Minireview

Role of multiple cellular proteases in the execution of programmed cell death

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Abstract A family of mammalian homologues of the *Caenorhabditis elegans* cell death protein Ced-3 has been recently discovered. These mammalian proteins encode novel cysteine proteases with homology to the interleukin-1β converting enzyme (ICE). Although several studies support a role for one or more of these proteases in mediating apoptosis, their mechanism of action is far from understood. The presence of multiple mammalian ICE-like proteases, with apparently similar apoptotic function indicates that, despite its conservation during evolution, the cell death pathway is much more complex in mammals than in the worm. In addition to ICE-like proteases, several other proteases of different cleavage specificities have been implicated in apoptosis. There is now a growing body of evidence suggesting that apoptosis involves the activation of a cascade of proteases. This article summarises the presently available evidence and discusses how multiple proteases might be required in the effector phase of cell death.

Key words: Apoptosis; Cysteine proteases; Interleukin-1β converting enzyme; Nedd2/Ich-1; prICE/CPP32/Yama/apopain; TX/ICE$_{rel}$II/Ich-2; ICE$_{rel}$III; Mch2

1. Introduction

More than two decades ago Kerr et al. [1] described the distinctive ultrastructure features of physiological cell death (apoptosis). Apoptosis is now known to play key roles in development and homeostasis, and understanding the molecular mechanisms of cellular suicide has been an area of intense research for the past few years. Using classical genetic methods, several genes that play a role in programmed cell death of the worm *Caenorhabditis elegans* were identified [2]. Two of these, *ced-3* and *ced-4* were shown to be essential for cell death to occur, while *ced-9* negatively regulates the function of *ced-3* and *ced-4* (reviewed in [3]). The Ced-9 protein is homologous to the mammalian Bcl-2 family of apoptosis regulatory proteins [3]. Although no mammalian homologues of Ced-4 have been discovered, the *ced-3* gene product is homologous to a growing list of mammalian proteins similar to interleukin-1β-converting enzyme (ICE), a cysteine protease required for the processing of pro-IL-1β [4–7]. Currently, the ICE family of cysteine proteases includes Nedd2/Ich-1 [8,9], prICE/CPP32/Yama/apopain [10–13], TX/Ich-2/ICE$_{rel}$II [14–16], ICE$_{rel}$III [16] and Mch2 [17].

Apoptosis is an event that requires dismantling of the cellular architecture, therefore the involvement of proteases is not surprising. Even before the discovery of *ced-3* and its mammalian counterparts, it had been shown that a number of cellular proteins including poly(ADP-ribose) polymerase (PARP), lamin B, topoisomerases, protein kinase C β1, cytosolic phospholipase A_2 and histone H1 are degraded during apoptosis [18]. More recently, the product of ademotous polyposis coli gene (APC) [19], terminin antigen [20], U1-70kD [21], fodrin [22] and β-actin [23] have been shown to be cleaved in cells undergoing apoptosis. Cytotoxic T-cells produce a series of serine proteases (granzymes) which are necessary for target cell killing by the induction of apoptosis (reviewed in [24]). Protease inhibitors of different specificities including serine and cysteine protease inhibitors, have been shown to inhibit apoptosis which illustrates that apoptosis may require the activation of several classes of proteases [18, 25–33].

The genetic regulation of apoptosis has been a topic of several recent reviews and the possible role of ICE-like proteases in apoptosis has also been discussed to some extent [34–37]. The aim of this article is examine the recent functional data on the ICE-family of proteins and to speculate how these members may act in alliance with non-ICE-like proteases in the execution of the apoptotic pathway.

