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Review

Caspase structure, proteolytic substrates, and function during apoptotic cell death

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Abstract

Caspases play an essential role during apoptotic cell death. These enzymes define a new class of cysteine proteases and comprise a multi-gene family with more than a dozen distinct mammalian family members. The discrete and highly limited subset of cellular polypeptides that are cleaved by these proteases is sufficient to account for the majority of cellular and morphological events that occur during cell death. In some cases, caspases also play a contributory role in escalating the propensity for apoptosis, and in doing so may exacerbate disease pathogenesis.

Keywords: apoptosis; caspase; protease; neurodegeneration

Abbreviations: ICE, interleukin-1β converting enzyme; caspase, cysteinyl aspartate-specific proteinase; CED, product of cell-death-abnormal gene

Proteases in apoptosis (the ICE age)

ICE (interleukin-1β converting enzyme; caspase-1) is the prototypical caspase and was initially identified as the protease responsible for the proteolytic maturation of proIL-1β to its pro-inflammatory, biologically active form.[1,2] When originally discovered, ICE defined a new class of cysteinyl proteases that was distinguishable from other cysteine protease families based on general structural organization and the absolute requirement for aspartic acid in the P_1 position of the scissile bond. A key role for ICE in inflammation had been largely established, which was quickly secured by inhibitor studies and by the phenotype of ICE-deficient mice, but other potential biological functions were not evident. At approximately the same time, a genetic pathway for cell death was being defined in the nematode *C. elegans*.[3] One of the genes in this genetic pathway, *ced-3*, encoded a protein that was essential for all 131 programmed cell deaths that occurred during hermaphrodite development. When *ced-3* was cloned and sequenced, it was found to be a *C. elegans* homologue of mammalian ICE.[4,5] This and other evidence strongly implicated ICE (or related family members, as it turned out) in a similar mammalian cell death pathway. Importantly, this collective information also demonstrated an essential role for specific proteolysis in apoptotic cell death, which led to the identification of proteolytic 'victims' of the caspases and shed light on the biochemical events that occurred as a consequence of their cleavage. The importance of *C. elegans* genetics in defining the mammalian cell death pathway is underscored by the fact that the individual cell death components, their molecular ordering and cellular functions have been largely conserved throughout evolution[6] (Figure 1). In addition to caspases, other proteases also contribute to the apoptotic cell death pathway. The serine protease granzyme B, for example, has been well established as a caspase activator during CTL-mediated killing, and owing to its P_1 Asp bias can function as a caspase surrogate when caspases themselves are inoperative.[7–9] The calpains have also been implicated in apoptosis,[10] although their precise role remains to be determined.

The mammalian caspase gene family and functional sub-families

The caspase gene family[11] thus far contains at least 14 mammalian members, of which 11 human enzymes are known (Figure 2). A phylogenetic analysis indicates that the gene family is composed of two major sub-families which are related to either ICE (caspase-1) or to the mammalian counterparts of CED-3. Further subdivisions can be made depending on whether the proenzymes harbour short prodomains (caspases-3, -6, -7) or long prodomains (the remainder). Alternatively, these proteases can be subdivided on the basis of their substrate specificities which has been defined using a positional scanning combinatorial substrate library.[12,13] Using the latter technique, the proteases fall into only three specificity subgroups (Figure 3). As expected from earlier studies, the major specificity determinant is the S_4 subsite (most of the enzymes are promiscuous at P_2 and P_3, although they prefer His or Ile in P_2 and prefer Glu in P_3). Group I caspases (1, 4, 5, 13) are tolerant of liberal substitutions in P_4 but prefer bulky hydrophobic amino acids such as Tyr or Trp. This preference is consistent with their role in cytokine processing but does not support a substantial role in apoptosis since none of the polypeptides that are cleaved during apoptosis contain hydrophobic residues in P_4. The group II caspases (2, 3, 7) are substantially more stringent in S_4, requiring a P_4 Asp. This specificity and stringency is nearly indistinguishable from that of *C. elegans* CED-3. The preferred cleavage motif (DExD) for group II caspases appears in many proteins that are cleaved during cell death, consistent with group II caspases being the major effectors of cell death. Group III caspases (6, 8, 9, 10), on the other hand, prefer branched chain aliphatic amino acids in P_4; residues

that are found at the maturation site of most group II and group III caspases. This specificity is consistent with the group III enzymes being upstream activators of the group II effector caspases (Figure 4). This molecular ordering of group III and group II caspases has been upheld in several cases and is best exemplified by caspase-8-mediated activation of caspase-3 and -7 in the CD95 (Fas, APO-1) system,[14,15] and caspase-9-mediated activation of caspase-3 in the APAF-1/cytochrome *c* pathway[16] (both of these examples have been substantiated in caspase-8 or -9 knock-out mice as well).[17–20] One possible exception to this general ordering based on substrate specificity, which remains unresolved, is whether caspase-6 (a group III caspase) plays an effector role (e.g. lamin proteolysis)[21] instead of or in addition to a putative activation role. Another exception may be caspase-2 which appears to be a self-activating effector caspase.[12,22,23]

