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Protease Activation during Apoptosis: Death by a Thousand Cuts?

Minireview

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The concept that cell death can be a regulated process under molecular control is now well established. Much evidence also indicates that the molecular machinery for death has been well conserved through evolution. Until recently, efforts to identify components of this machinery were focused primarily on identifying endonucleases capable of cleaving DNA at internucleosomal sites, since this type of DNA degradation is a well-established characteristic of most, but not all, forms of apoptosis. However, observations from studies using enucleated cells as well as cell-free systems indicate that the central components of the cell death machinery, which we will call the executioner, are most likely localized to the cytosol. Furthermore, several lines of evidence indicate that proteases, particularly those of the emerging interleukin-1β (IL-1β)-converting enzyme (ICE) family, are good candidates for regulators or components (or both) of the executioner.

Proteolysis and Apoptosis

One of the early indications that proteases could be important triggers for apoptosis came from studies on proteins found in the cytoplasmic granules of cytotoxic T lymphocytes and natural killer cells, both of which kill by binding to target cells and inducing apoptosis within—the so-called kiss of death. Purification of the constituents of cytotoxic T lymphocyte and natural killer granules yielded perforin, a pore-forming protein, as well as a series of serine proteases, one of which, granzyme B/fragmentin-2, has the unusual property of cleaving at Asp residues (Shi et al., 1992). Exposure of target cells to purified preparations of perforin/granzyme B in combination was sufficient to induce apoptosis.

More direct evidence that proteases may be centrally involved in the regulation of the cell death process has come from studies on the nematode Caenorhabditis elegans. A series of genes that control various elements of the programmed cell death process in this worm have been identified, two of which, *ced-3* and *ced-4*, are required for cell death during development. Subsequently, *ced-3* was found to exhibit significant homology to *Ice* (Yuan et al., 1993), which converts the 33 kDa protease form of IL-1β to the active 17.5 kDa form, once again by cleaving after Asp residues (Thornberry et al., 1992). Ectopic expression of *Ice* in fibroblasts resulted in apoptosis, suggesting that *Ice* is both functionally as well as structurally homologous to *ced-3* (Miura et al., 1993). These observations have been closely followed by the discovery of several more *Ice/ced-3* homologs: *Nedd-2/Ich-1* (Kumar et al., 1994; Wang et al., 1994), *CPP32/Yama* (Fernandes-Alnemri et al., 1994; Tewari et al., 1995), *Tx/Ich-2* (Faucheu et al., 1995; Kamens et al., 1995), and *Mch-2* (Fernandes-Alnemri et al., 1995) in which the pentameric peptide, QACRG, surrounding the putative actived site Cys, is completely conserved (Table 1). As with ICE, overexpression of each of these proteases in various cell types results in apoptosis.

A third line of evidence for a role for proteases in apoptosis comes from studies that have explored the effects of diverse protease inhibitors on apoptosis induced by a variety of agents. These studies have implicated calpain I, a calcium-dependent protease, in activation-induced apoptosis (Sarin et al., 1993), as well as apoptosis induced by dexamethasone, low level irradiation, or macromolecular synthesis inhibition (Squier et al., 1994).

In addition, a number of proteins, including but not restricted to poly(ADP-ribose) polymerase (PARP) (Kaufmann et al., 1993; Lazebnik et al., 1994), the 70 kDa protein component of the U1 small nuclear ribonucleoprotein (Casciola-Rosen et al., 1994), lamin B1 (Neamati et al., 1995; Voelkel-Johnson et al., 1995; Lazebnik et al., 1995), α-fodrin (Martin et al., 1995), topoisomerase I (Voelkel-Johnson et al., 1995), and β-actin (Kayalar et al., 1995), have been reported to become degraded in association with the onset of apoptosis, thereby providing direct evidence for the activation of one or more proteases during this process.

Proteases as Components of the Executioner

The observation that overexpression of ICE or its homologs results in apoptosis may not be all that significant by itself, particularly given the fact that introduction of practically any protease into cells in a deregulated manner is likely to provoke considerable damage and result in cell death. This is illustrated by studies in which delivery of either chymotrypsin, proteinase K, or trypsin into the cytoplasm of several different cell types resulted in apoptosis within a few hours (Williams and Henkart, 1994).

