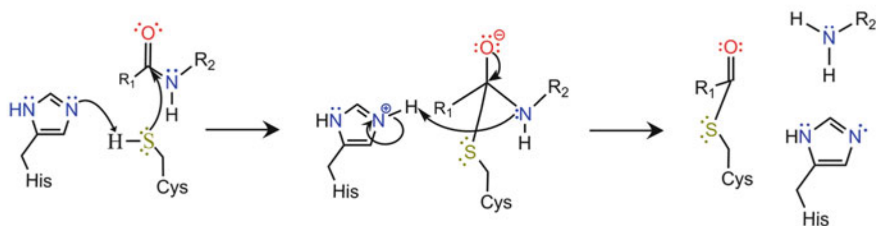
3.2 Catalysis

Proteases all have some mechanistic features in common. The trigonal planar peptide bond of the substrate is forced into a tetrahedral intermediate [33]. As this tetrahedral intermediate forms, a nucleophile attacks the carbonyl carbon of the peptide bond. Then, the amino nitrogen of the leaving group is protonated.

Caspases contain a catalytic dyad consisting of a cysteine and a histidine [33]. Based on the catalytic mechanism accepted for cysteine proteases, the mechanism for caspases has been thought to be as follows (See Fig. 2.3a): First, the catalytic histidine abstracts a proton from the catalytic cysteine. The catalytic cysteine acts

A: Typical cysteine protease mechanism

1. Formation of covalent adduct



2. Hydrolysis of covalent adduct

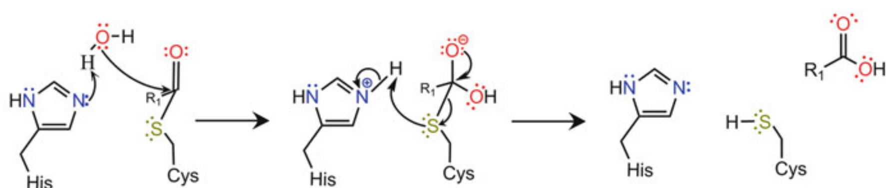
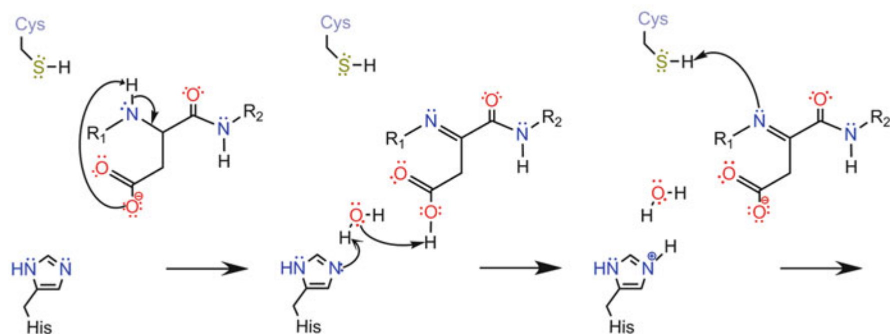


Fig. 2.3 Two proposed mechanisms of caspase catalysis (Adapted from Miscione et al. [34])

