Caspases: killer proteases

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Caspases (cysteinyl aspartate-specific proteinases) mediate highly specific proteolytic cleavage events in dying cells, which collectively manifest the apoptotic phenotype. The key and central role that these enzymes play in a biochemical cell-suicide pathway has been conserved throughout the evolution of multicellular eukaryotes.

APOPTOTIC CELL SUICIDE is a fundamentally important biological process that is required to maintain the integrity and homeostasis of multicellular organisms. Inappropriate apoptosis, however, underlies the aetiology of many of the most intractable of human diseases, including neurodegenerative disorders and cancer[1].

In only the last few years, many of the molecules that participate in a conserved biochemical pathway that mediates the highly ordered process of apoptotic cell suicide have been identified. At the heart of this pathway are a family of cysteine proteases, the 'caspases', that are related to mammalian interleukin-1β converting enzyme (ICE/caspase-1) and to CED-3, the product of a gene that is absolutely necessary for apoptotic suicide in the nematode *Caenorhabditis elegans*.

It appears that the role of these proteases in cell suicide is to disable critical homeostatic and repair processes, as well as to cleave key structural components, resulting in the systematic and orderly disassembly of the dying cell. Rapid advances in our understanding of the biology of cell suicide, and of these proteases in particular, has unveiled exciting new perspectives on the life and death of cells within complex multicellular organisms.

Proteases are at the heart of a conserved cell-death pathway

Caspases were first implicated in apoptosis by genetic analysis in the nematode *C. elegans*. The deletion or mutation of a single gene, *CED-3*, resulted in abolition of all 131 programmed cell deaths that would otherwise occur during hermaphrodite development. The finding that the product of the *CED-3* gene is related to mammalian ICE/caspase-1 strongly suggested that this family of proteases plays a critical role in the biochemical events governing apoptosis in both nematodes and mammals[2–5]. This hypothesis has been substantiated by several observations.

First, specific members of the caspase family have been identified as the enzymes responsible for the proteolysis of key proteins that are known to be selectively cleaved at the onset of apoptosis[6]. Second, potent peptide-based and macromolecular inhibitors of these proteases prevent apoptosis *in vitro*, in whole cells and in *in vivo* models of development and stroke. Third, post-translational activation of several caspases has been shown to correlate with and be necessary for the appearance of the apoptotic phenotype. Finally, and most compelling, mice deficient in caspase-3 (CPP32, apopain, Yama) have a striking defect in the extensive apoptosis that occurs during brain development.

Taken together, these results constitute overwhelming evidence that members of this family play a critical role in mammalian apoptosis, as CED-3 does in nematodes, and that this component of the cell-death pathway has been conserved throughout evolution.

ICE as a caspase prototype

ICE/caspase-1 is the cysteine protease responsible for the proteolytic conversion of the 31 kDa inactive cytokine precursor, pro-interleukin 1β (prolL-1β), to its 17.5 kDa active form[2,3,7,8]. As a consequence, this enzyme has been the subject of intense investigation as an attractive target for the treatment of IL-1-driven inflammatory diseases such as rheumatoid arthritis. More recently, caspase-1 has also been implicated in the proteolytic maturation of interferon-γ inducing factor (IGIF), an 18 kDa cytokine that stimulates T-cell production of interferon-γ[9,10]. So far, many of the paradigms that have been established for caspase-1 also hold true for its homologues (including CED-3) with the notable exception of substrate specificity and consequent physiological role.

Caspase-1 cleaves prolL-1β at Asp116–Ala117 to generate the mature, biologically active form of this cytokine. The most distinctive catalytic feature of this protease is a near absolute requirement for Asp in the substrate P_1 position. Substitution of this residue in macromolecular and peptide substrates results in >100-fold decrease in k_{cat}/K_m. The enzyme has an equally stringent requirement for four amino acids amino-terminal to the cleavage site. By contrast, there do not appear to be any functionally important specificity subsites on the carboxy-terminal side of the scissile bond, typical of other cysteine proteases. In caspase-1, hydrophobic amino acids are preferred in the P_4 position, although other amino acids are also reasonably well tolerated.

Caspase-1 is synthesized as a dormant 45 kDa proenzyme that resides in the cell cytoplasm, and is proteolytically activated by an unknown mechanism in response to various inflammatory challenges. The active form is composed of two subunits, one of 20 kDa and one of 10 kDa, both of which are derived from the 45 kDa proenzyme by excision at Asp–x sites. Because cleavage after a P_1 Asp is a molecular fingerprint for the involvement of caspases, it is possible that caspase-1 is either autolytically activated or that other family members are responsible.

The caspase family of cysteine proteases

When ICE/caspase-1 was first purified, and its cDNA cloned and sequenced in 1992, it was found to be unrelated to any known protein[2,3]. The finding that it was similar to CED-3 prompted an intense search for other mammalian homologs and, to date, ten proteases of human origin have been identified (Fig. 1a)[11–31].

The unified nomenclature for these proteases that has recently been adopted[11], using the term 'caspase' as a root for serial names, embodies two distinguishing features of this enzyme family: they are cysteine proteases and they are specific for cleavage after Asp residues. A phylogenetic analysis of the caspases suggests that these enzymes may

