MINIREVIEW. HUMAN CASPASES: ACTIVATION, SPECIFICITY AND REGULATION
Cristina Pop and Guy S. Salvesen

Program in Apoptosis And Cell Death Research, The Burnham Institute for Medical Research, 10901 N. Torrey Pines Rd, La Jolla CA 92037, USA
Address correspondence to Guy S. Salvesen, The Burnham Institute for Medical Research, 10901 N. Torrey Pines Rd, La Jolla CA 92037, Phone: 858-646-3100; Fax: 858 646-3197; E-mail: gsalvesen@burnham.org

Caspases are intracellular proteases that propagate programmed cell death, proliferation and inflammation. Activation of caspasess occurs by a conserved mechanism subject to strict cellular regulation. Once activated by a specific stimulus, caspases execute limited proteolysis of downstream substrates to trigger a cascade of events that culminates in the desired biological response. Much has been learned of the mechanisms that govern the activation and regulation of caspases, and this review provides an update of these areas. We also delineate substantial gaps in knowledge of caspase function, which can be approached by techniques and experimental paradigms that are currently undergoing development.

Introduction

Ever since the CED3 gene was found to be required for developmental cell death in C. elegans following a genetically encoded pathway (1), a great deal of effort has been spent to accumulate information about the molecular mechanism of the form of programmed cell death known as apoptosis. Apoptosis studies have been extrapolated to many other species beside C. elegans only to discover that caspases - CED3-like molecules - and much of the molecular machinery responsible for apoptosis are conserved in metazoan organisms,- reviewed in (2,3). The function of caspases was mainly established by a combination of biochemical experiments in vitro and gene deletion studies in mice (4). About the same time that CED3 was discovered, the interleukin-1β processing enzyme (now caspase-1) was found to be involved in pro-inflammatory cytokine processing. It was clear that caspases could prosecute two radically different biological processes: apoptosis or inflammation, but probably not both. However, recent reports suggest roles for apoptotic caspases in proliferation, differentiation, or migration (5,6). In the last few years, substantial progress has been made in delineating the fundamental properties that govern caspase activation, specificity, and regulation, and this review will focus on recent advances in these areas. Many recent reports on the influence of caspase activity by phosphorylation or ubiquitination are currently lacking in mechanistic explanations, and therefore will not be covered in this review.

Caspases are almost never associated with non-specific degradative processes, but rather with signaling events. Caspases transmit downstream signals by specific limited cleavage of key cellular components that galvanize a certain pathway. The name caspase is a contraction of cysteine-dependent aspartate specific protease: 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 (4,7). 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 biotic kingdoms. Caspases are widely expressed, with the exception of caspase-14, which is limited to keratinocytes (8).

Structural Organization

Many available crystal structures demonstrate that caspases are organized in a similar manner. Caspase zymogens are single-chain proteins, with N-terminal pro-domains preceding the conserved catalytic domains (Figure 1A). They occur either as monomers or dimers, a crucial property that defines their activation mechanism (Figure 1B). During activation and/or maturation, the catalytic domain is cleaved to a large (α) and a small subunit (β) that interact intimately with each other. In the active form, a caspase is a dimer of catalytic domains of αββ'α'
symmetry, with two active sites per molecule. Although the two catalytic domains of the executioner caspase-7 are equal and independent (9), there is evidence that the active sites of caspase-1 may be linked such that occupation of one site promotes activity of the second site (10). The large subunit contains the catalytic dyad residues Cys and His, while the small subunit supplies several residues that form the substrate-binding groove. The unstructured regions linking the pro-domains and catalytic domains or linking the two subunits are often the subject of (auto)proteolysis during maturation (Figure 1A).