2. Multiple mammalian homologues of Ced-3

Among the six mammalian homologues of Ced-3 currently known, ICE is the best studied as it was first identified for its role in the processing of pro-interleukin-1β (pIL-1β) to active IL-1β. The crystal structure showed that the active form of ICE is a $(p20+p10)_2$ tetramer consisting of two heterodimers of the p20 and p10 subunits [38,39]. Earlier suggestions as to a role for ICE in apoptosis came from studies showing that overexpression of ICE results in cell death and that ICE inhibitors such as cowpox virus CrmA [40] can suppress apoptosis induced by various stimuli [7,41–44]. Mice lacking ICE develop normally, but have defective IL-1β processing and Fas-mediated apoptosis [45,46]. Significantly though, ICE (−/−) mice do not show a *lpr* phenotype, characteristic of mice carrying a mutation in the *fas* gene. Clearly, ICE alone is not sufficient to mediate apoptosis.

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Abbreviations: ICE, interleukin-1β converting enzyme; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; DCI, 3,4-dichloroisocoumarin; TLCK, *N*-tosyl-L-lysyl chloromethyl ketone; TAME; *N*-tosyl-L-arginine methyl ester; YVAD-CMK, Tyr-Val-Ala-Asp-chrolomethyl ketone; PARP, poly(ADP-ribose) polymerase; IL-1β, interleukin-1β; pIL-1β, pro-IL-1β.

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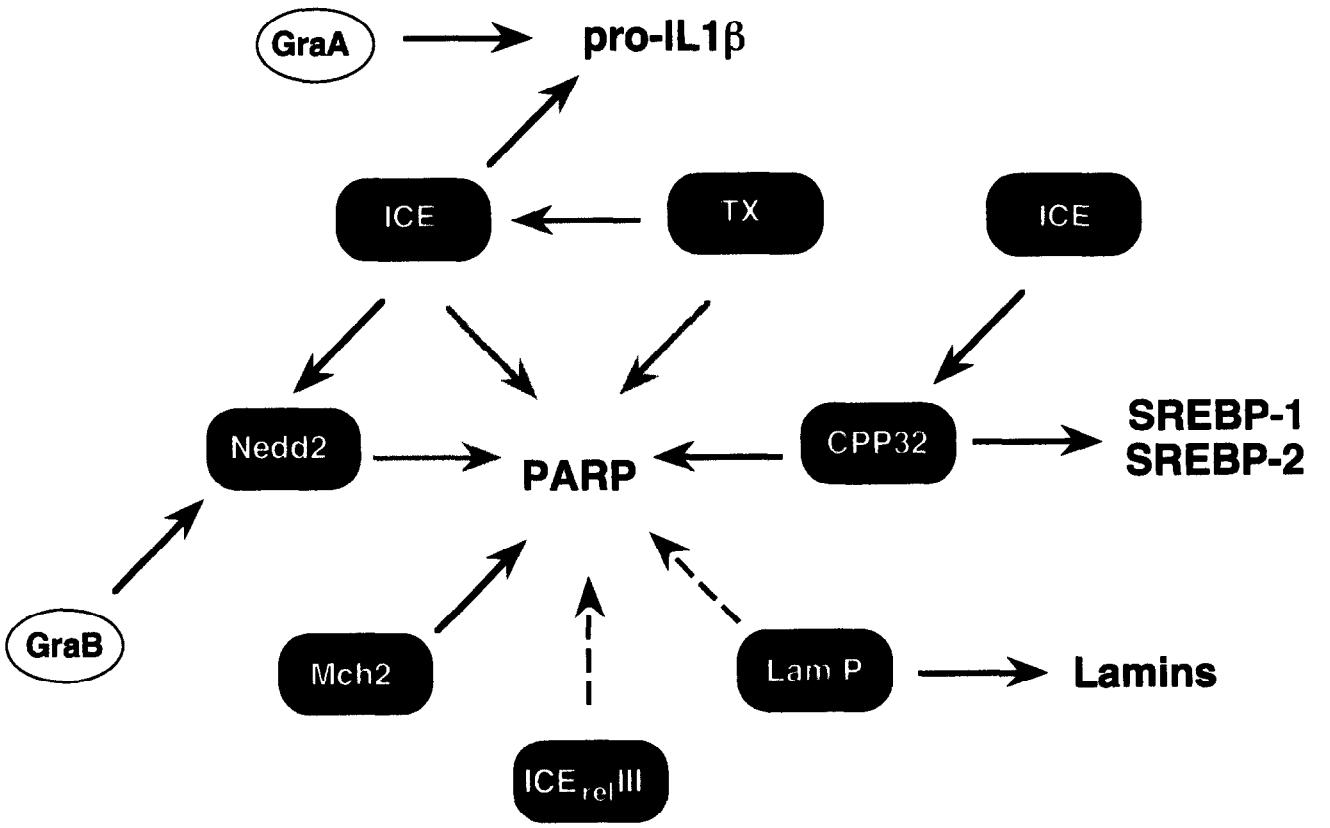

