PART I GENERAL PRINCIPLES OF CELL DEATH

1 Human Caspases – Apoptosis and Inflammation Signaling Proteases

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1. PROTEASE SIGNALING IN APOPTOSIS AND INFLAMMATION

In 1992 two groups independently reported the identity of a human protease responsible for activating the precursor of interleukin-1β, naming it interleukin-1β converting enzyme (ICE). [1,2] Several months later, one of the key genes governing the commitment to apoptosis in *Caenorhabditis elegans* – *CED3* – was demonstrated to show homology with ICE.[3] These publications initiated a successful search by many groups over the ensuing years for mammalian ICE homologs that should govern cell death. Today these proteases are known as caspases.[4] Of the 11 caspases in humans, 7 are considered to be involved primarily in apoptosis, three are considered to be involved primarily in proinflammatory cytokine activation, and one is involved in keratinocyte differentiation (Figure 1-1). How cells learned to employ closely related proteases to execute two opposing phenotypes – apoptosis and inflammation – is strongly debated, and this chapter incorporates some discussion on this tricky issue.

Confirmation of the important roles of the caspases in the inflammatory cytokine response comes from gene ablation experiments in mice. Animals ablated in caspase-1 or -11 are deficient in cytokine processing,[5,6] but without any overt apoptotic phenotype. The phenotypes of the apoptotic caspase knockouts are often gross and are sometimes antiapoptotic and vary from early embryonic lethality (caspase-8), to perinatal lethality (caspase-3 and -9),[7,8,9] to relatively mild with defects in the process of normal oocyte ablation (caspase-2).[10] Techniques in biochemistry and cell biology have allowed us to place the apoptotic caspases in two converging pathways (Figure 1-2). This core pathway probably represents a minimal apoptotic program, but almost certainly the apparent simplicity is complicated by cell-specific additions that help to fine tune individual cell fates. Moreover, recent evidence clearly implicates caspase-8 in nonlethal roles in the control of cell proliferation.[11,12]

1.1. Apoptosis and limited proteolysis

Apoptosis is a mechanism to regulate cell number and is vital throughout the life of all metazoan animals. Although several different types of biochemical events have been recognized as important in apoptosis, perhaps the most fundamental is the participation of the caspases.[13,14,15,16,17]

The name *caspase* is a contraction of cysteine-dependent aspartate-specific protease[4]; thus their enzymatic properties are governed by a dominant specificity for protein substrates containing Asp and by the use of a Cys side chain for catalyzing peptide bond cleavage. The use of a Cys side chain as a nucleophile during peptide bond hydrolysis is common to several protease families. However, the primary specificity for Asp turns out to be very rare among proteases throughout the biotic kingdoms. Of all known mammalian proteases, only the caspase activator granzyme B, a serine protease, has the same primary specificity.[18,19] Caspases cleave a number of cellular proteins,[20] and the process is one of limited proteolysis in which a small number of cuts, usually only one, are made. Sometimes cleavage results in activation of the protein, sometimes in inactivation,[21] but never in degradation, because their substrate specificity distinguishes the caspases as among the most restricted of endopeptidases. This is an important distinction from the other well-known proteolytic signaling system – the proteasome – which permits signaling by wholesale destruction of regulatory proteins such as