**Classification**

For the purposes of this review we distinguish the human caspases based on their presumptive function and location in signaling pathways (Figure 2A). Additional criteria include: pro-domain length, substrate preference (4), and phylogenetic relationships (11). For several years, caspases were simply divided into “apoptotic” and “pro-inflammatory”, and this classification remains useful to some extent, although most apoptotic candidates (caspase-2, 3, 6, 7, 8, 9, 10) have had at least one non-apoptotic role attributed to them (12). Similarly, typical “non-apoptotic” members such as caspase-1, 4, 5 are proposed to induce “pyroptosis”, a form of death associated with massive activation of inflammatory cells (13). The only truly remaining non-apoptotic human candidate may be caspase-14 - a mediator in keratinocyte differentiation (8).

Within the apoptotic sub-group, the terms “initiators” or “apical” caspases versus “executioners”, “effector” or “downstream” caspases distinguish the caspases that initiate the cascade (caspase-8, 9, 10) from those that of those that are activated by the initiators to execute apoptosis (caspase-3, 6, 7). Initiators are further divided into caspases participating in the extrinsic (caspase-8 and -10) or intrinsic apoptotic pathway (caspase-9). Using these definitions, it is hard to classify caspase-2, which displays combined features (Figure 2A) (14). Classification according to pro-peptide length coincides with the mechanism of activation (see below). Usually, caspases with long pro-domain (~100 residues) activate by dimerization (inflammatory caspases, apical caspases and caspase-2), while caspases with short pro-domain (<30 residues) activate by cleavage of the catalytic domain (caspase-3, 6, and 7).

Lastly, classification of caspases based on synthetic substrate preference, though illuminating in terms of catalytic mechanisms (15), likely does not reflect the real caspase substrate preference in vivo (16-19), and provides inaccurate information for discriminating among caspase activities (20). Thus, extreme caution is warranted in applying the intrinsic tetrapeptide preferences to predict the targets of individual caspases.

**Activation Mechanisms**

As in any multi-step proteolytic pathway, downstream caspases are activated by proteolysis, but upstream ones, having no protease “above” them, must respond to an activating signal by another mechanism. Initially it was thought that all caspases were activated by proteolysis, but over the last few years it has become clear that this is a minor mechanism in caspase activation, pertaining principally, at least in humans, to the three executioner caspases 3, 6 and 7 (4). Where structural information is available, the conformations of zymogens are quite similar, as are the conformations of active forms. But the mechanisms that deliver the zymogen→active transition are substantially different between initiators and executioners.

**Initiator caspases – activation by dimerization.** In the off state, initiator caspases are inert monomers that require homodimerization for activation (Figure 1B). Physiologically, 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 pro-domains like death effector domains (DEDs) of caspase-8 and 10, and caspase recruitment domains (CARDs) of caspase-1, 2 and 9. The recruitment enforces a local increase in caspase concentration and generates activity by proximity-induced dimerization (21). Each apical caspase has its own activation platform: the DISC (death inducing signaling complex) recruits and activates caspase-8 and 10, the apoptosome activates caspase-9, while 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 (Figure 2A). In some cases, specific adaptor proteins incorporated in the activation complex may direct the signaling towards different pathways. For example, under certain conditions, caspase-2 and caspase-8 can trigger either cell death or NF-kB survival pathway, although little mechanistic data have been put forward for the latter event (22,23).

The inflammatory caspases are probably activated by a similar induced dimerization mechanism. The multi-protein activation platforms are called inflammasomes with affinity for the CARD pro-domains of caspase-1, 4 and 5 (24). However, it is not clear whether the activation mechanism of inflammatory caspases occurs by enforced homo-dimerization or it is the result of hetero-dimerization 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 (25).

Executioner caspases – activation by cleavage.

Short pro-domain (executioner) caspases occur as inactive dimers that require cleavage of the catalytic domain to become active (Figure 1). The first step in activation – dimerization – has already occurred shortly following their synthesis and thezymogens are restrained by a short linker that separates the large and small subunits of the catalytic domain. The most illuminating evidence for the activation of executioner caspases comes from the crystal structures of the zymogen form of caspase-7 (26,27), which reveal the molecular details of catalytic groove formation upon activation. Proteolytic processing of the linker allows rearrangement of mobile loops equivalent to the initiator caspases, favoring formation of the catalytic site (4). In vivo, upstream processors 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 (28,29).