B. Simulated caspase mechanism

1. Formation of covalent adduct



2. Hydrolysis of covalent adduct

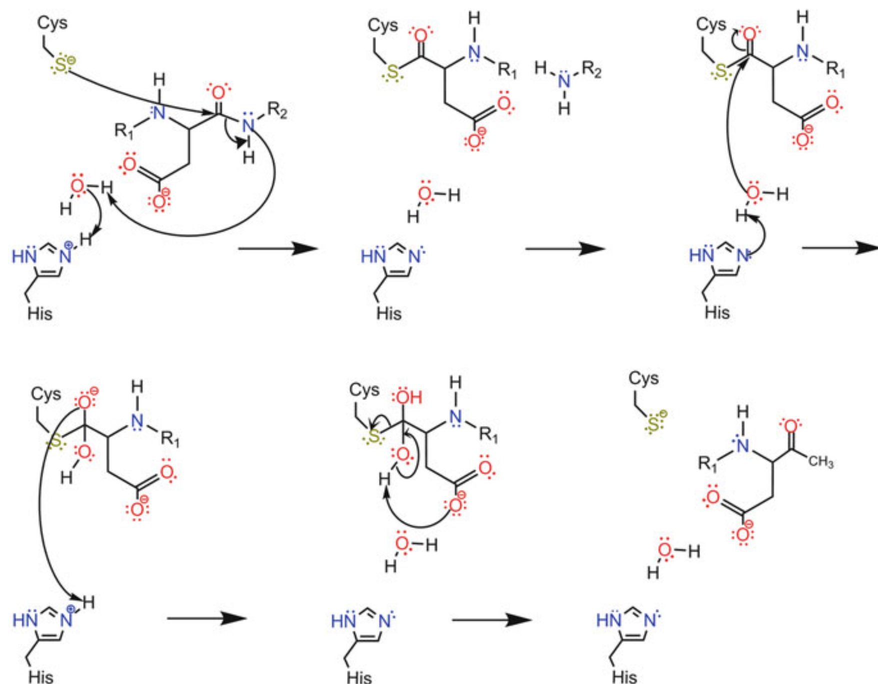


Fig. 2.3 (continued)

as the nucleophile to form a covalent tetrahedral intermediate with the peptide substrate. Once the cysteine has bound, the histidine donates the proton to the amino moiety of the peptide leaving group. The peptide bond is cleaved, with the N-terminal part of the peptide remaining covalently attached to the cysteine while

the C-terminal part of the peptide leaves. Finally, hydrolysis frees the N-terminal part of the peptide and re-protonates the catalytic histidine.

An oxyanion hole, a pocket in the enzyme that hydrogen bonds to the carbonyl oxygen of the substrate, is also thought to be key for catalysis [33]. It is formed by the backbone nitrogens of a conserved glycine (238 in caspase-1) and the catalytic cysteine (285 in caspase-1). The oxyanion hole is thought to be important for polarizing and stabilizing the scissile carbonyl group [34].

However, there are some problems with the proposed mechanism. The 6–7 Å distance between the two catalytic residues is larger than found in most proteases, and makes direct hydrogen transfer unlikely [33]. Molecular dynamics simulations have shown that the catalytic residues cannot exist as a charged pair prior to catalysis [35]. Therefore, the deprotonation of the cysteine likely occurs during catalysis. Also, the histidine residue is not in an optimal location for protonating the amino leaving group [36].

A DFT study of the first part of the catalytic process (Fig. 2.3b, part 1) has been carried out for caspase-7 [34]. Miscione and coworkers found that first, a proton is transferred from the backbone nitrogen of the P1 aspartate to the carboxylate group of the P1 aspartate. In the second step, a proton is transferred from the aspartate to a water molecule, and from that water to the catalytic histidine. In the third step, a proton is transferred from the catalytic cysteine to the backbone nitrogen of the P1 aspartate. The overall result of these first three steps is the protonation of the catalytic histidine and the deprotonation of the catalytic cysteine. In a fourth step, the catalytic cysteine nucleophile attacks the carbonyl carbon of the substrate to form a tetrahedral intermediate, the peptide bond is cleaved, and a proton is transferred from the catalytic histidine to a second water, which transfers a proton to the amino nitrogen of the leaving group.

A QM/MM simulation focused on the hydrolysis of the covalent adduct (Fig. 2.3b, part 2) [37]. In the reaction scheme proposed by Sulpizi and coworkers, the catalytic histidine deprotonates a water molecule, which attacks the scissile carbonyl carbon (as in the original proposed mechanism). Then the proton from the catalytic histidine is abstracted by the now negatively-charged carbonyl oxygen, such that a diol is formed. A second water molecule interacts with the catalytic histidine and one of the diolhydroxy groups. Finally, a proton is transferred from that diol hydroxyl group to the P1 aspartate residue, causing cleavage of the covalent adduct. If this is true, it could more cogently explain the specificity for a P1 aspartate residue.