More direct evidence that ICE, an ICE-like protease, or both are required for apoptosis stems from studies that have explored the effects on apoptosis of CrmA, an inhibitor of ICE encoded by the cowpox virus, or specific tetrapeptide (YVAD) ICE inhibitors. For example, CrmA-expressing Rat1 fibroblasts were protected from death owing to serum withdrawal (Wang et al., 1994). Overexpression of *crmA* also prevented apoptosis due to engagement of the Fas (CD95) receptor or to exposure to tumor necrosis factor α (Tewari and Dixit, 1995; Enari et al., 1995;

Table 1. ICE Family Proteases Implicated in Apoptosis

Protease	Active Site	Substrates
ICE	QACRG	Pro-Il-1β, pro-ICE, pro-YAMA
CED-3	QACRG	?
ICH-1/NEDD-2	QACRG	?
CPP32/YAMA	QACRG	PARP
TX/ICH-2	QACRG	Pro-ICE, pro-TX
MCH-2	QACRG	PARP

Los et al., 1995). In line with these observations, tetrapeptide inhibitors of ICE activity dramatically inhibited cell death due to Fas ligation (Enari et al., 1995; Los et al., 1995). In addition, rapid increases in ICE or ICE-like activity are observed within minutes of engagement of Fas (Los et al., 1995).

Further evidence for the involvement of an ICE-like protease in apoptosis comes from studies on the short form of the *Ice/ced-3* homolog *Ich-1/Nedd-2*. Whereas the long form of *Ich-1*, *Ich-1L*, induced apoptosis of Rat1 fibroblasts, overexpression of *Ich-1S* actually suppressed serum withdrawal-induced apoptosis in the same cells (Wang et al., 1994). This suggests either that ICH-1S competes for the same downstream targets of ICH-1L or other ICE-like proteases but does not cleave them or that ICH-1S somehow inactivates the proteases themselves, possibly by becoming incorporated into oligomeric complexes with full-length forms of ICH-1 or other ICE family proteases (Gu et al., 1995).

It has also recently been demonstrated that cleavage of PARP, one of the proteins known to be cleaved in association with apoptosis, can be catalysed by CPP32/YAMA, an ICE-like protease (Tewari et al., 1995; Fernandes-Alnemri et al., 1995). This cleavage was prevented by CrmA, both in intact cells as well as in vitro, whereas a CrmA point mutant that had lost the ability to interact with CPP32/YAMA as well as ICE was ineffective in this regard (Tewari et al., 1995). These observations are important since they not only identify an ICE homolog as being responsible for proteolysis of a protein known to be degraded during apoptosis, but they also raise the possibility that CrmA blocks apoptosis in the cases mentioned earlier, not by neutralizing ICE, but by utilizing its ability to neutralize CPP32/YAMA or other ICE-like proteases. Similar conclusions could also be drawn regarding the YVAD inhibitor studies described above. In this context, it is also interesting to note that whereas ICE is capable of processing CPP32/YAMA to its active form, the reverse is not true (Tewari et al., 1995). It is therefore tempting to speculate that in studies in which ectopic expression of ICE resulted in apoptosis (Miura et al., 1993), this may have been due to activation of CPP32/YAMA in these cells.

Recent data derived from *Ice* knockout mice would seem to suggest that ICE itself is not an essential requirement for cell death, except perhaps for Fas-related apoptosis of thymocytes (Kuida et al., 1995), since these mice develop normally and cells from these animals are capable of undergoing apoptosis in vitro (Li et al., 1995; Kuida et al., 1995). The fact that several ICE homologs, with overlapping tissue distribution, are now known to exist suggests that either there is redundancy in the executioner or that an ICE-like protease such as CPP32/YAMA or TX/ICH-2, rather than ICE itself, is more centrally involved in the cell death process.

How Does Proteolysis Lead to the Stereotypic Changes Associated with Apoptosis?