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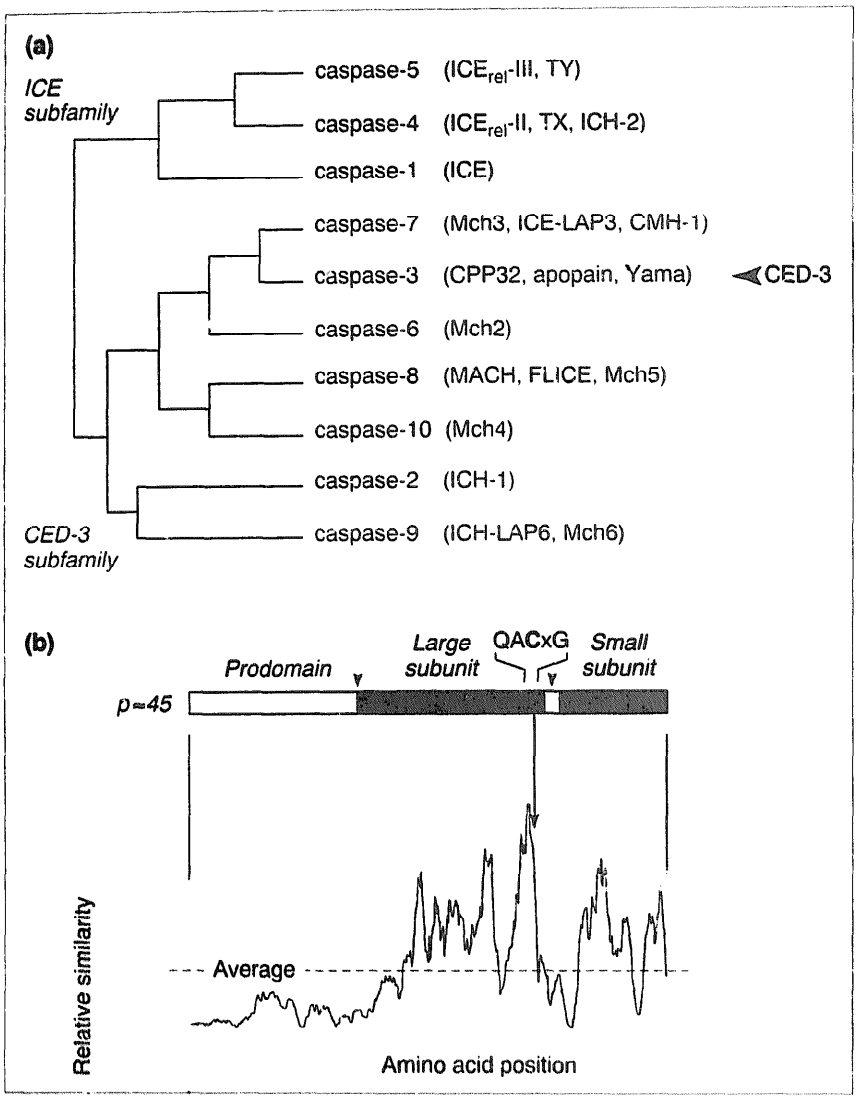

Figure 1

(a) The caspase family of ICE/CED-3-related cysteine proteases. Aliases for the proteases are shown in parentheses. (Phylogenic relationships were determined using the PILEUP algorithm of the Wisconsin GCG sequence analysis package.) Known members of the caspase family include *Caenorhabditis elegans* CED-3 and ten enzymes of human origin. Counterparts in other species (e.g. murine, guinea pig and *Drosophila melanogaster*) have also been reported (not shown). The proteins can be tentatively grouped into two subfamilies, based on their sequence homologies to ICE and CED-3. Functional subdivisions within the CED-3 subfamily also appear to exist. *C. elegans* CED-3 is most related to human caspase-3 (arrow). **(b)** All family members are synthesized as proenzymes that are processed at Asp–x sites (indicated by arrows) to generate the heterodimeric mature enzymes. Sequence conservation is high in regions contributing to substrate binding and catalysis (e.g. the absolutely conserved QACxG pentapeptide that contains the catalytic Cys) whereas prodomains are largely unrelated.

be grouped into two, possibly three, subfamilies. Accumulating evidence indicates that members of the ICE subfamily (caspases-1, -4 and -5) predominantly play a role in inflammation whereas members of the CED-3 subfamily (caspases-2, -3 and -10) are largely (if not exclusively) involved in apoptosis.

All of the known caspases appear to have similar proenzyme organization (Fig. 1b). Most of the currently known members of the caspase family were identified by molecular cloning, but in two cases (caspase-1 and caspase-3) the mature active enzymes have also been purified from cultured human cells by chromatographic techniques[2,15]. Their proenzyme organizations have been deduced from their cDNA sequences, amino-terminal peptide sequences and their precise molecular masses. Caspase-1 and -3 proenzymes are highly similar except that the prodomain of caspase-3 is much smaller than that in caspase-1 (27 vs. 119 residues, respectively) and the two subunits that make up the active form of caspase-3 (p17 and p12) are not separated in the proenzyme by a linker peptide as their counterparts are in caspase-1 (p20 and p10). In both cases, however, all maturation sites are at Asp–x motifs.

Overall this structural information has provided a reasonable foundation for predicting the equivalent maturation sites within the proenzymes of other caspase family members.

Structure and catalytic mechanism

The three-dimensional crystal structures of the mature forms of caspase-1 (ICE)[32,33] and caspase-3 (CPP32, apopain, Yama)[34] in complex with tetrapeptide inhibitors have been determined, resulting in the identification of the amino acids important in binding and catalysis, and securing the relationship of these enzymes to their homologues.

A comparison of the primary sequences of these proteins shows that all of the residues implicated in catalysis, in stabilization of the oxyanion intermediates, and in recognition of the P_1 Asp, are conserved. Thus, it is clear that a cysteine protease mechanism and a stringent requirement for Asp in the P_1 position of substrates are common to all family members. By contrast, those residues that are implicated in binding the P_4 residue vary substantially, and appear to be key determinants of the distinct substrate preferences of the various caspase isoforms. For example caspase-1 prefers hydrophobic amino acids in P_4, in both macromolecular and peptide-based substrates. Conversely, the consensus sequence for cleavage of macromolecular and peptide substrates by caspase-3 is DxxD (a motif found at the cleavage site of many proteins cleaved during apoptosis), consistent with a caspase-3 requirement for Asp in P_4. The structural basis for these distinct specificities are evident in a comparison of their three-dimensional structures.

The tertiary and quaternary structure of caspases-1 and -3 are remarkably similar, at least superficially (Fig. 2a,b). Both structures show the large and small subunits intimately associated, with each contributing crucial residues to the active site to form a single, heterodimeric catalytic domain. In both crystals, two heterodimers associate to form a tetramer, and there is some evidence that this is the catalytically active form of the enzyme in solution.

Several details of the active site are also highly conserved between these two

proteases. First, the catalytic machinery involves a diad composed of a cysteine sulphydryl group (Cys285) in close proximity to a histidine imidazole group (His237), both of which reside on the larger subunit. Second, in analogy with other cysteine proteases, these enzymes appear to stabilize the oxyanion of the tetrahedral transition state through hydrogen-bonding interactions with the backbone amide protons of Cys285 and Gly238. Finally, four residues, two from each subunit, appear to be involved in stabilization of the P_1 Asp of substrates (Arg179, Gln283, Arg341, Ser347). In both structures, there are less-extensive contacts with P_2 and P_3 amino acids.