Fig. 1. Known, possible and unknown relationships between various proteases implicated in apoptosis. Proteases in black boxes represent ICE-like proteases. Granzyme A (GraA) and granzyme B (GraB) are cytotoxic T-cell specific proteases. Established (continuous lines) and possible (dashed lines) interactions between various proteases and between proteases and their substrates are shown by arrows. PARP has been placed in the centre because it can be cleaved by several members of the ICE family. The cleavage of PARP by ICE, TX and Nedd2 occurs at relatively high concentrations [48] and this is shown by light-coloured arrows. Since ICE is not expressed in all cell types and ICE mutant mice show normal apoptosis, the function of ICE in cells lacking ICE may be carried out by proteases most similar to ICE, i.e. $ICE_{rel}II$ (TX) and $ICE_{rel}III$. This would imply that one or both of these proteases process pro-Yama/CPP32, pro-Mch2 and pro-Nedd2 (these possible interactions are not shown in the figure).

Overexpression of all ICE-like proteases induces apoptosis in transfected cells. Conversely, the expression of either antisense *Nedd2* or a smaller form of Nedd2 protein (Nedd2s/Ich-1s) partially inhibits cell death [9,47]. prICE (protease resembling ICE) was originally identified as a protease activity responsible for the cleavage of PARP in an in vitro apoptosis system [10]. CPP32 was identified as another ICE homologue [11] that was recently shown to be responsible for the cleavage of PARP and was renamed Yama/apopain [12,13]. It has been suggested that CPP32/Yama/apopain represents the cysteine protease activity originally defined by Lazbenik et al. as prICE in chicken cell extracts [10,12,13], although Mch2 can also cleave PARP [17]. The common feature among known ICE-family members is that cleavage by these proteases requires the presence of an Asp residue in the P_1 position, a property shared by cytotoxic T-cell specific granzyme B that is unique among serine proteases [24]. As such, the residues involved in catalysis and those which make up the Asp binding pocket are conserved in all ICE-like proteins.

3. Non-ICE proteases in apoptosis

ICE-like proteins are not the only proteases implicated in apoptosis. Many of the earlier observations illustrating the involvement of proteases in apoptosis came from studies using protease inhibitors. Several classes of protease inhibitors such as *N*-tosyl-L-lysyl-chloromethylketone (TLCK), *N*-tosyl-L-phenylalanyl-chloromethylketone (TPCK), tosyl-argenine methyl ester (TAME) and 3,4-dichloroisocoumarin (DCI) prevent apoptosis-associated DNA cleavage in various cellular systems [25–33] (Table 1). Some of these protease inhibitors might also inhibit ICE-like proteases directly or their activity may lie in inhibition of proteases upstream or downstream of ICE (see below).