Although apoptosis was described almost 25 years ago, it is remarkable that we still know so little concerning the mechanistic aspects of the most dramatic features of this process—condensation of the nucleus, degradation of the chromatin, and pronounced blebbing of the plasma membrane. One particularly attractive feature of the idea that proteases could be key components of the executioner is that it is relatively easy to conceive how activation of one or more proteases could directly produce at least some of the structural changes seen during apoptosis. A scheme showing known and hypothetical roles for proteases in apoptosis is shown in Figure 1.

Activation of a protease can lead to processing and activation of additional molecules of the same or other proteases, leading to an amplified protease cascade. This is a common theme in protease function, and such protease cascades are central to the processes of clotting and complement activation. Among the proteases associated with apoptosis, at least three are known to interact in this way. Thus, TX/ICH-2 can process both pro-TX and pro-ICE (Faucheu et al., 1995), and ICE processes both pro-ICE and pro-YAMA/CPP32 (Thornberry et al., 1992; Tewari et al., 1995). It is also possible that the activation of these or related proteases contributes to the activation of another protease, calpain, that is often induced during apoptosis (Sarin et al., 1993; Squier et al., 1994; Martin et al., 1995). The result may be an escalating cascade of proteases.

Proteolytic cleavage of specific substrates may further contribute to the process of apoptosis in several ways: through structural changes, by activation of other effector molecules such as nucleases (e.g., processing of proforms of a protein), or by removal of an inhibitor (by analogy with degradation of IκB to release NF-κB). Some of the effects would be expected to be cell type specific, if a relevant substrate were expressed in a tissue-specific

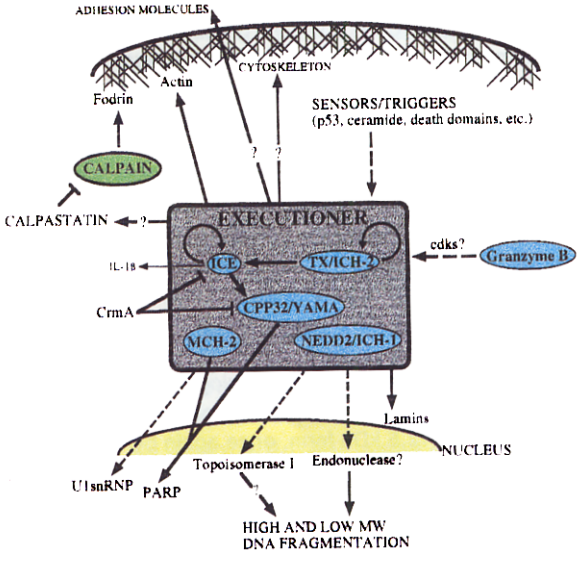

Figure 1. Proteases as Central Components of the Executioner

Summarized are known (solid lines) and putative (broken lines) interactions between proteases and their substrates that take place during apoptosis. The central box places the known ICE family proteases within the executioner. Although some of these proteases are known to interact, there is currently no evidence that they act as a complex.

manner. Since apoptosis tends to be morphologically similar among different cells, we would predict that the most important substrates are fairly ubiquitous and are evolutionary conserved, at least at their cleavage sites.

Relatively few targets for proteolysis during apoptosis have been identified. Some of these fit the above predictions and might have important roles in apoptosis. At this time, however, the role of these substrates in apoptosis remains largely hypothetical.

Some of the most characteristic changes associated with apoptosis affect the nucleus, and proteolysis could play a major role here. For example, lamin B1 degradation during apoptosis could lead to collapse of the chromatin due to the loss of attachment points on the nuclear matrix (Neamati et al., 1995). Recent observations suggest that an ICE-like protease, distinct from the PARP-cleaving protease CPP32/YAMA, is responsible for degradation of the lamins during apoptosis (Lazebnik et al., 1995). Interestingly, while inhibition of lamin-cleaving protease activity does not interfere with chromatin margination or DNA degradation, it does prevent the collapse of the chromatin into discrete blobs and break-up of the nucleus that typifies late stage apoptosis (Lazebnik et al., 1995). It is also possible that proteases could contribute to DNA fragmentation by activation of endonucleases; a partially characterized 24 kDa protease has been shown to induce the formation of characteristic DNA ladders when added to isolated nuclei, but this protease does not itself exhibit any nuclease activity (Wright et al., 1994). In addition, the cleavage of PARP by CPP32/YAMA or MCH-2 (Tewari et al., 1995; Fernandes-Alnemri et al., 1995) inhibits most of its DNA repair activity (Kaufmann et al., 1993), and this may also contribute to the demise of the cell.