Although, at a glance, the caspase-1 and -3 structures are nearly indistinguishable, a close examination reveals several subtle but important differences that have profound biological consequences. The most significant is the presence of a surface loop in caspase-3 (corresponding to residues Phe380–Phe389), which does not have a counterpart in caspase-1 (Fig. 2c,d). This loop forms one side of the S_4 subsite, and it is now clear that the distinct specificities of these two enzymes derive almost entirely from differences in this subsite. In caspase-1, S_4 is a large and shallow hydrophobic depression on the surface of the enzyme that easily accommodates a tyrosyl sidechain. In caspase-3, this subsite is significantly smaller and is analogous to a highly constrained socket into which the carboxylate sidechain of the P_4 Asp snugly fits. It is clear that a hydrophobic amino acid such as Tyr could not be accommodated in this site. In addition, there is an extensive network of hydrogen bonds that serve to stabilize the P_4 Asp. The geometry and the chemical nature of this subsite explain this enzyme's strict requirement for Asp in the P_4 position of substrates and inhibitors, a prediction supported by the finding that all known substrates for caspase-3 are cleaved at the sequence DxxD. Several of the features responsible for the distinct specificities of caspases-1 and -3 are conserved among closely related homologs (Fig. 2e) suggesting that some of these enzymes have similar substrate preferences.

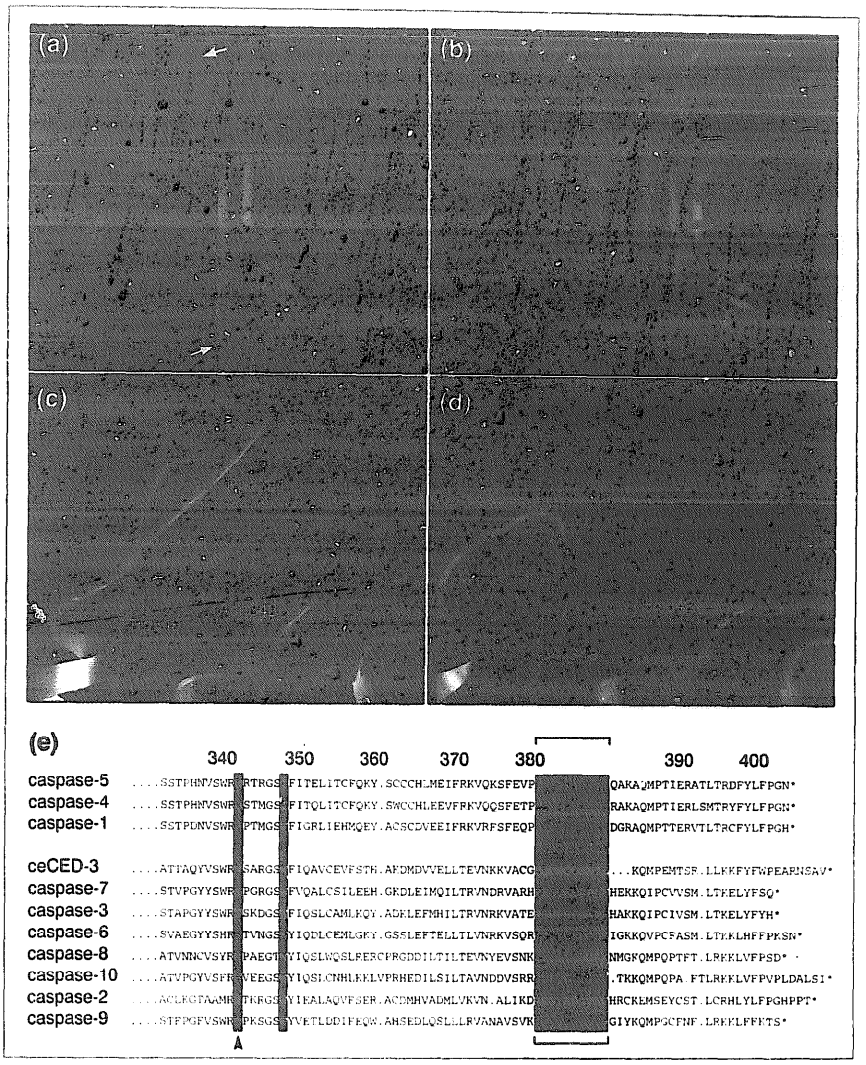

Figure 2

X-ray crystal structures of **(a)** caspase-3 and **(b)** caspase-1. The tetramers are composed of two heterodimers (blue/red and green/pink), which, in turn, are each composed of a large and small subunit. Within each heterodimer, the two subunits are intimately associated to form a single catalytic domain in which both contribute key residues to the active site. The two structures are shown in complex with appropriate tetrapeptide aldehyde inhibitors (shown in yellow) bound in the active site. The most obvious difference between the two structures is the presence of a loop in caspase-3 (indicated with arrow) that does not have a counterpart in caspase-1. Details of the S_4 subsite of **(c)** caspase-3 and **(d)** caspase-1. S_4 is the most crucial determinant of specificity between the two enzymes. In caspase-3, S_4 is a narrow pocket whose geometry is determined by Trp348 and the amino acids that comprise the loop. This subsite contains several residues appropriately positioned to hydrogen-bond with the P_4 Asp found in endogenous substrates of this enzyme (Asn342, Phe381B). By contrast, S_4 in caspase-1 is a shallow, hydrophobic depression that easily accommodates the hydrophobic amino acids found in the P_4 position of substrates for this enzyme. **(e)** Alignment of S_4 specificity determining region of caspases. A comparison of the sequences in this region suggests that the three features responsible for the near absolute specificity of caspase-3 for Asp in S_4 [loop (green), Trp348 (red), hydrogen-bond donor (blue) in the position of Asn342] are conserved among closely related homologs. Numbering corresponds to caspase-1 primary sequence.

This has recently been confirmed: a positional scanning substrate combinatorial library was used to determine the tetrapeptide specificities of nine of the ten known caspases and *C. elegans* CED-3 (Refs 35, 36). The results divide these enzymes into three distinct groups (Table I). Caspases-1, -4 and -5 (group I) all prefer the tetrapeptide sequence WEHD. By contrast, the optimal peptide recognition motif for caspases-2, -3, -7 and CED-3 (group II) is DExD. Caspases-6, -8 and -9 (group III) prefer the sequence (I/L/V)ExD. Within each group, the amino acid preferences are remarkably similar, in some cases identical, strongly suggesting that at least some of these enzymes have redundant functions.