Although physiologic allosteric regulators of caspases are yet to be discovered, a cysteine protease from *V. Cholerae* that is distantly related to caspases utilizes a strategic mechanism of allosteric activation induced by the host inositol hexaphosphate (30). The possibility of caspase activation by allostery 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 (10).

Proteolytic Maturation

Caspase activation is frequently followed by (auto)proteolytic cleavages called maturation events. Maturation is often an optional, chronologically distinct event that should not be confused with activation per se. Most maturation involves trimming/removal of the pro-domain or cleavage of the inter-subunit linker. Importantly, in the absence of an activation process, maturation is unable to generate enzymatic activity (4,32). 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 is not without an effect at the cellular level. For instance, dimerization in the absence of maturation produces a form of active caspase-8 capable of signaling T cell proliferation and activation, but not cell death, which requires cleaved caspase-8 (31). Mechanistically, this auto-cleavage greatly stabilizes the caspase-8 catalytic domain, potentially enabling activity to linger in the cytosol once the protease is released from the DISC (32). But it is not known whether simple stabilization by maturation could explain the contrasting functions of caspase-8 mentioned above, and this is a fruitful avenue for research.

Maturation cleavage of the caspase-9 inter-subunit linker by caspase-3 sets the grounds for caspase-9 regulation by the endogenous inhibitor XIAP (33) by exposing new epitopes necessary for XIAP binding. A clear role remains to be established for some maturation events, and it is entirely possible that these events are simply cleavage of innocent by-standers resulting from caspase activity. The take home message is that caspase maturation is a distinct process from activation, important for generating caspase stability or signaling downstream regulatory events.
Specificity

The most salient feature of caspase specificity usually retained by readers is that caspases cleave after Asp residues (to be read as “any Asp”). The truth is that many other requirements need to be met to turn a peptide/protein into a good caspase substrate. No black-and-white rules exist to define Asp-containing peptides as “substrates” or “non-substrates”, but rather as “bad”, “intermediate” and “good” caspase substrates (16). 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 (34); 2) the P_1’ residues is small and uncharged (Gly, Ser, Ala) (Figure 2B) (35); and 3) P_4-P_3-P_2 residues are complementary for interactions with the catalytic groove. Optimal residues in P_4-P_3 turn a mediocre substrate (XXXXD/G) into an excellent caspase substrate. For example, executioners cleave very efficiently DEVD/G peptides but much less efficiently WEHD/G peptides (Figure 2B), while exactly the opposite is true for inflammatory caspases (15). In the case of natural protein substrates, two more rules apply: 4) the substrate cleavage site (P_2-P_1’) is exposed to the aqueous environment. This suggests that “loops” or “turns” of natural substrate fold are prone to be proteolyzed; 5) Caspases and their substrates co-localize, a common sense rule.

In the early days of caspase investigation it was suggested that the sum total of proteolytic events of endogenous proteins by caspases delineates apoptosis. An unexpectedly large number of proteins have been reported to be in vivo caspase substrates (16,36). Targeted proteomics approaches reveal in the order of 400 cellular proteins that are cleaved in a caspase-specific manner following induction of apoptosis in cell culture (18,19,37). But how to separate the cleavage events that cause apoptotic function/morphology from those innocent bystander events that are inevitable given the complexity of the human proteome? It turns out to be very difficult to do this in a scientifically rigorous manner. The list of annotated caspase substrates continues to increase, but most candidates lack functional evidence linking cleavage to a role in apoptosis. In principle, meticulous investigation of cleavage site mutants in cells and animals will help to remove irrelevant “bystander” substrates from the list of caspase substrates. By removing the bystanders, it will be possible to gain a more realistic understanding of how caspases drive apoptotic cell death, and this promises to be another fertile area of future research.