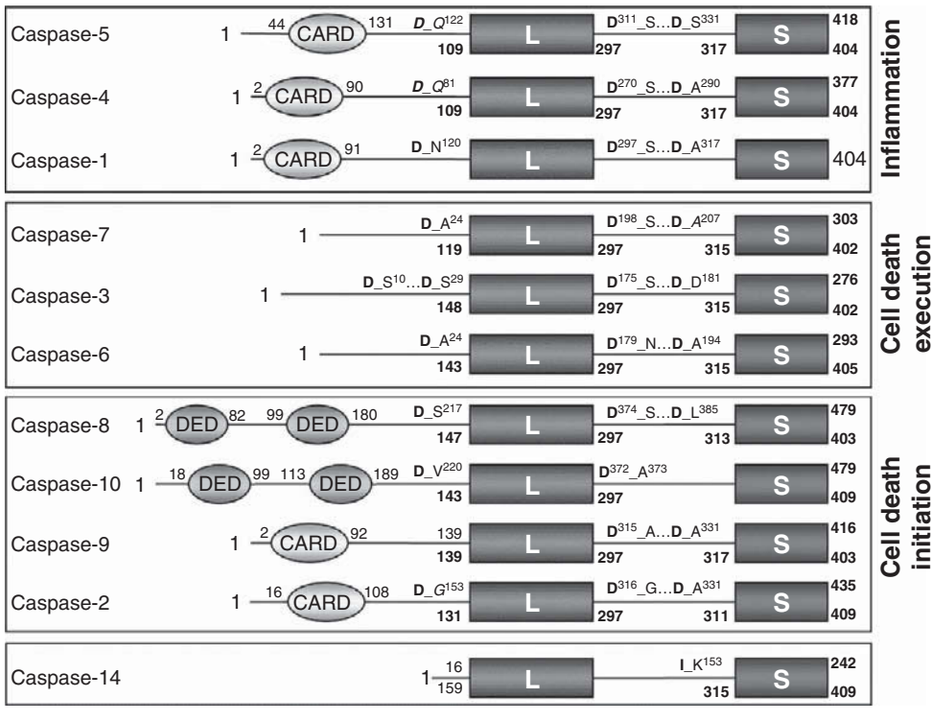

Figure 1-1. Domain organization of human caspases. Human caspases have been grouped according to their sequence similarities. Notice that sequence homology divides caspase-1 to -10 into three subfamilies, in accord with the physiologic distinction between inflammatory, initiator, and effector caspases. In contrast to the widespread distribution of these family members, caspase-14 is mainly found in the epidermis, may be involved in keratinocyte differentiation, and is not activated in vivo at an Asp residue. The positions of suspected (*italics*) or known maturation cleavage sites are given, with the P_1 aspartate residue indicated by "D." Numberings correspond either to the Swiss-Prot entries, with the exception of caspase-10, for which the sequence of the more commonly expressed isoform 10/a is given, or to the caspase-1–based numbering convention. The ovals are the recruitment domains, and the catalytic domain is designated in bars for the large and small subunit. Reproduced with permission from Fuentes-Prior, P. and Salvesen, G. S. (2004). The protein structures that shape caspase activity, specificity, activation and inhibition. *Biochem J* 384, 201–32. © The Biochemical Society (http://www.biochemj.org).

IκB in nuclear factor kappa B (NFκB) signaling and PDS1 in anaphase promotion.[22]

1.2. Caspase evolution

The first bona fide apoptotic caspase, Ced3, was identified in *C. elegans*, and it appears that this organism requires only one caspase to execute apoptosis.[3] Initially it was thought that as the complexity of primitive cell death pathways developed, so apparently did the number of caspases. *Drosophila* species have 7 caspases,[23] and humans have 11. But this simple picture needs to be revised in light of the presence of multiple caspases in organisms far more primitive than *C. elegans*. It is likely that *C. elegans* and *Drosophila* may have lost genes that encode the more complex apoptotic network of more primitive animals. For example, the primitive sea anemone contains 10 caspases, whereas deuterostomes have seen an expansion to 42 members of the caspase family.[24] Presumably, the multifaceted apoptotic response is more ancient than previously thought, because this expansion is not restricted to caspases but is also found in Bcl2 family members.

Mapping the inherent substrate specificity of caspases has allowed some broad consensuses to be recognized.[25] These consensuses also allow apoptotic caspases to be distinguished from proinflammatory caspases, because the latter have a rather distinct specificity that presumably allows them to carry out their job without threatening cell viability. Interestingly, there seems to have been a parallel evolution of apoptotic caspases along with their substrates. Consensus caspase targets in humans such as nuclear lamins and poly (ADP)ribose polymerase have easily recognizable caspase cleavage sites in *Drosophila*, but apparently not in organisms such as yeast and plants, which lack an apoptotic pathway. Indeed, although yeast and plants are known to employ programmed cell death, they do not contain