Inhibitors

Several classes of reversible and irreversible inhibitors have been described for cysteine proteases, and these strategies have been applied to develop highly potent inhibitors for several caspase family members[37]. Reversible inhibitors include aldehydes, ketones and nitriles. Irreversible inhibitors are of the

Table I. Specificities and proposed biological functions for caspases

Specificity group[a]	P_4–P_1 specificity[b]	Consensus	Proposed role
Group I			
Caspase-1 (ICE)	WEHD		
Caspase-4 (ICE_{rel}-II, TX, ICH-2)	(W/L)EHD	WEHD	Maturation of multiple pro-inflammatory cytokine
Caspase-5 (ICE_{rel}-III, TY)	(W/L)EHD		
Group II			
Caenorhabditis elegans CED-3	DETD		
Caspase-3 (CPP32, apopain, Yama)	DEVD	DExD	Cleavage of DxxD apoptotic substrates
Caspase-7 (Mch3, ICE-LAP3, CMH-1)	DEVD		
Caspase-2 (ICH-1)	DEHD		
Group III			
Caspase-6 (Mch2)	VEHD		Activation of group-II cespases, activation of other group-III caspases, cleavage of non-DxxD apoptotic structures
Caspase-8 (MACH, FLICE, Mch5)	LETD	(IVL)ExD	
Caspase-9 (ICE-LAP6, Mch6)	LEHD		
Granzyme B	IEPD		

[a]An intimate understanding of the tetrapeptide specificities of the caspases was obtained using a combinatorial approach[35,36]. The results divide these enzymes into three major groups, and provide insights into their biological roles in inflammation and apoptosis. The three groups can be largely distinguished by their P_4 preferences, a crucial determinant in caspase specificity. Group-I enzymes prefer bulky hydrophobic residues at P_4, group-II enzymes have a strict requirement for Asp at P_4 and group-III enzymes prefer branched-chain aliphatic amino acids in this position.
[b]x represents any amino acid.

general structure peptide-CO-CH_2-X, where X is a halide ion (e.g. chloromethylketones, fluoromethylketones), -N_2 (diazomethylketones), -OCOR [(acyloxy) methylketones], or -OR [a-(pyrazoloxy) methylketones and (phosphinyloxy) methylketones]. The most potent in these classes contain a peptide-recognition element corresponding to that found in endogenous substrates. For example, the tetrapeptide aldehyde similar to the cleavage site in proIL-1β, Ac-YVAD-CHO, is a highly potent caspase-1 inhibitor (K_i = 0.76 nM). Similarly, Ac-DEVD-CHO, whose peptide component is identical to the P_4–P_1 sites in at least two endogenous caspase-3 substrates, is an excellent inhibitor of this enzyme (K_i = 0.35 nM). Not surprisingly, Ac-YVAD-CHO is a poor inhibitor of caspase-3 (K_i = 10 μM), reflecting the constrained geometry of the S_4 subsite in this enzyme. Conversely, Ac-DEVD-CHO is a reasonable caspase-1 inhibitor (K_i = 17 nM), presumably because of the large size of S_4 in this family member.

Three naturally occurring protein inhibitors have also been described. Cytokine response modifier A (CrmA) is a 38 kDa serpin from cowpox virus that appears to facilitate viral infection through both inhibition of the host inflammatory response, and inhibition of apoptosis[38]. CrmA has been evaluated as an inhibitor of both caspase-1 and caspase-3, and found to exhibit very different potencies against the two enzymes. It is a potent inhibitor of caspase-1 (K_i = 4 pM, $k_{on} = 1.7 \times 10^7\ M^{-1}\ s^{-1}$) and a weak caspase-3 inhibitor (K_i > 100 nM, $k_{on} = 1 \times 10^4\ M^{-1}\ s^{-1}$) (Ref. 15).

The poor inhibition observed with caspase-3 can be explained by the stringent specificity of this enzyme for Asp in S_4, as CrmA requires recognition at the sequence LVAD for inhibition. Recently, this serpin has been shown to be a potent inhibitor of caspases other than caspase-1, including caspase-8 (MACH, FLICE, Mch5)[39], and it is likely that this is the mechanism by which CrmA prevents Fas-mediated apoptosis.

Another viral gene product, a 35-kDa protein from baculovirus (p35), also appears to attenuate apoptosis through inhibition of the caspases[40,41]. Synthesis of p35 has been shown to prevent cell death in insect cells, in *C. elegans* and in mammalian systems. It has been shown to be an irreversible inhibitor of several family members, including caspases 1–4 and CED-3. The IAP (inhibitor of apoptosis) gene family comprise a third group of polypeptides that also prevent cell death in a variety of species. One family member, XIAP, has been shown to be a direct inhibitor of group-II caspases (e.g. caspase-3 and -7), with K_i values < 1 nM, but a poor inhibitor of group I and III caspases (e.g. caspase-1, -6 and -8)[42]. Unlike viral CrmA and p35, the IAPs are endogenous mammalian cell-death suppressors (although they also have viral counterparts) and their importance has been demonstrated in the human disease, spinal muscular atrophy, where mutations occur in the neuronal IAP gene (NAIP)[43]. The ability of this protein to inhibit cell death induced by a variety of stimuli in phylogenetically diverse organisms is further evidence for an evolutionarily conserved role for the caspases in a conserved cell-death pathway.

Biological roles of caspases: caspase knockouts

In the nematode *C. elegans*, a single caspase has been identified (CED-3) and its central importance to the apoptotic machinery has been well-established by the abolition of developmental and stress-induced apoptosis in worms lacking CED-3 or harboring non-functional CED-3 mutants[4]. Multiple caspases (at least three) have been identified in *Drosophila*[44,45], and one of these gene products has been shown to be necessary for normal development (DCP-1).

In mammals, four knockout mice have been generated (caspases 1–3 and mICH-3) and these animals have helped define the role of individual caspase family members *in vivo*. As predicted, caspase-1-deficient mice cannot generate mature IL-1β, but, surprisingly, do not produce IL-1α either (the biological basis of this phenomenon is not understood)[7,8]. They also have substantially reduced levels of serum IGIF and interferon-γ, presumably because of reduced caspase-1-dependent maturation of IGIF[9,10]. These animals are resistant to lipopolysaccharide-induced endotoxic shock, and they substantiate the importance of caspase-1 in inflammatory disorders such as rheumatoid arthritis.