4 Functions

4.1 Apoptosis

The activation of caspases commits the cell to apoptosis. The main hallmarks of apoptosis include rounding of cells and retraction from neighbors, membrane blebbing to form vesicles called apoptotic bodies, nuclear fragmentation, chromatin

condensation, hydrolysis of genomic DNA to approximately 200 bp fragments, and translocation of phosphatidylserine (PS) to the external surface of cells as an "eat me" signal to phagocytes. The apoptotic caspases are necessary for conferring all of these phenotypes.

In addition to the systematic dismantling of the cell, caspases are also involved in producing "find-me" signals to cause chemotaxis of phagocytes to apoptotic cells [38–40]. The recruitment of phagocytes keeps cells from releasing their contents into extracellular space and activating an immune response which could be harmful to the tissue.

When the number of apoptotic cells is too great for consumption by phagocytes, secondary necrosis can occur. When this happens, the cell releases its contents into extracellular space. However, immune cells are somehow able to recognize the cells undergoing apoptosis (and secondary necrosis) differently from necrotic cells. This is likely due to the actions of caspases. Caspases keep danger-associated molecular patterns (DAMPs) and alarmins from being activated [41]. This can be thought of as a "tolerate me" signal.

Caspases are also involved in turning off transcription and translation [42]. This keeps any infecting viral particles from replicating using the host's machinery. They also fragment the Golgi, ER, and mitochondria [43, 44].

4.2 Inflammatory Response

In contrast to the actions of apoptotic caspases, which systematically dismantle the cell to avoid an immune response, the actions of inflammatory caspases lead to cell lysis and activation of the immune response in a process called pyroptosis [45]. In order to activate an immune response, caspases cleave cytokine IL-1β and IL-18 to produce the mature form [46].

In addition to activation of cytokines, procaspase-1 is also able to activate the pro-inflammatory transcription factor NF-κB [47]. Rather than using its catalytic activity, the CARD domain of procaspase-1 binds to a CARD domain in the kinase RIP2, which is involved in NF-κB activation.

4.3 Other Functions

Caspase expression is kept below a certain threshold required for apoptosis by IAPs (inhibitor of apoptosis proteins). At these subthresholdlevels they are able to play roles that are neither apoptotic nor inflammatory. Caspase-3 activity was found to be important for differentiation of erythroblasts, [48] skeletal muscle, [49] bone marrow stromal stem cells, [50] and neural stem cells [51].

Caspase-3 has several other non-apoptotic functions in nerve cells. In addition to neural cell differentiation, caspase-3 has also been implicated in neuronal migration and plasticity, [52] axon pruning, and synapse elimination [53].

Caspases have been shown to play a role in cell migration and invasion under certain circumstances [54]. They can also induce neighboring cells to proliferate to replace dying cells in a process called apoptosis-induced proliferation [55]. These roles for caspases have implications for cancer: moderate activation of caspases could, in fact, cause cancer to progress rather than regress [54, 55].

In addition to its apoptotic function, caspase-8 has an anti-apoptotic function when it forms a heterodimer with $FLIP_L$ (a protein similar to caspase-8 but lacking a catalytic site) [56]. This protein complex is able to activate the NF-κB signaling pathway leading to proliferation [57]. In another pro-survival capacity, the caspase-8/$FLIP_L$ complex is also able to inhibit RIPK3-dependent necrosis [56].

5 Substrates and Inhibitors

5.1 Synthetic Substrates and Substrate Specificity

Caspase substrate specificity is determined by a series of 4–5 substrate residues which bind to the active site of the caspase. These residues are named P1-P4 or P5, with P1 always being an aspartate residue (Fig. 2.4). The P4 residue is especially important in determining specificity for a given caspase [58].

Because of this 4–5 residue contribution to specificity, substrates used for measuring caspase activity typically have a tetrapeptide preceded by an acetyl group (Ac) on the N terminus and followed by a fluorophore on the C terminus: for example, Ac-DEVD-AFC. When the peptide is cleaved by the caspase, the fluorophore is released and activity can be determined by fluorescence. Some typical fluorophores include AMC (7-amino-4-methylcoumarin) and AFC (7-amino-4-trifluoromethylcoumarin). Addition of p-nitroanilide (pNA) instead of a fluorophore to the C-terminus allows caspase activity to be determined colorimetrically.