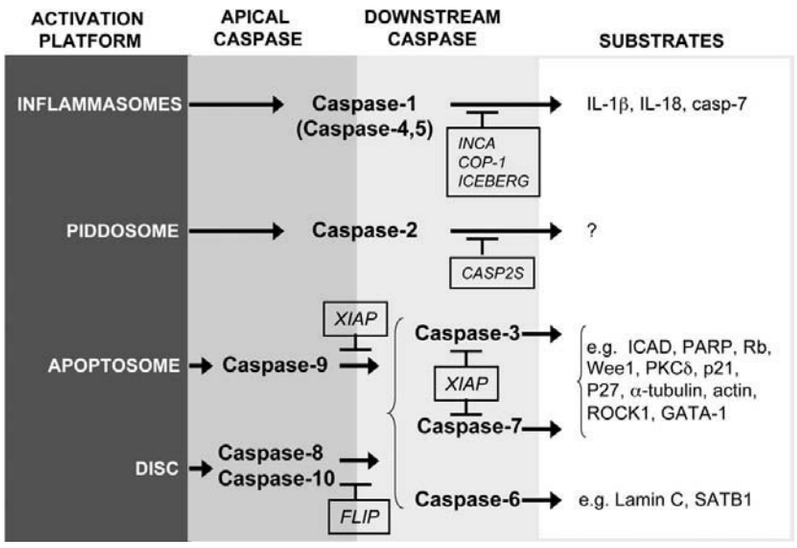

Figure 1-2. Activation pathways and substrates. An oligomeric protein platform activates an apical caspase, which then cleaves specific caspases. The apoptotic apical caspases require an intermediary step through the direct activation of downstream caspases, creating a two-step pathway that amplifies the apoptotic signal and allows for additional regulation points. The inflammatory caspases seem to use no intermediate, although the biochemical relationship of caspase-1, -4, and -5 is still obscure. A few caspase substrates are shown, but many are yet to be discovered. Caspase inhibitors, shown in boxes, regulate the activation pathways. Note that, although the inflammatory caspases are placed on a separate pathway, activated by recruitment to inflammasomes, they are able to activate caspase-7 during acute inflammation models in vitro, leading to the suggestion that the inflammatory network may feed into the apoptotic network in this manner.[86] Reproduced with permission from Pop, C. and Salvesen, G., Human caspases: activation, specificity, and regulation. *J Biol Chem* 284, 21777–21781. From the American Society for Biochemistry and Molecular Biology.

caspases, and the concept of apoptosis in these forms of life is probably wrong. They use other mechanisms to kill off their cells, such as the hypersensitive response in plants.[26,27]

2. ACTIVATION MECHANISMS

Akin to the classic multistep proteolytic pathway of coagulation, downstream caspases are activated by proteolysis, but upstream ones, having no protease "above" them, must respond to an activating signal by another mechanism. Some time ago it was thought that all caspases were activated by proteolysis, but it has become clear that this is a minor mechanism in caspase activation, pertaining principally, at least in humans, to the three executioner caspase-3, -6, and -7. Structural information reveals that the conformations of zymogens are quite similar, as are the conformations of active forms. But the mechanisms that enforce the zymogen-to-active transition are substantially different between initiators and executioners.

2.1. Initiator caspases – activation by dimerization

In the latent state, initiator caspases are inert monomers that require homodimerization for activation. In vivo, dimerization is facilitated by caspase recruitment to oligomeric activation platforms that assemble subsequent to an apoptotic signal. Adaptor molecules from the activation platform specifically bind caspase prodomains such as the death effector domains (DEDs) in caspase-8 and -10 and caspase activation and recruitment domains (CARDs) in caspase-1, -2, and -9. The recruitment enforces a local increase in caspase concentration and generates activity by proximity-induced dimerization.[28] Each apical caspase has its own activation platform: the death-inducing signaling complex (DISC) recruits and activates caspases-8 and 10 and the apoptosome activates caspase-9 (Figure 1-2).

2.2. The activation platforms

The DISC formed by the Fas receptor, Fas-associated death domain protein (FADD), and caspase-8 is a highly oligomeric network of homotypic protein interactions that comprise the death domains of Fas and FADD. The crystal structure of the Fas/FADD complex shows a tetrameric arrangement of four FADD death domains bound to four Fas death domains. Fas appears to act as a mechanistic switch that prevents accidental DISC assembly yet allows for highly processive DISC

formation and clustering on ligation of the cognate death receptor.[29,30]