An important aspect that needs to be kept in mind is that caspase substrate specificity overlaps, therefore “specific” artificial substrates/inhibitors for caspases do not exist. Figure 2B shows caspase-3 and 8 substrate preferences when an artificial peptide library is used. We can appreciate that IETD/G, theoretically “preferred” for caspase-8, could also be cleaved by caspase-3 as judged by the synthetic library data (Figure 2B). However, extrapolating to data from real protein samples, a protein containing IETD/G should be a really very good substrate for caspase-3, indistinguishable from caspase-8 activity. When the activity is measured in cell lysates, high caspase-3 concentration masks the activity of other caspases, even if a “preferred” artificial substrate is used (20). Future attempts to divide caspase specificity in complex mixtures may follow the use of biotinylated probes that enable tagging of individual classes of proteases (38), or a combination of live-cell reporters and flow cytometry coupled with more selective caspase inhibitors (39).

Regulation

Because proteolysis is irreversible, activation of caspases in cells is tightly regulated. To prevent unwanted physiological responses, cells employ three back-up strategies: caspase inhibition, caspase degradation and decoy inhibitors. Nature’s solution to inhibiting proteases is often to take advantage of the substrate-binding cleft, occupying it with a chain segment that mimics a good substrate. Such examples are found in some viruses, where caspase inhibitors act to defeat the hosts’ measures to control infection (40). The two best characterized viral caspase inhibitors, CrmA (cytokine response-modifier A) from the cowpox virus and p35/p49 proteins produced by baculoviruses are both active site directed “suicide” inhibitors. They achieve rapid inhibition of caspases in a relatively non-selective manner, a
consequence of their requirement to present substrate-like sequences to the caspase, which as explained above, is a non-specific strategy (41). A much more selective mechanism is to be found in the human caspase regulator XIAP (X-linked inhibitor of apoptosis). This multi domain protein efficiently and selectively inhibits caspase-9 (via its BIR3 domain) and caspase-3 and 7 (via its BIR2 domain). These domains of XIAP combine two specific, but relatively weak, interactions with their target caspases, to achieve specific tight inhibition by a two-site mechanism, and are therefore mechanistically distinct to the viral inhibitors mentioned above (42). Other inhibitors of apoptosis (IAPs) related to XIAP (cIAP1, cIAP2, ILP-2, ML-IAP, survivin etc) do not directly inhibit caspases (42). Intriguingly, no caspase inhibitor has been identified in C. elegans until recently when Csp3, a protein with a predicted caspase homology domain, was shown to prevent spontaneous activation of CED3 by a mechanism affecting the assembly of caspase heterotetramer (43), in principle similar to the inhibition of caspase-9 by XIAP.

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”. FLIP (ELICE inhibitory protein), a pseudo-caspase-8 with a nonfunctional catalytic domain, precludes caspase-8 recruitment to the DISC. Similarly, caspase-1 related proteins like COP1, INCA or ICEBERG bind to the caspase-1 pro-domain via CARD-CARD interactions and prevent its recruitment to inflammasomes (44).

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 (45), and it has been suggested that the proteins responsible for their rapid removal are IAPs (mentioned above). In addition to the defining BIR domain, many IAPs also contain RING and UbA domains that are involved in ubiquitin ligation (46,47). Although it is somewhat controversial as to 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 demonstrated elevated caspase activity in certain cells, implying a physiological requirement of XIAP ubiquitin-ligase activity for caspase removal (48).

Future perspectives

Although we have introduced apoptosis as a major function of caspases, there is mounting evidence that apoptotic caspases also have vital survival functions in drosophila and mammalian cells - reviewed in (49). Why and how has nature taken a lethal strategy (propagation of apoptosis) and utilized it for the opposite – namely survival and proliferation? Arguments such as local restriction of activity, differential control of activity, and operation as signaling molecule in the absence of enzymatic activity have all been raised as possibilities. Perhaps a regulatory process called “compensatory proliferation” - communication between cells that are able to undergo proper division only when the amount of death in the population is adequate - could help to unify the apparently contradictory roles of caspases (50). Future research will reveal how the non-apoptotic function of caspases is regulated in healthy cells, and thus the mechanisms that distinguish the pro-death versus pro-survival capacities of caspases present a fruitful area of future research.