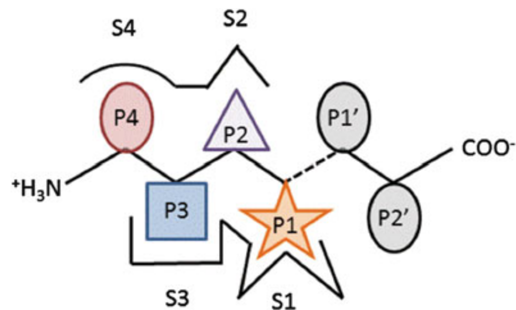


Fig. 2.4 Caspase substrate binding site (Adapted from Stennicke and Salvesen [33])

A positional scanning combinatorial library approach has been used with these synthetic substrates to determine the substrate specificity for most of the mammalian caspases [58, 59]. Caspases-3 and -7 share the same substrate specificity: DEVD. The optimal sequence for caspase-1 is WEHD, and the optimal sequence for both caspase-4 and caspase-5 is (W/L) EHD. The optimal sequence for caspase-2 is DEHD, for caspase-6 is VEHD, for caspase-9 is LEHD, for caspase-8 is LETD, and for caspase-10 is LE(Nle)D (Nle = norleucine).

The P1-P4 residues fit into the S1-S4 pockets in the active site of the caspase. The S1 pocket, consisting of R179, R341, and Q283 (caspase-1 numbering), is nearly 100 % conserved; its basicity and its size make it ideally suited for binding an aspartate residue [60].

The S2 pocket of caspases-3 and -7 is formed by aromatic residues and accommodates small aliphatic amino acids [61]. A substitution of a valine or alanine in place of a tyrosine opens up the S2 subsite to larger residues in the initiator and inflammatory caspases.

The S3 pocket consists of main-chain interactions with R341 (caspase-1 numbering) [61]. In caspases-8, and -9, nearby basic residues enhance the binding of glutamic acid residues to the S3 subsite [27, 62, 63].

The S4 subsite of inflammatory caspases is long, shallow, and hydrophobic, accommodating bulky aromatic side chains such as a tryptophan [59]. On the other hand, in apoptotic caspases, a tryptophan (214 in caspase-3) reduces the size of the subsite, causing a preference for an aspartate or a small aliphatic residue in the S4 pocket [60]. An asparagine in caspases-2 and -3 or a glutamine in caspase-7 enhances interaction with a P4 aspartate [60].

Caspase-2 requires a P5 residue to occupy a S5 subsite [60]. The reason for this specificity may be that binding of a small hydrophobic residue to this subsite may enhance the burial of a P4 aspartate [64].

5.1.1 Endogenous Substrates

To date more than 700 substrates of caspases have been catalogued [65]. A searchable database can be found at http://bioinf.gen.tcd.ie/casbah/. Caspase substrates are involved in conferring an apoptotic phenotype to cells. They are also involved in producing “find-me” and “tolerate-me” signals during apoptosis.

5.1.1.1 Substrates Involved in the Apoptotic Phenotype

The following are some of the substrates of caspases which are involved in producing the apoptotic phenotype. The rounding of cells is likely in part due to caspase cleavage of components of actin microfilaments and microtubular proteins [42]. Retraction of cells from their neighbors likely facilitates phagocytosis and is caused in part by caspase cleavage of components of focal adhesion sites, components of cell-cell adherens junctions, cadherins, and desmosome-associated

proteins [42]. Caspase cleavage of Rho effector ROCK1 which regulates movement of the actin cytoskeleton is a factor in blebbing and nuclear fragmentation [42]. Nuclear fragmentation also involves caspase cleavage of lamins A, B, and C [66]. Chromatin condensation is caused by caspase cleavage of Mst1 kinase [67]. Hydrolyisis of genomic DNA to small fragments is carried out by caspase-activated DNAse (CAD/DFF) [68]. Translocation of PS to the external surface of the cell is also caspase-dependent, but not fully understood [69].