Caspase-9 activation requires the cofactor Apaf-1, which, in the absence of an apoptotic stimulus, exists in a monomeric form.[31] In the presence of cytochrome c, and either 2′-deoxy adenosine triphosphate (ATP) or ATP, the AAA+ ATPase domain of Apaf-1 oligomerizes to form a wheel-shaped signaling platform, the apoptosome.[32] Each of these signaling platforms has the ability to recruit its cognate caspase through homotypic interactions of the N-terminal domain on the caspase.[28] Indeed, replacing the recruitment domain of caspase-9 onto caspase-8 allows caspase-8 to be activated at the apoptosome – giving support to the general induced proximity model for apical caspase activation by dimerization.[33]

The PIDDosome may be involved in the activation of caspase-2, although in the latter case, scant structural evidence is available to substantiate this proposed mechanism. In some cases, specific adaptor proteins incorporated in the activation complex may direct the signaling toward different pathways. For example, under certain conditions, caspase-2 and caspase-8 can trigger either cell death or NFκB survival pathway, although few mechanistic data have been put forward for the latter event.[34,35]

The inflammatory caspases are probably activated by a similar induced dimerization mechanism. The multiprotein activation platforms are called inflammasomes, with affinity for the CARD pro-domains of caspase-1, -4, and -5.[16] However, it is not clear whether the activation mechanism of inflammatory caspases occurs by enforced homodimerization or it is the result of heterodimerization with other components of the inflammasome, such that caspase-1 could heterodimerize with caspase-5, as has been seen for the caspase-8/FLIP heterocomplex, for example.[36] The structural and mechanistic aspects learned from work on various apoptosomes should prove interesting for a closely related family of proteins – the NOD-like receptors (NLRs). The NLRs encompass a glut of acronyms in the literature, including NOD (nucleotide-binding oligomerization domain), NALP (NLR-, LRR-, and PYD-containing proteins), NACHT (domain that is present in NAIP, CIITA, HET-E, and TP1), PAN (pyrin- and NACHT-domain family), and CATERPILLAR (CARD, transcription enhancer, R (purine)-binding, pyrin, lots of LRR) proteins. NLRs are key mediators in the innate immune system and are linked to inflammation, host defense, and a number of inflammatory diseases.[37,38,39,40,41,42,43] All NLR proteins share a domain that is closely related to the NB-ARC AAA+ ATPase domain of Apaf-1.[38] Like Apaf-1, they are thought to sense a signal – possibly a bacterial product – leading to their activation and the subsequent generation of activity in inflammatory caspases (caspase-1, -4, or -5) or kinases. These proteins can also be considered soluble receptor counterparts to the widely investigated Toll-like receptor transmembrane receptor family. Their domain architecture closely resembles the one seen in Apaf-1. Most NLR proteins possess leucine-rich repeats (LRRs) that are responsible for ligand binding in place of the WD40s of Apaf-1. It has been proposed that the NACHT domains, very much like Apaf-1, can form oligomeric inflammasomes to activate their target proteins via adaptor domains, such as CARD or pyrin domains.[44] Although detailed structural information about this process is not available yet, it is hypothesized that this family represents soluble receptors that use AAA+ related domains to assemble in a manner like that of Apaf-1 and thus activate their targets by oligomerization.[45]

2.3. Executioner caspases – activation by cleavage

Short pro-domain (executioner) caspases occur as inactive dimers that require cleavage at a position within the catalytic domain to become active (Figure 1-1). The first step in activation, dimerization, has already occurred shortly after their synthesis, and the zymogens are restrained by a short linker that separates the large and small subunits of the catalytic domain. The clearest evidence for the activation of executioner caspases comes from the crystal structures of the zymogen and active forms of caspase-7,[46,47,48] which reveal the molecular details of catalytic site formation on activation. Proteolytic processing of the linker allows rearrangement of mobile loops equivalent to the initiator caspases, favoring formation of the catalytic site.[13] In vivo, the upstream activators of effector caspases are the apoptotic initiators (caspase-8, -9, -10) and the lymphocyte-specific serine protease granzyme B. Caspase-14, a short pro-domain caspase, requires both cleavage and dimerization for in vitro activation, although the natural activator has yet to be identified.[15,49]

Although physiologic allosteric regulators of caspases are yet to be discovered, a cysteine protease from *Vibrio cholerae* that is distantly related to caspases uses a mechanism of allosteric activation induced by the host inositol hexaphosphate.[50] The intriguing possibility of caspase activation by an as yet undiscovered allosteric mechanism in vivo is suggested by the finding that the activity of caspase-1, -3, and -7 can be modulated in vitro by using ligands that bind next to the dimer interface, far away from the active site.[51]

2.4. Proteolytic maturation

Caspase activation is frequently followed by (auto) proteolytic cleavages called maturation events, often optional, chronologically distinct events, which are functionally distinct from activation. Most maturation involves trimming or removal of the pro-domain or cleavage of the inter-subunit linker. It is important to note that, in the absence of an activation process, maturation is unable to generate enzymatic activity. Caspases do not activate by pro-domain removal, an activation mechanism used by many other proteases.