4. Substrate specificities, what is currently known

ICE has the ability to self-process [4]. In addition, ICE can mediate the processing of pro-Yama [12] and pro-Nedd2 (N.L.H., J.A. Trapani and S.K., unpublished). $TX/ICE_{rel}II$ has thus far been the only protease shown to cleave pro-ICE, although neither $TX/ICE_{rel}II$ nor $ICE_{rel}III$ cleave the ICE substrate pIL-1β efficiently [15,16]. Similarly, prICE does not cleave pIL-1β [13]. A recent study shows that ICE, TX and Nedd2 when overexpressed in COS cells can cleave PARP [48]. It is noteworthy that in in vitro experiments 50–100-fold higher concentration of purified ICE was required to cleave PARP than to process pro-IL-1β [48]. In addition to cleaving PARP, CPP32 also cleaves sterol regulatory element-binding proteins SREBP-1 and SREBP-2 [49]; however, the significance of this

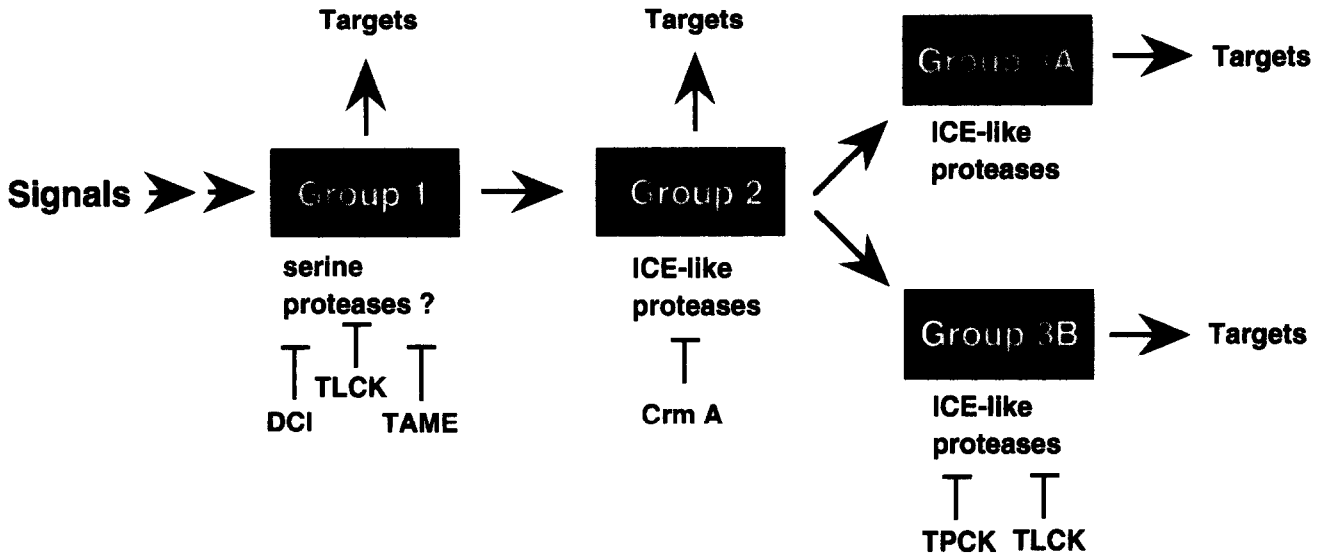

Fig. 2. A hypothetical model showing the role of various groups of proteases in apoptosis. The model is based on the assumption that the execution phase of apoptosis involves serial activation of different classes of proteases. Each group of proteases is represented as a black box. The rationale behind placing the various groups in the order shown is as follows. Proteases in group 1 are probably involved in the activation of ICE-like proteases. This group involves TLCK, DCI and TAME inhibitable proteases, which are known to be required for early apoptotic events (Table 1 and references therein). TLCK, DCI and TAME do not inhibit all ICE-like proteases, but processing of Nedd2 is inhibited by TLCK. TLCK also inhibits PARP cleavage (Table 1). Group 2 proteases include ICE-like proteases capable of activating other downstream ICE-like proteases. This group may include ICE itself (and perhaps its closest homologues $ICE_{rel}II$ and $ICE_{rel}III$), which is strongly inhibited by CrmA. CTL-specific serine protease granzyme B could also be classified as a member of the Group 2. Granzyme B can activate pro-Nedd2 and is inhibited by CrmA [53]. ICE is known to activate pro-Nedd2 and pro-Yama/CPP32, two ICE-like proteases, probably downstream of ICE (Group 3A). Activated group 3A proteases can then cleave nuclear targets such as PARP. Group 2 proteases may also activate member(s) of other group(s) of ICE like proteases such as LamP (Group 3B). The function of LamP is blocked by TLCK and TPCK, but it is not clear whether these inhibitors directly interact with LamP [52]. Group 3B proteases also act on nuclear targets such as lamins. Ac-YVAD-CMK inhibits all events associated with apoptosis, probably by inhibiting several Group 2 and Group 3 proteases (not shown). The cumulative action of the protease cascade culminates in events characteristic of apoptotic cell death.