5.1.1.2 Substrates Involved in Other Aspects of Apoptosis

Caspases are also involved in producing "find-me" signals to cause chemotaxis of phagocytes to apoptotic cells. Caspase-3 cleaves calcium-independent phospholipase A2, causing phosphatidylcholine in the membrane to become hydrolyzed to produce lysophatidylcholine (LPC) [38]. The C-terminal fragment of endothelial monocyte-activating polypeptide II (EMAPII) is produced by caspase-dependent proteolysis and acts as a "find-me" signal to attract monocytes [39]. Caspase-dependent cleavage of the membrane channel pannexin-1 causes release of modest amounts of ATP, which may also act as a "find-me" signal [40].

Caspases also function to keep danger-associated molecular patterns (DAMPs) and alarmins from being activated. This function can be thought of as a "tolerate-me" signal, and is important for avoiding autoimmunity [41]. As mentioned above, caspase activation leads to hydrolysis of genomic DNA (which acts as a DAMP) into short fragments [68]. Additionally, the alarmin IL-33 is inactivated by caspase-3/-7-dependent proteolysis [70].

5.1.2 Synthetic Inhibitors

5.1.2.1 Active Site Inhibitors

Active-site inhibitors bind in the place of substrate and are therefore competitive inhibitors. These inhibitors can be peptidic or nonpeptidic and can bind reversibly or irreversibly.

Peptidic inhibitors can have as few as one amino acid (for example, Boc-Asp-FMK), but typically have four (for example, Ac-DEVD-FMK) [71]. Peptides linked to leaving groups such as halomethylketones [for example, chloromethylketone (CMK) and fluoromethylketone (FMK)], acylomethylketones, and (phosphinyloxy) methyl ketones bind irreversibly, whereas peptides linked to non-leaving groups such as aldehyde (CHO) bind reversibly. The electrophilic carbonyl of the aldehyde or ketone binds to the catalytic cysteine, inhibiting it.

Several different peptidomimetics have been designed as inhibitors for caspases. These include urazolopyrazine-based β-strand peptidomimeticsdesigned as inhibitors for caspase-3 and caspase-8, [72] hydantoin-based peptidomimetics as inhibitors of caspase-3 [73], dipeptidylaspartylfluormethylketones with unnatural

amino acids [74], 1-(2-acylhydrazinocarbonyl)-cycloalkyl carboxamides, [75] 8,5-fused bicyclic compounds, [76] and peptidomimetics containing a caprolactam ring [77].

Non-peptide inhibitors have also been discovered. These include isatins, [78, 79] indole aspartyl ketones, fuchsone derivatives, and pyrrolo[3,4-c]quinolone-1,3-diones [80].

5.1.2.2 Allosteric Inhibitors

Caspases-3 and -7 were found to contain an allosteric site at the dimer interface [81]. The drugs FICA and DICA form disulfide bonds with cysteines in the dimer interface of those caspases and inactivate the protein. The structural changes brought about by binding of these drugs involves massive loop rearrangements to a structure very similar to that of the proenzyme.

Mutation of valine 266 to a histidine at the dimer interface of caspase-3 also caused allosteric inactivation of the protein [28]; however, the structural changes brought about by the mutation were much more subtle than those that occurred upon binding of FICA or DICA [82]. Instead of conversion to a structure like that of the proenzyme, inactivation may be caused by a series of steric clashes, disordering of loop L1, and/or destabilization of helix 3.

A drug called compound 34 was found to bind to cysteines near the dimer interface of caspase-1 [83]. Similarly to the binding of FICA and DICA, the inactive structure was like that of the proenzyme.

Another set of allosteric inhibitors was found to inhibit caspases-3, -7, -8, and -9 [84]. A crystal structure with caspase-7 indicates that one and likely all of these compounds binds to the dimer interface. One of the compounds, Comp-A, inhibits dimerization of caspase-8; however, caspase-7 remained a dimer upon binding of the drug. As with FICA and DICA inhibition, the inhibited form was similar to that of the zymogen. However, these new compounds are reversible inhibitors, unlike FICA and DICA.

One of the urazolering peptomimetic inhibitors which bind at the active site was also found to bind near the dimer interface of caspase-8 [72]. Some of the interacting residues of caspase-8 are Tyr334, Thr337, Glu396, and Phe399.