Caspase-1, however, does not appear to play an important non-redundant role in apoptosis, because these mice develop normally and the *ex vivo* response of cells to various apoptotic stimuli is indistinguishable from cells derived from wild-type animals. One possible exception is a subtle resistance to apoptosis induced by Fas ligation in *ex vivo* thymocytes; however, the lack of an *in vivo* phenotype indicating apoptotic failure, such as lymphoproliferative or autoimmune disorder, does not support a substantial role for caspase-1 itself in apoptosis.

The most striking apoptotic phenotype reported to date is in caspase-3-knockout mice[46]. Abnormalities are primarily confined to failed neural apoptosis (predominantly neurons), leading to substantial alterations in brain structures. The overall brain mass in these mice was markedly larger than that of wild-type mice and exhibited a variety of hyperplasias, ectopic cell masses (e.g. in the

Table II. Proteolytic substrates for caspases during apoptosis[a]

Site[b]		Cleaved protein	Proposed role	Predicted consequence of cleavage	Refs
↓					
DEVD G	Caspase-3	PARP	DNA repair (stress)	Disable DNA repair	c
DEVD N		DNA-PKcs	DNA repair (ds)	Disable DNA repair	d
DGPD G		U1-70K snRNP	pre-mRNA splicing	Reduce productive transcripts	e
DxxD x		hnRNP-C	pre-mRNA splicing	Reduce productive transcripts	f
DEPD S		SREBP (+)	Sterol biosynthesis	Elevate sterols (engulfment)	g
DELD S		D4-GDI	Sustain Rho-GTPase	Cytoskeletal disassembly	h
DxxD x		Huntingtin	(Unknown, essential)	Metabolic distress	i
DETD S		DFF-45 site I (+)	DNA fragmentation	Dismantle genome	j
DAVD T		DFF-45 site II (+)			
DEVD G		DNA-RC C140	DNA replication	Halt DNA replication	k
DMQD N		PKC δ (+)	Cell-cycle progression	G2–M arrest	l
DSID S		Rb	Cell-cycle progression	G1 arrest	m
DVPD C		HDM2/MDM2	p53 modulation	p53 nuclear entry	n
DQTD S		FAK	Regulate cell adhesion	Cell detachment/migration	o
DSLD L		NuMA	Nuclear structure integrity	Nuclear disassembly	p
xxxD x		Pro-caspase (+)		Protease activation	
DMQD N		α-Fodrin	Cortical cytoskeleton	Disassembly	q
ELPD G		Actin	Cytoskeletal microfilaments	Disassembly	r
SRVD G		Gas2	Cytoskeletal microfilaments	Disassembly	s
VEID N	Caspase-6	Lamins	Nuclear envelope mesh	Disassembly	t
P_4 P_1					

[a]Proteins that are currently believed to be cleaved by caspases during apoptosis are listed along with their normal cellular function and predicted or demonstrated contribution to the apoptotic phenotype.

[b]Cleavage sites, indicated with the arrow, have been determined by protein sequencing or mutational analysis (except for NuMA and α-fodrin, which are sites corresponding to the molecular mass of cleavage products). The specificity determining P_4 residue and the essential P_1 Asp are in red. Proteins that have been validated as caspase-3 substrates are indicated and similar evidence exists for the role of caspase-6 in lamin cleavage.

[c]Kaufmann, S. H. *et al.* (1993) *Cancer Res.* 53, 3976–3985, Lazebnik, Y. A. *et al.* (1994) *Nature* 371, 346–347; [d]Casciola-Rosen, L. A., Anhalt, G. J. and Rosen, A. (1995) *J. Exp. Med.* 182, 1625–1634, Casciola-Rosen, L. A. *et al.* (1996) *J. Exp. Med.* 183, 1957–1964, Teraoka, H. *et al.* (1996) *FEBS Lett.* 393, 1–6, Le Romancer, M. *et al.* (1996) *J. Cell Sci.* 109, 3121–3127, Song, Q. *et al.* (1996) *EMBO J.* 15, 3238–3246; [e]Casciola-Rosen, L. A. *et al.* (1994) *J. Biol. Chem.* 269, 30757–30760; [f]Waterhouse, N. *et al.* (1996) *J. Biol. Chem.* 271, 29335–29341; [g]Wang, X. *et al.* (1995) *J. Biol. Chem.* 270, 18044–18050, Wang, X. *et al.* (1996) *EMBO J.* 15, 1012–1020; [h]Na, S. *et al.* (1996) *J. Biol. Chem.* 271, 11209–11213; [i]Goldberg, Y. P. *et al.* (1996) *Nat. Genet.* 13, 442–449; [j]Liu, X. *et al.* (1997) *Cell* 89, 175–184; [k]Ubeda, M. and Habener, J. F. *Proc. J. Biol. Chem.* (in press), Song, Q. *et al.* (1997) *Biochem. Biophys. Res. Commun.* 233, 343–348; [l]Emoto, Y. *et al.* (1995) *EMBO J.* 14, 6148–6156, Ghayur, T. *et al.* (1996) *J. Exp. Med.* 184, 2399–2404; [m]An, B. and Dou, Q. P. (1996) *Cancer Res.* 56, 438–442, Jänicke, R. U. *et al.* (1996) *EMBO J.* 15, 6969–6978, Chen, W-D. *et al.* (1997) *Oncogene* 14, 1243–1248, Tan, X. *et al.* (1997) *J. Biol. Chem.* 272, 9613–9616; [n]Chen, L. *et al.* (1996) *Proc. Am. Assoc. Cancer Res.* A-25; [o]Crouch, D. H., Fincham, V. J. and Frame, M. C. (1996) *Oncogene* 12, 2689–2696; [p]Hsu, H-L. and Yeh, N-H. (1996) *J. Cell Sci.* 109, 277–288, Casiano, C. A. *et al.* (1996) *J. Exp. Med.* 184, 765–770; [q]Martin, S. *et al.* (199?) *J. Biol. Chem.* 270, 6425–6428, Vanags, D. M. *et al.* (1996) *J. Biol. Chem.* 271, 31075–31085, Cryns, V. L. *et al.* (1996) *J. Biol. Chem.* 271, 31277–31282; [r]Mashima, T. *et al.* (1995) *Biochem. Biophys. Res. Commun.* 217, 1185–1192, Chen, Z. *et al.* (1996) *Cancer Res.* 56, 5224–5229, Mashima, T. *et al.* (1997) *Oncogene* 14, 1007–1012, Song, Q. *et al.* (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 157–162, Brown, S. B., Bailey, K. and Savill, J. (1997) *Biochem. J.* 323, 233–237; [s]Brancolini, C., Benedetti, M. and Schneider, C. (1995) *EMBO J.* 14, 5179–5190; [t]Lazebnik, Y. A. *et al.* (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 9042–9046, Orth, K. *et al.* (1996) *J. Biol. Chem.* 271, 16443–16446, Takahashi, A. *et al.* (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 8395–8400, Rao, L., Perez, D. and White, E. (1996) *J. Cell Biol.* 135, 1441–1455.