observation in the context of apoptosis remains obscure. All these new findings indicate that although there is some functional overlap within the ICE family of proteases, certain target specificities may be quite distinct between them. Interestingly, pro-Nedd2 can be processed by purified granzyme B (N.L.H., J.A.T. and S.K., unpublished), a characteristic not shared by

Table 1
The effects of various protease inhibitors on apoptosis

Protease inhibitor	Target proteases	Effect on apoptosis	Ref.
TAME	Serine proteases	Inhibits TNF induced apoptosis and partly inhibits drug induced apoptosis	25, 28, 31, 33
TPCK	Chymotrypsin-like proteases	Inhibits drug induced, Fas induced, TNF induced and γ-irradiation induced apoptosis in a range of cell types. Inhibits proteolysis of PARP in response to cell treatment with drugs or γ-irradiation.	25 27, 29 31, 33
TLCK	Trypsin-like proteases	Inhibits TNF induced and drug induced apoptosis in a range of cell types. Inhibits both proteolysis of PARP and apoptosis in response to cell treatment with drugs or γ-irradiation.	25, 27, 29, 31, 33
DCI	Serine proteases	Inhibits drug induced and Fas mediated apoptosis. Inhibits proteolysis of pIL-1β by granzyme A.	26, 30, 43, 51
YVAD-CHO, YVAD-CMK	ICE-like proteases	Inhibits proteolysis of pIL-1β by ICE and cleavage of PARP induced in apoptotic nuclei. Inhibits Fas-mediated apoptosis.	4, 10, 30, 42, 43
CrmA	ICE-like proteases and Granzyme B	Inhibits proteolysis of pIL-1β by ICE and proteolysis of PARP by prICE/CPP32/Yama. Inhibits apoptosis induced by activation of Fas or TNF receptors, serum or growth factor withdrawal. Inhibits activity of granzyme B.	9, 12, 40, 41–44, 53
Iodo-acetamide	Cysteine proteases	Inhibits cleavage of PARP and morphological changes of apoptosis in isolated nuclei induced by apoptotic cell extracts and apoptosis induced by drug treatment and γ-irradiation in a range of cell types.	10, 13, 27

ICE [50]. This may suggest a role for Nedd2 in cytotoxic T-cell mediated killing of target cells. These recent observations indicate that there is extensive interaction between the members of the Asp specific proteases (Fig. 1). In addition, there is some evidence that the processing of these zymogens to active enzymes may involve proteases other than ICE-like proteins and granzyme B. For instance, the processing of Nedd2 is blocked by TLCK and TPCK, which inhibit trypsin-like and chymotrypsin-like proteases, respectively (N.L.H., J.A.T. and S.K., unpublished). As previously mentioned, the natural substrate of ICE is pIL-1β, which is cleaved to generate active IL-1β. Curiously, granzyme A has been recently shown to process pIL-1β [51] and although the site at which granzyme A cleaves pIL-1β differs from that of ICE, the processed IL-1β is biologically active [51]. This further reinforces the view that there is functional redundancy even between different classes of proteases.

As discussed before, a number of cellular proteins are degraded during apoptosis. It is now becoming clear that some of these targets are cleaved by ICE-like proteases. Nuclear lamins are degraded by an ICE-like protease termed LamP [52]. Since nuclear lamina degradation is a hallmark of the apoptotic nuclei, activation of LamP might be a crucial event in the terminal phase of apoptosis. As yet LamP has not been cloned and it remains to be seen whether it represents an already known member of the ICE-family.