Caspase-2 was allosterically inhibited through binding of a designed ankyrin repeat protein (DARPin) [85]. Binding causes the caspase to be fixed in an inactive conformation different from that of the proenzyme.

A novel allosteric site was found on caspase-6 [86]. Phage display produced a peptide pep419 which binds near helix 2 and causes tetramerization and therefore inactivation of caspase-6. Interestingly, it was found that at pH 8, the zymogen of caspase-6 is a tetramer in solution, whereas at pH 5.5, the zymogen is a dimer, but can be induced to form a tetramer through the binding of pep419 or a related peptide pep420. The pH changes in the cell brought about by apoptosis could potentially lead to dissociation of caspase-6 tetramers to the dimeric form, leading to activation of the protein.

5.1.3 Endogenous Inhibitors

Both viral and endogenous inhibitors can block caspase activity by competing for binding to activation complexes. Viral inhibitors target caspase activity of their host cells in order to counter an immune response. Several γ-herpesviruses and molluscipoxvirus use v-FLIPs to block caspase access to the DISC. Similarly, endogenous $FLIP_s$ blocks procaspase-8 recruitment to DISC, [87] and ICEBERG blocks caspase-1 recruitment to form the inflammasome [88].

Most protease inhibitors bind to the protease and block substrate access [60]. Suicide inhibitors are cleaved and cause a conformational change to occur in either the inhibitor or the protease. Although it is typically a serine protease inhibitor, the serpinCrmA is also able to inhibit caspases-1, [89] -8, [90] and -9, [91] likely by forming a covalent attachment with the caspase and undergoing a conformational change upon cleavage of the scissile P1–P1′ bond to place the caspase on the "bottom" of the inhibitor [92]. Similarly, the baculovirus protein p35 becomes covalently attached to the catalytic cysteine, the scissile bond is cleaved, but the protein is not liberated because it blocks the hydrolytic water from gaining access to the active site [93, 94].

Anothercategory of inhibitors are IAPs (inhibitor of apoptosis proteins). They were first discovered using baculovirus lacking a functional *p35* gene [95]. They contain a 70–80 residue Zn^{2+} binding module named BIR. The most well-studied is X-linked inhibitor of apoptosis protein (XIAP) [96].

XIAP targets caspases in two different ways. A linker to the BIR1 domain and The BIR2 domain of XIAP target effector caspases-3 and -7 [97]. Residues in the active site, particularly in loop L1 make critical contacts with the inhibitor. Loop L2′ also makes contacts with XIAP. The necessity of ordered active site loops and cleaved intersubunit linker to form L2′ mean that XIAP only binds the active caspase rather than the inactive procaspase.

Unlike XIAP binding to the effector caspases, BIR3 and RING of XIAP target initiator caspase-9 [97, 98]. Also, instead of binding to the active site, it binds to the dimer interface of the monomer and blocks dimer formation. Loop L2′ of caspase-9 binds BIR3 in a similar manner to how loop L2′ of caspase-3 binds BIR2. The pocket where loop L2′ of caspase-9 binds BIR3 is called the Smac (second mitochondrial activator of caspases) pocket because Smac can also bind there to derepress caspase activation.

Caspase activity is also controlled endogenously through the use of post-translational modifications. The RING domain of XIAP acts as an E3 ubiquitin ligase toubiquitylate effector caspases-3 and -7, leading to proteasomal degradation [99, 100]. Sumoylation of procaspase-2 [101] and caspase-8 [102] likely leads to localization of the protein in the nucleus.

Phosphorylation is a third post-translational modification which affects caspase activity. p38-MAPK phosphorylates S150 of caspase-3, inhibiting it [103]. Phosphorylation by PKC-δ at an as yet unknown site, on the other hand, enhances caspase-3 activity [104]. PAK2 phosphorylates caspase-7 at three sites,

decreasing its activity [105]. For caspase-9, ERK phosphorylates T125, [106] c-Abl phosphorylates Y153, [107] and Akt phosphorylates S196, [108] leading to decreased activity of the protein.

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