cortex, cerebellum, striatum and hippocampus), disorganized cell deployment and duplicated brain structures (e.g. in the cortex and optic stalk). The entire phenotype, including the lack of pycknotic cell clusters in regions of the developing brain where neurogenic cell death is usually prominent (e.g. along ventricles, the optic stalk and spinal cord), is consistent with failed developmental apoptosis.

Strikingly, these defects do not extend to other tissues and organ systems. For example, lymphocytes from caspase-3-deficient mice are indistinguishable from wild-type mice in their *ex vivo* responsiveness to multiple and diverse apoptotic stimuli, and a caspase-3-like proteolytic activity is present in these cells. Similarly, other major organs, including heart, lung, liver, kidney, spleen and testis, are largely unaffected in these mice. These results suggest that caspase-3 plays a dominant, non-redundant role in neurogenic apoptosis, whereas the effector function of caspase-3 in the apoptosis that occurs in other cell types and organ systems is sufficiently supported by other caspase isoforms.

Collectively, the four caspase-knockout mice demonstrate both redundancy and non-redundancy in the biological processes attributable to these enzymes and, furthermore, indicate that individual members of the caspase superfamily function in a tissue selective manner.

Proteolytic victims of caspases during apoptosis

Although the morphological changes that occur in cells undergoing apoptotic suicide are striking, there is not the wholesale proteolysis of cellular constituents that the gross ultrastructural changes might suggest. Instead, a discrete number of specific proteins appear to be targeted for proteolytic cleavage once the cell-death pathway has been initiated[47–49]. Several of these proteolytic 'victims' have been identified and in each case the contribution that their cleavage makes to the orderly process of cell suicide is logical (Table II). In every case, proteolysis occurs after an Asp residue, and there is compelling evidence that one or more caspases are involved.

Polypeptides known to be cleaved during apoptosis include enzymes involved in genome function, such as poly(ADP-ribose) polymerase, the 460-kDa catalytic subunit of DNA-dependent protein kinase, the 70-kDa protein component of the U1 small ribonucleoprotein, heteronuclear ribonucleoproteins C, and the 140-kDa component of the DNA replication complex C. Regulators of cell-cycle

progression, including the retinoblastoma gene product and the δ-isoform of protein kinase C, are also cleaved during apoptotic cell death. The mouse (or human) double minute 2 protein, which binds to and retains p53 in the cytoplasm, is cleaved, which may allow p53 entry into the nucleus, p53-dependent arrest at G1 and the transcriptional activation of pro-apoptotic genes such as Bax.

Structural proteins of both the nucleus and cytoskeleton that are cleaved include the lamins, actin, Gas2 and α-fodrin/non-erythroid spectrin. Nuclear and cytoskeletal disassembly during apoptosis is also facilitated, at least in part, by the cleavage of NuMA (nuclear mitotic associated protein) and D4 G-protein dissociation inhibitor. DNA fragmentation is triggered by the activation of DFF (DNA fragmentation factor), which occurs after caspase-mediated proteolysis of the 45-kDa component of the heterodimeric DFF complex.

Cells that are embedded in organized tissue structures need to be detached and this appears to be facilitated in part by the cleavage of the 125-kDa focal adhesion kinase (FAK) and the adenomatous polyposis coli (APC) gene product. (In the latter case, APC cleavage during apoptosis has been demonstrated, but the involvement of a caspase has not been examined.)

Finally, sterol accumulation at the plasma membrane and the asymmetric redistribution of phosphatidylserine into the outer leaflet of the plasma membrane are putative engulfment markers that identify dying cells and their apoptotic corpses to phagocytes. Sterol regulatory element-binding proteins (SREBP-1 and -2) become activated by caspase-mediated cleavage during apoptosis, and although a specific cleavage event has not been associated with phosphatidylserine redistribution, caspase inhibitors have been shown to block it.

The net effect of these and other as-yet-unidentified cleavage events are to: (1) halt cell-cycle progression; (2) disable homeostatic and repair mechanisms; (3) initiate the detachment of the cell from its surrounding tissue structures; (4) disassemble structural components; and (5) mark the dying cell for engulfment by other cells such as macrophages.

It is unlikely that cleavage of any single protein is a necessary requirement for cell death. This has been demonstrated, for example, in the subtle phenotype of PARP-deficient mice. Instead, the collective effect of multiple cleavage events – but of a limited number of key proteins – culminates in the highly ordered and systematic process of apoptotic death.

Despite the apparent specificity of cleavage events that occur during apoptosis, it is also possible that some proteolytic victims are 'innocent bystanders' and are of no substantial importance to the cell-death process. Nevertheless, it is clear that most known cleavage events disable important cellular processes or break down structural components of the cell, and there is also emerging evidence for some cleavage products taking an active role in the cell-death process as well (DFF, SREBP and PKC δ, for example).

Specific caspase inhibitors have been shown to prevent the cleavage of several of these proteins in apoptotic cell cytosols and in whole-cell models of apoptosis, providing compelling evidence that caspases are involved in these events. Although, in most cases, the responsible enzyme(s) has not been firmly established, a comparison of the cleavage-site sequences (Table II) with their known peptide specificity (Table I) suggests the following.

First, the specificity of all of the group-II caspases, included *C. elegans* CED-3, implies that all of these enzymes are reponsible for proteolysis of the proteins that are cleaved at DxxD sites during the effector phase of apoptosis. This conclusion is supported by several independent lines of evidence implicating caspase-3, in particular, in these cleavage events. For example, purification of the activities responsible for specific PARP and SREBP cleavage resulted in the isolation of caspase-3. Other group-II caspases may be tissue-specific isoforms, or may have redundant functions within the same cell type, consistent with the phenotype of caspase-3 null mice.