5. Interactions between various ICE-like proteases

Despite substantial achievements in the past year, the role of proteases in apoptosis remains poorly understood. The discovery of multiple ICE-like proteases in mammalian cells has complicated the picture somewhat. Considering that most of the ICE-family members are expressed in all cell types, cell-specific function seems unlikely. Since ICE mutant mice show an apparently normal phenotype (except deficient cytokine processing and to some extent a defect in Fas-mediated apoptosis), ICE is either not required in the apoptosis pathway or there is functional redundancy between members of the ICE family. The closest structural homologues of ICE, TX/ICE$_{rel}$II and ICE$_{rel}$III (and perhaps all other members) do not process pIL-1β efficiently. This means that the only undisputed function of ICE is the processing of pIL-1β. Other ICE-like proteins, Nedd2, CPP32, TX, Mch2 and ICE$_{rel}$III, also induce apoptosis when overexpressed. Since endogenous levels of these proteins are usually low and not induced under apoptotic conditions, the physiological relevance of these experiments is not clear. The most convincing data on the role of these proteases in apoptosis has come from inhibitor studies. Both CrmA and tetrapeptide ICE inhibitors (e.g. Ac-YVAD-CMK) strongly inhibit apoptosis (Table 1). These two inhibitors probably inhibit all ICE-like proteases to a certain extent, therefore one cannot distinguish which of the proteases mediates apoptosis. Yama/CPP32 has been claimed to be the 'true' homologue of Ced-3, but this may be partially because its known downstream target PARP, is cleaved in apoptotic cells [20,26]. Since Mch2 also cleaves PARP [17] and ICE, TX and Nedd2 can mediate PARP cleavage when present in high enough concentrations [48], it is simplistic to assume that mammalian apoptotic pathway utilises a single Ced-3 homologue. Furthermore, purified CPP32 alone is not sufficient to provoke apoptotic changes in the cell-free system indicating that other cytoplasmic components may be required [13].

6. Role of multiple proteases in apoptosis: a model

How might several proteases fit together in the apoptosis pathway? Since the targets of most of the proteases are still unknown, and some of the proteases are yet to be identified, it is not possible to formulate a unified model which would accommodate all the findings; however, some general conclusions can be drawn. One possibility is that several proteases are triggered simultaneously but independently of each other in response to an apoptotic signal and as such no hierarchy between various proteases would exist. As reviewed above, emerging data suggest that some of the ICE-members can be processed by others. Since some of these interactions are essentially unidirectional, e.g. ICE can process Nedd2 and prICE, but the reciprocal does not occur, we favour the model in which various proteases interact with each other in some hierarchical fashion (Fig. 2). Inhibitor studies also suggest that different classes of proteases act at different stages during the process of apoptosis [29,30]. Broadly speaking, the process of apoptosis might involve several groups of proteases including proteases required for the activation of ICE-like proteases (s) and ICE-like protease(s). Within the ICE-family of proteases, there may be two or more sub-groups. In a normal cell, various proteases may reside in discreet cellular compartments, and the role of early acting proteases might be to make it possible for the ICE-like proteases to interact with each other. Once the first group of ICE-like proteases have been activated, they will in turn activate the next sub-groups of proteases which act on various nuclear targets (Fig. 2). Cells undergoing apoptosis also exhibit profound cytoplasmic changes which are probably also mediated by selective proteolysis of certain structural proteins. Therefore, some of these proteases may also target certain proteins in the cytoplasm.

The discovery of ICE-like proteases heralded a significant advancement in the understanding of the biochemical mechanism of apoptosis and it is now evident that apoptosis involves a cascade of proteolytic events. Further elucidation of the proteases and their substrates in the apoptotic cascade will be vital to understanding the execution of cell death.

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