As a source of new epitopes and arrangements, maturation has several important consequences at the cellular level. For instance, dimerization in the absence of maturation generates a form of active caspase-8 that can signal T-cell proliferation and activation, but not cell death. The apoptotic role of caspase-8 appears to require cleaved caspase-8 in vivo.[52] Mechanistically, this auto-cleavage greatly stabilizes the caspases-8 catalytic domain, potentially enabling activity to linger in the cytosol once the protease is released from the DISC.[53] But it is not known whether simple stabilization by maturation could explain the contrasting functions of caspase-8 mentioned previously.

Maturation cleavage of the caspase-9 inter-subunit linker by caspase-3 lays the grounds for caspase-9 regulation by the endogenous inhibitor X-linked IAP (XIAP)[54] by exposing new epitopes necessary for XIAP biding. A clear role remains to be established for some maturation events, and it is entirely possible that most of these events are simply cleavage of innocent bystanders resulting from caspase activity. However, caspase maturation is a distinct process from activation, important for generating caspase stability or signaling downstream regulatory events.

3. CASPASE SUBSTRATES

The most essential feature of caspase substrate recognition is that caspases cleave after Asp residues. But other requirements need to be met to turn a peptide/protein into a good caspase substrate, both in vitro and in vivo. A peptide of sequence P_4-P_3-P_2-P_1-P_1', with P_1-P_1' as scissile bond, is a caspase substrate when (1) the P_1 residue is Asp – with the notable exception of the *Drosophila* caspase Dronc, a caspase-9 relative, which cleaves in vitro just as well after Glu[55]; (2) the P_1' residue is small and uncharged (Gly, Ser, Ala)[56]; and (3) P_4-P_3-P_2 residues are complementary for interactions with the catalytic groove.[25] Optimal residues in P_4-P_2 turn a mediocre substrate (XXXD/G) into an excellent caspase substrate. For example, executioner caspases prefer DEVD/G peptides to WEHD/G peptides, whereas the opposite is true for inflammatory caspases.[25] In the case of natural protein substrates, two more rules apply: (1) the substrate cleavage site (P_4-P_1') is exposed to the aqueous environment (this suggests that "loops" or "turns" of natural substrate fold are prone to be proteolyzed); and (2) caspases co-localize with their substrates.

At one time it was suggested that the sum total of proteolytic events of endogenous proteins by caspases defines apoptosis.[57,58] An unexpectedly large number of proteins have been reported to be in vivo caspase substrates.[21,59] Focused proteomics approaches reveal at least 400 cellular proteins that are cleaved in a caspase-specific manner after induction of apoptosis in cell culture.[60,61,62] However, it is far from clear, and indeed rather unlikely, that all of these cleavages are required for apoptosis. Separating the cleavage events that cause apoptotic function/morphology from the collateral bystander events that are inevitable given the complexity of the human proteome turns out to be very difficult. Although the list of annotated caspase substrates continues to lengthen, most candidates lack functional evidence linking cleavage to a role in apoptosis. In principle, in-depth investigation of cleavage site mutants in cells and animals will help to gain a more realistic understanding of how caspases drive apoptotic cell death and which substrates are part of the apoptotic or inflammatory response.