Second, the specificities of group-III caspases resemble activation sites within several caspase proenzymes, implicating these enzymes as upstream components in a proteolytic cascade that serves to amplify the death signal (see below). Their specificities, however, are also consistent with cleavage of the lamins, suggesting that these group members may have a broad role in apoptosis. The specificity of group-I caspases, while consistent with their role in cleavage of pro-inflammatory cytokines, does not support a role for these enzymes in cell death, in that hydrophobic amino acids (such as Trp or Tyr) are not observed in the P_4 position of proteins so far known to be cleaved during this process.

An unusual caspase-mediated cleavage event appears to contribute to the pathogenesis of Huntington's disease (HD)[49]. Selective, genetically-determined neuronal cell death is a recognized feature of HD. The mutation underlying the disease is an expansion of a CAG trinucleotide repeat located in the 5′ region of a novel gene (*Hdh*), which encodes an essential protein (Huntingtin; 350 kDa) of unknown function. The CAG expansion results in an extended polyglutamine stretch at the amino terminus of the polypeptide, which manifests the disorder when it exceeds 35 residues. Downstream of the polyglutamine stretch lies a remarkable cluster of five DxxD motifs, which are cleaved by caspase-3 both *in vitro* and in apoptotic cells. The length of the polyglutamine stretch directly affects the susceptibility of the Huntingtin protein to caspase-3 cleavage at this cluster. The resulting amino-terminal fragment, containing the expanded polyglutamine stretch, is cytotoxic and itself provokes apoptosis. A likely scenario is that a vicious cycle evolves in susceptible neurons when polyglutamine expansion leads to elevated susceptibility of Huntingtin to caspase-3 cleavage and generation of a cytotoxic amino-terminal fragment. The accumulation of this fragment likely provokes further caspase-3 activation, which may result in more Huntingtin cleavage and accelerated neuronal apoptosis.

Caspase proenzyme activation

The regulation of caspase activity predominantly occurs at the level of proenzyme processing and maturation. Dormant caspase proenzymes are converted to catalytically competent heterodimeric proteases by cleavage at Asp–x bonds. Available evidence suggests that caspase activation *in vivo* occurs by at least two distinct mechanisms.

The results of several investigations suggest that at least some of the caspases are downstream components in a proteolytic cascade, and are activated by either other caspases or by proteases with a similar specificity. Much of this evidence has been obtained in *in vitro* studies of caspase activation, where various mature caspases have been shown to correctly process other caspase proenzymes. Consistent with this, the proteolytic specificity of group-III caspases [(IVL)ExD] closely matches the activation sites found between the large and small subunits of several caspase proenzymes[36]. The serine protease granzyme B has also been shown to catalyse the activation of several effector caspases *in vitro*, leading to speculation that this

is one of its primary roles in cytotoxic lymphocyte-mediated cell death[50,51]. Further evidence for a proteolytic cascade has been obtained using both peptide-based and macromolecular inhibitors of caspases, which have been shown to prevent the proteolytic activation of both caspase-3 and caspase-7.

There is also accumulating evidence to suggest that some caspases are activated instead by intermolecular autoproteolysis. First, in heterologous expression systems, recombinant caspase proenzymes are autolytically processed to their mature forms when synthesized at sufficiently high levels, and mutation of the catalytic cysteine prevents this processing. Second, isolated caspase proenzymes can be autoactivated when concentrated by ultrafiltration.

The most compelling evidence that intermolecular autoproteolysis occurs *in vivo* has been obtained in the Fas (Apo-1, CD95) model of apoptosis[25,26]. In this system, Fas–receptor ligation results in formation of a signaling complex that includes the receptor, the FADD/MORT1 adapter protein and the caspase-8 proenzyme. The interaction between the intracellular domain of Fas and FADD/MORT1 is mediated via dimerization of two homologous regions in the two proteins, termed the death domain (DD). FADD/MORT1, in turn, appears to associate with the proenzyme form of caspase-8 through dimerization of a domain known as the death-effector domain (DED), with a similar region that occurs in the prodomain of caspase-8.

Similar events have been demonstrated for the tumor-necrosis-factor receptor (TNF-R1), except that an additional adapter molecule, TRADD, facilitates the receptor–FADD/MORT1 interaction. Furthermore, the recruitment, dimerization and activation of caspase-2 (ICH-1) may be mediated by RAIDD, a bipartite adapter molecule that associates with the RIP kinase, which also associates with TNF-R1 (Ref. 52).

