Caspases are intracellular proteases that propagate programmed cell death, proliferation, and inflammation. Activation of caspases 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 minireview 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.

Ever since the ced-3 gene was found to be required for developmental cell death in Caenorhabditis 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 (CED-3-like molecules) and much of the molecular machinery responsible for apoptosis are conserved in metazoan organisms (reviewed in Refs. 2 and 3). The function of caspases was established mainly by a combination of biochemical experiments in vitro and gene deletion studies in mice (4). About the same time that CED-3 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 minireview 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 minireview.

Caspases are almost never associated with nonspecific 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. Caspasezymogens are single-chain proteins, with N-terminal prodomains preceding the conserved catalytic domains (Fig. 1A). They occur either as monomers or dimers, a crucial property that defines their activation mechanism (Fig. 1B). During activation and/or maturation, the catalytic domain is cleaved to a large (α) and a small (β) subunit 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, whereas the small subunit supplies several residues that form the substrate-binding groove. The unstructured regions linking the prodomains and catalytic domains or linking the two subunits are often the subject of (auto)proteolysis during maturation (Fig. 1A).

**Classification**

For the purposes of this minireview, we distinguish the human caspases based on their presumptive function and location in signaling pathways (Fig. 2A). Additional criteria include prodomain length and 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, and -10) have had at least one non-apoptotic role attributed to them (12). Similarly, typical “non-apoptotic” members such as caspase-1, -4, and -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 subgroup, the terms “initiators” and “apical” caspases versus “executioners” and “effector” or “downstream” caspases distinguish the caspases that initiate the cascade (caspase-8, -9, and -10) from those that are activated by the initiators to execute apoptosis (caspase-3, -6, and -7). Initiators are further divided into caspases participating in the extrinsic (caspase-8 and -10) or intrinsic (caspase-9) apoptotic pathway. Using these definitions, it is hard to classify caspase-2, which displays combined features (Fig. 2A) (14). Classification according to propeptide length coincides with the mechanism of activation (see below). Usually, caspases with a long prodomain (∼100 residues) activate by dimerization (inflammatory caspases, apical caspases, and caspase-2), whereas caspases with a short prodomain (<30 residues) activate by cleavage of the catalytic domain (caspase-3, -6, and -7).

Finally, classification of caspases based on synthetic substrate preference, although 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 multistep 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, caspase-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 (Fig. 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 prodomains such as death effector domains of caspase-8 and -10 and CARDs² 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 recruits and activates caspase-8 and -10; the apoptosome activates caspase-9; and 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 (Fig. 2A). 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 -8 can trigger either the cell death or NF-κB 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 multiprotein activation platforms are called inflammasomes, with affinity for the CARD prodomains of caspase-1, -4, and -5 (24). However, it is not clear whether the activation mechanism of inflammatory caspases occurs by enforced homodimerization or 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 (25).

**Executioner Caspases: Activation by Cleavage**—Short prodomain (executioner) caspases occur as inactive dimers that require cleavage of the catalytic domain to become active (Fig. 1). The first step in activation, dimerization, has already occurred shortly following their synthesis, and the zymogens 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, and -10) and the lymphocyte-specific serine protease granzyme B. Caspase-14, a short prodomain caspase, requires both cleavage and dimerization for in vitro activation, although the natural activator has yet to be identified (28, 29).

Although physiological allosteric regulators of caspases are yet to be discovered, a cysteine protease from *Vibrio 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 prodomain or cleavage of the intersubunit linker. Importantly, in the absence of an activation process, maturation is unable to generate enzymatic activity (4, 32). Caspases do not activate by prodomain 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 autocleavage 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 intersubunit 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 bystanders 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 P₄-P₃-P₂-P₁-P₁’, with P₁-P₁’ as scissile bond, is a caspase substrate when 1) the P₁ 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 P₁’ residue is small and uncharged (Gly, Ser, Ala) (Fig. 2B) (35); and 3) P₄-P₃-P₂ residues are complementary for interactions with the catalytic groove. Optimal residues in P₄-P₃-P₂ turn a mediocre substrate (XXX(D/G)) into an excellent caspase substrate. For example, executioners cleave

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²The abbreviations used are: CARD, caspase recruitment domain; DISC, death-inducing signaling complex; FLIP, FLICE inhibitory protein; XIAP, X-linked inhibitor of apoptosis; IAP, inhibitor of apoptosis; BIR, baculovirus IAP repeat.
MINIREVIEW: Human Caspases

DEV(D/G) peptides very efficiently but WEH(D/G) peptides much less efficiently (Fig. 2B), whereas 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$_{4}$-P$_{1}$) is exposed to the aqueous environment. This suggests that “loops” or “turns” of the natural substrate fold are prone to be proteolysed. 5) Caspases and their substrates co-localize, a commonsense 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 on the order of 400 cellular proteins that are cleaved in a caspase-specific manner following induction of apoptosis in cell culture (18, 19, 37). How can the cleavage events that cause apoptotic function/morphology be separated 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. Fig. 2B shows caspase-3 and -8 substrate preferences when an artificial peptide library is used. We can appreciate that IET(D/G), theoretically “preferred” for caspase-8, could also be cleaved by caspase-3 as judged by the synthetic library data (Fig. 2B). However, extrapolating to data from real protein samples, a protein containing IET(D/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 backup 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 baculovi-
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.

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.

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