An important aspect that needs to be kept in mind is that no specific artificial substrates/inhibitors for caspases exist. One can appreciate that IETD/G, theoretically preferred for caspase-8, could also be cleaved by caspase-3, as judged by the synthetic library data.[25,56] However, extrapolating to data from real protein samples, a protein containing IETD/G should be a very good substrate for caspase-3, indistinguishable for caspase-8 activity. When the activity is measured in cell lysates, the high caspase-3 concentration masks the activity of other caspases, even if a so-called preferred artificial substrate is used.[63] Future attempts to divide caspase specificity in complex mixtures may follow the use of biotinylated probes that enable tagging of individual classes of proteases[64] or a combination of live-cell reporters and flow cytometry coupled with more selective caspase inhibitors.[65]

4. REGULATION BY NATURAL INHIBITORS

The first level of regulating proteolytic pathways is by zymogen activation, but an equally important level is achieved by the use of specific inhibitors that can govern the activity of the active components. The

endogenous inhibitors of caspases, those present in mammalian cells, are members of the inhibitor of apoptosis (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, with few caveats,[66] agree with the biologic function of the inhibitors (reviewed in references 67, 68). Although IAPs and CrmA would be expected to regulate mammalian caspases in vivo, p35 would never be present normally in mammals, because it is expressed naturally by baculoviruses.

The best-characterized endogenous caspase inhibitor is the 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 reference 69). 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 caspase-3, -7, and -9 (reviewed in reference 70). Despite earlier claims, other members of the IAP family probably do not directly inhibit caspases (reviewed in reference 71) and have functions in addition to caspase inhibition because they have been found in organisms such as yeast, which neither contain caspases nor undergo apoptosis.[72,73]

IAPs contain one, two, or three baculovirus IAP repeat (BIR) domains, which represent the defining characteristic of the family. The first BIR domain (BIR1) binds to TRAF2 and regulates interactions with tumor necrosis factor receptor complexes,[74,75] the second BIR domain (BIR2) of XIAP specifically targets caspases 3 and 7 ($K_i \approx 0.1$–1 nM), and the third BIR domain (BIR3) specifically targets caspase 9 ($K_i \approx 10$ nM). This led to the general assumption that the BIR domain itself was important for caspase inhibition. Surprisingly, structures of BIR2 in complex with caspase-3 and -7 and BIR3 in complex with caspase-9 revealed the BIR domain to have almost no direct role in the inhibitory mechanism. Many of the important inhibitory contacts are made by the flexible region preceding the BIR domain.[76,77,78,79] Interestingly, the mechanism of inhibition of caspase-9 by the BIR3 domain requires cleavage in the inter-subunit linker to generate the new sequence NH_2-ATPF.[54] In part this explains the cleavage of caspase 9 during apoptosis, which, as previously described, is not required for its activation. Paradoxically, it seems required for its inactivation by XIAP.

Significantly, neither CrmA-like nor p35-like inhibitors, which operate by mechanism-based inactivation,67 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 caspase-3 and -7 requires a nonstandard interaction with the extended 381 loop that is specific to these two caspases (reviewed in reference 67). Possibly, the 381 loop has evolved to achieve substrate specificity in the executioner caspases.[80] However, 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 sophistication, IAPs, but not CrmA nor p35-like proteins, are subject to negative regulation by IAP antagonists that go by the names of Hid, Grim, Reaper, and Sickle in *Drosophila* and Smac/Diablo and HtrA2/Omi in mammals (reviewed in references 69, 70).

Inhibition by decoy proteins uses proteins structurally related to caspase pro-domains, competing for the same adaptors within activation platforms. Thus, from a semantic viewpoint, they are not inhibitors, but rather "activation preventers." FLICE inhibitory protein (FLIP), a pseudo-caspase-8 with a nonfunctional catalytic domain, precludes caspase-8 recruitment to the DISC. Similarly, caspase-1–related proteins such as COP1, INCA, or ICEBERG bind to the caspase-1 pro-domain via CARD–CARD interactions and prevent its recruitment to inflammasomes.[81]

The final mechanism of caspase regulation involves degradation via the proteasome, an eventual fate suffered by many cellular proteins. Activated caspases are ephemeral species inside the cell and have a more dynamic turnover than the inactive zymogens,[82] and it has been suggested that the proteins responsible for their rapid removal are IAPs (mentioned previously). In addition to the defining BIR domain, many IAPs also contain really interesting new gene (RING) and ubiquitin-associated domains that are involved in ubiquitin ligation.[83,84] Although it is somewhat controversial regarding whether these domains target the IAPs themselves, or cargoes such as caspases, the IAPs are currently the clearest candidates for removal of active caspases before they reach an apoptotic threshold. Consistent with this is the observation that mice with a deleted XIAP RING domain have elevated caspase activity in a subset of cells, implying a physiologic requirement of XIAP ubiquitin-ligase activity for caspase removal.[85]

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