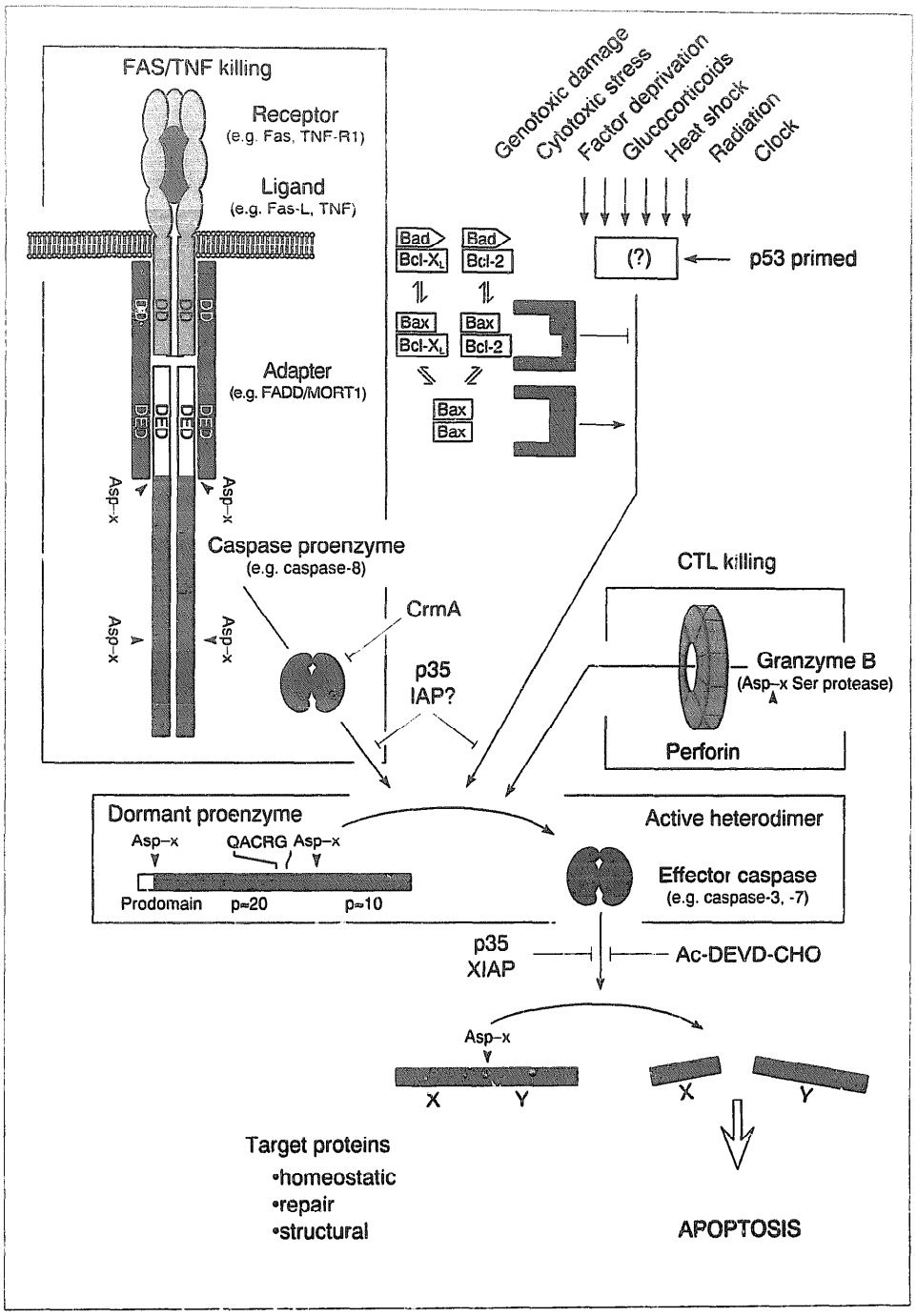

Figure 3

Biochemical events governing apoptotic cell suicide. Various pro-apoptotic stimuli result in the conversion of effector caspases from their dormant proenzyme forms to active heterodimers. These enzymes in turn cleave a discrete subset of key polypeptides, which collectively manifest the apoptotic phenotype. Multiple activation mechanisms feed into this common cell-death pathway. Diverse apoptotic stimuli provoke cell death by an unknown mechanism, which is under the control of the Bcl-2 family of dimerizing proteins. Members of the tumor-necrosis-factor receptor (TNF-R1) family bypass regulation by Bcl-2 family members by directly activating caspases that are recruited to the receptor complex following ligand binding. The granule serine protease, granzyme B, is delivered by cytotoxic T lymphocytes (CTL) and natural killer cells, and mediates cell suicide by activating endogenous caspases within the target cell.

Collectively, these observations are consistent with the hypothesis that, in the signaling complex, two or more caspase proenzymes are in close proximity and are activated by an intermolecular mechanism. Studies in a yeast expression system have confirmed an essential role for the prodomain in the dimerization and activation of caspase-1 (Ref. 53).

Taken together, these results so far suggest that effector caspases (e.g. caspases-2, -3 and -7) are mediators of a common

Thanatopsis

How doth the busy little cell
Tread the line 'twixt heav'n and hell?
Spinning threads of gene commands,
Repairing errors in the strands,
Cleaving daughters by mitosis
Without invoking apoptosis?
For life is but a tight-rope span,
A tenuous state for cell, or man:
With many a killer O_2 radical
from oxidations mitochondrial[1].

At least we now have consolation –
Death's biocomic explanation –
Our time to shuffle from the scene's
Dictated by gerontogenes[2].
And as we age 'tis rather nice
To learn that dying's put on ICE[3]:
The senile scramblings of our brains
Are simply due to apopains'
Aberrant acts, or morbid mutant[4]:
Morituri te salutant!

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cell-death pathway that is triggered in response to a variety of stimuli (Fig. 3). These proteases appear to be responsible for the cleavage of a number of proteins that are involved in maintaining normal cellular functions, and of several structural proteins, leading to the observed apoptotic phenotype. In Fas-mediated apoptosis, activation of the effector caspases appears to be mediated by caspase-8, which most likely undergoes dimerization and intermolecular autoproteolysis to generate its mature form.

In cytotoxic lymphocyte-mediated cell death, granzyme B appears to be responsible for activation of these caspases in target cells. No doubt, other mechanisms of effector-caspase activation exist in the apoptosis that occurs in different cell types, and in response to diverse stimuli. It is these latter activation mechanisms that appear to be under the control of the Bcl-2 family of dimerizing proteins and are the least understood, although recent studies have provided some clues.

In *C. elegans*, three central components of the cell-death pathway have been identified: CED-9 (an anti-apoptotic Bcl-2-like protein), CED-3 (the *C. elegans* caspase) and CED-4 (a pro-apoptotic protein upstream of CED-3 but of unknown function)[5]. Recently, CED-4 has been shown to bind simultaneously to both CED-9 and CED-3, establishing a potential link between anti- and pro-apoptotic proteins in nematodes[54–57]. CED-4 can bridge a similar interaction between mammalian caspases having long prodomains (e.g. caspase-1 or -8, but not caspase-3 or -7) and Bcl-X_L, and some evidence exists that an endogenous mammalian protein can also perform this task. These studies thus establish the possibility that a functional counterpart of CED-4 exists in mammalian cells and that its principal role is to activate caspase proenzymes. When bound to Bcl-X_L or similar proteins, the CED-4 counterpart may not be available to engage in caspase proenzyme activation (e.g. through subcellular re-distribution).

Concluding remarks

The central role of caspases in the biochemical events that mediate the apoptotic phenotype has been well substantiated through several biochemical and genetic approaches. Rapid advances in our understanding of the enzymology and biochemical role of these enzymes has contributed to entirely new perspectives on the events that control and mediate mammalian cell death. Despite these major advances, this complex field remains fertile ground for further studies to advance our understanding of this important physiological process and to develop therapeutics that will modulate these enzymes for the control of human diseases where inappropriate apoptosis contributes or accounts for disease pathogenesis.

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Owing to space limitations, we cannot cite many of the fine articles that have contributed substantially to this field.

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