We have described the explosion of putative caspase substrates revealed by focused proteomics approaches, yet there is no facile way to distinguish the primary targets that mediate caspase-driven proteolysis from the collateral proteolytic events of no mechanistic importance. Development of strategies to expose the biologically relevant substrates is another fertile area of research.

And finally, small molecules that inhibit caspases in a specific manner are lacking from the pharmacological armory, as are putative small molecule caspase activators. We have described how recent biochemical and structural advances have demonstrated the role of allostery in caspase activity. We expect rapid developments in small molecule assay and design to guide the way to the specific control of caspase activity.
REFERENCES

FIGURE LEGENDS

**Fig. 1.** A. Caspase organization. A pro-domain precedes the catalytic domain, composed of two covalently linked subunits. Sites for (auto)-proteolysis at Asp residues are indicated. B. Activation mechanisms. Initiators are monomers that activate by pro-domain mediated dimerization. Executioners are dimers that activate by cleavage of intersubunit linkers. Following activation, additional proteolytic events mature the caspases to more stable forms, prone to regulation.

**Fig. 2.** A: 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. A few caspase substrates are shown, but many are yet to be discovered. Caspase inhibitors, shown in boxes, regulate the activation pathways. B: Substrate specificity of caspases. The figure plots amino acid preferences in the P4-P1' positions of caspases. The total height of each position is proportional to sequence conservation, while the height of residues in each position plots the relative frequency of each amino acid at the position (http://weblogo.berkeley.edu/logo.cgi). The caspase-3 and caspase-8 panels summarize peptide libraries exploring the P4-P1' substrate primary specificity determinants and reveal little overlap between caspases-
3 and 8 – taken from data in (15, 35). The apoptotic cells panel shows caspase substrates from natural proteins cleaved in apoptotic cells, revealing an almost total dropout in specificity of the P4 site, implying that selectivity for natural substrates may fail to obey the simplistic rules established with synthetic peptide library substrates – taken from data in (18).

ACKNOWLEDGEMENTS

We thank John Timmer for generating Figure 2B and for helpful discussions. This work was supported by grant CA069381 from the NCI.
Figure 1

A

Pro-domain linker
Inter-subunit linker

Pro
Asp
Large
Asp
Catalytic dyad
Small

B

INITIATOR

Dimerization
adaptors
Maturation

EFFECTOR

Cleavage
Inactive

ACTIVE

Regulation
**Figure 2**

### A

<table>
<thead>
<tr>
<th>Activation Platform</th>
<th>Apical Caspase</th>
<th>Downstream Caspase</th>
<th>Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammasomes</td>
<td>Caspase-1 (Caspase-4,5)</td>
<td></td>
<td>IL-1β, IL-18, casp-7</td>
</tr>
<tr>
<td>PIDDosome</td>
<td>Caspase-2</td>
<td></td>
<td>?</td>
</tr>
<tr>
<td>Apoptosome</td>
<td>Caspase-9</td>
<td>Caspase-3, Caspase-7</td>
<td>e.g. ICAD, PARP, Rb, Wee1, PKCδ, p21, P27, α-tubulin, actin, ROCK1, GATA-1</td>
</tr>
<tr>
<td>DISC</td>
<td>Caspase-8, Caspase-10</td>
<td>Caspase-6</td>
<td>e.g. Lamin C, SATB1</td>
</tr>
</tbody>
</table>

### B

![Casp-3/library](http://www.jbc.org/)

![Casp-8/library](http://www.jbc.org/)

![Apoptotic cells](http://www.jbc.org/)
Access the most updated version of this article at doi: 10.1074/jbc.R800084200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2008/12/31/jbc.R800084200.citation.full.html#ref-list-1