Other characteristic changes during apoptosis focus on the plasma membrane and cytoskeleton, as cells lose adherence, bleb, and fragment. Adherent cells can be readily removed from the substratum by limited proteolysis, and it is not unlikely that this is what happens when such cells undergo apoptosis. Blebbing and cellular fragmentation into apoptotic bodies depend upon actin polymerization (Cotter et al., 1992); thus, proteolytic cleavage of actin (Kayalar et al., 1995) and of the actin-associated protein fodrin (Martin et al., 1995) is likely to be relevant to these membrane changes. Further, the cleavage of actin by ICE destroys its ability to inhibit DNase I (Kayalar et al., 1995), which might then have effects at the level of the nucleus.

Specific proteolytic cuts of a limited number of substrates would be repaired in the cell as the substrates are replaced, and unless this proteolysis outpaces the replacement, we might expect the cell to survive (if such proteolysis is indeed central to the apoptotic process). We suggest, however, that this is exactly what happens, owing in part to the amplifying effect of the proteolytic cascade. Eventually, too many cuts in too many places result in a critical change in the cell, and it suddenly collapses into apoptotic death. In this scenario, the timing and exact details of death are not "programmed" in a sequence of defined steps, but are instead set in motion like a rock slide (an image from the concept of criticality). In the context of our discussion, perhaps a better analogy is the manner in which a large tent collapses if lines are cut at random: there are fluctuations and changes in shape, but the tent stays up until some critical moment when everything suddenly comes crashing down, a description that fits the process of apoptotic death.

Life and Death: A Delicate but Robust Balance

If ICE-like proteases are indeed components of the executioner or are involved in its control, then, by analogy with p53 and other genes known to regulate apoptosis positively, one would expect that a significant proportion of tumors should arise as a result of inactivation of ICE family proteases. By the same reasoning, *crmA* would be expected to synergize with known oncogenes, such as *myc*, to transform cells. The fact that neither of these predictions has been fulfilled to date may simply indicate that no one has yet looked. Alternatively, it may be that neither *crmA* nor ICE family protease defects can contribute significantly to the transformed state. If this proves to be the case, then there are several possible explanations; the most obvious of these is that ICE-like proteases are not components of the executioner. A more interesting possibility is that loss of control over the cell death machinery is likely to have such disastrous consequences for a multicellular organism that mechanisms to ensure that this does not occur have evolved. One such mechanism may be redundancy, the hobgoblin of gene knockout predictions. Thus, animals lacking a functional *Ice* gene may develop normally, not because this protein is irrelevant to apoptosis, but because other proteases can take its place (Li et al., 1995; Kuida et al., 1995).

A third scenario would be that in which all of the components of the executioner are required for cell growth as well as death. For proteases, we can readily envision the possibility that those that are elements of the executioner also process (say, at lower constitutive levels of activity) pro forms of proteins involved in normal cell function. Thus, mutations that interfere with the function of the executioner would simultaneously eliminate any growth advantage a cell may obtain from losing its ability to undergo apoptosis. An obvious prediction that can be made from this scenario is that no tumor can arise as a result of inactivating mutations involving the executioner and, by extension, that all tumors should be capable of undergoing apoptosis.

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Note Added in Proof

The biochemical purification of the active form of CPP32/YAMA (termed apopain) was recently described by Nicholson et al. (1995) (Nature *376*, 37–43). In addition, another member of the ICE family, ICE_{rel}III, has been reported by Munday et al. (1995) (J. Biol. Chem. *270*, 15870–15876), as well as one, ICE_{rel}II, that is identical to TX/ICH-2.