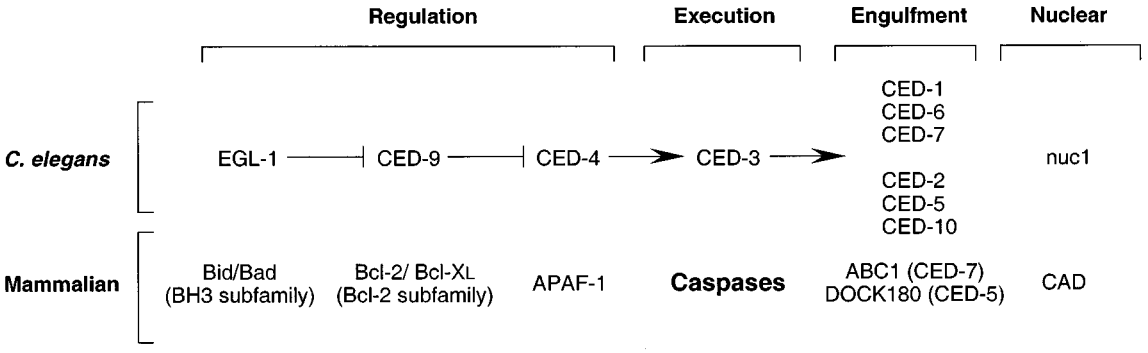

Figure 1 *C. elegans* cell death genetic pathway and mammalian counterparts

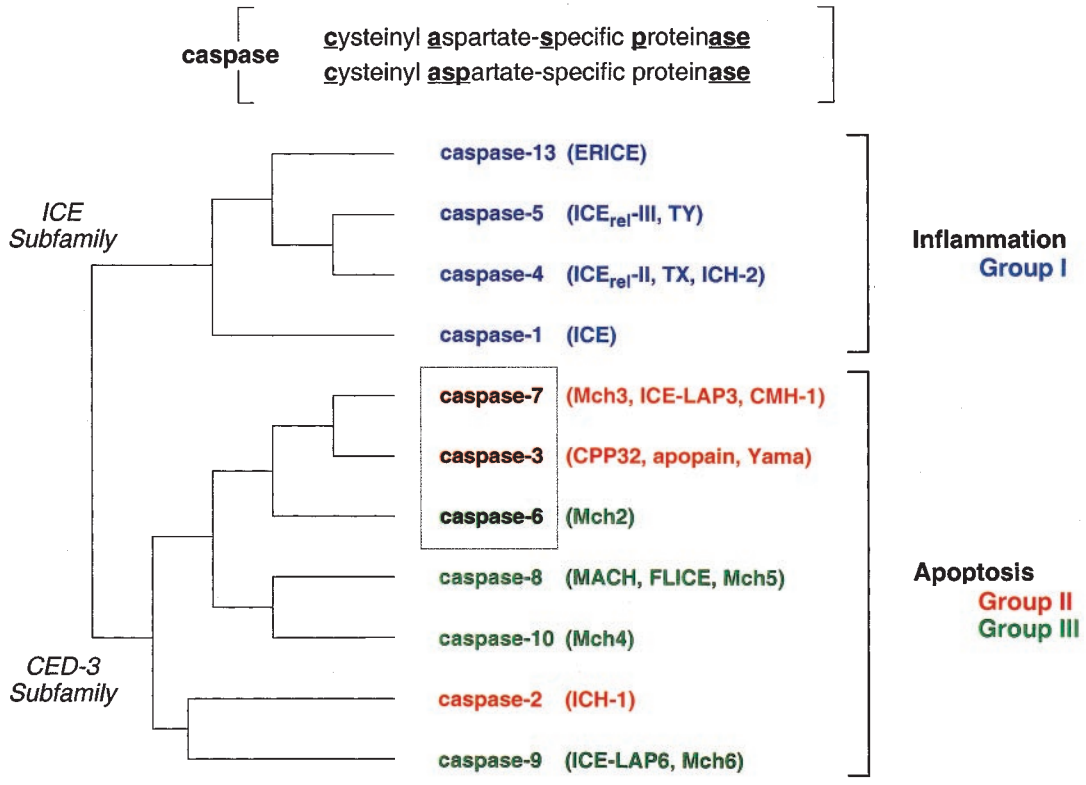

Figure 2 The human caspase gene family. Caspases segregate into two major phylogenic sub-families (ICE, CED-3). Based on their proteolytic specificities (see Figure 3), caspases further divide into three groups: group I enzymes (blue) mediate cytokine maturation whereas the apoptotic caspases are either group II (red) effectors of cell death or group III (green) upstream activators. Most caspases have long prodomains (>10 kDa) except for caspases-3, -6 and -7 (box) which have short peptidic prodomains (<30 aa). With the exception of caspase-13, the human chromosomal location for all of the caspases has been determined. At least two gene clusters have been identified, consistent with some caspases arising from tandem gene duplication. These include the caspases-1, -4, -5 gene cluster on 11q22.2-q22.3 and the caspases-8, -10, cFLIP/Usurpin gene cluster on 2q33-q34.[71,72] (cFLIP/Usurpin (aka CASH, Casper, CLARP, FLAME-1, I-FLICE, MRIT) is homologous to caspases -5, -8 and -10, except that substrate binding and catalytic determinants are absent, making it a dominant-negative death repressor.) The human counterparts of murine caspases-11, -12 and -14 have not yet been identified (although murine caspase-12 may be equivalent to human caspase-5)

General caspase structural features

Caspases are synthesized as catalytically-dormant tripartite proenzymes (Figure 5). Both the large and the small subunits, which together make up the active form of the enzyme, are liberated from the proenzyme by cleavage at Asp(P_1)-X(P_1') bonds. The presence of Asp at the maturation cleavage sites is consistent with the ability of caspases to auto-activate or to

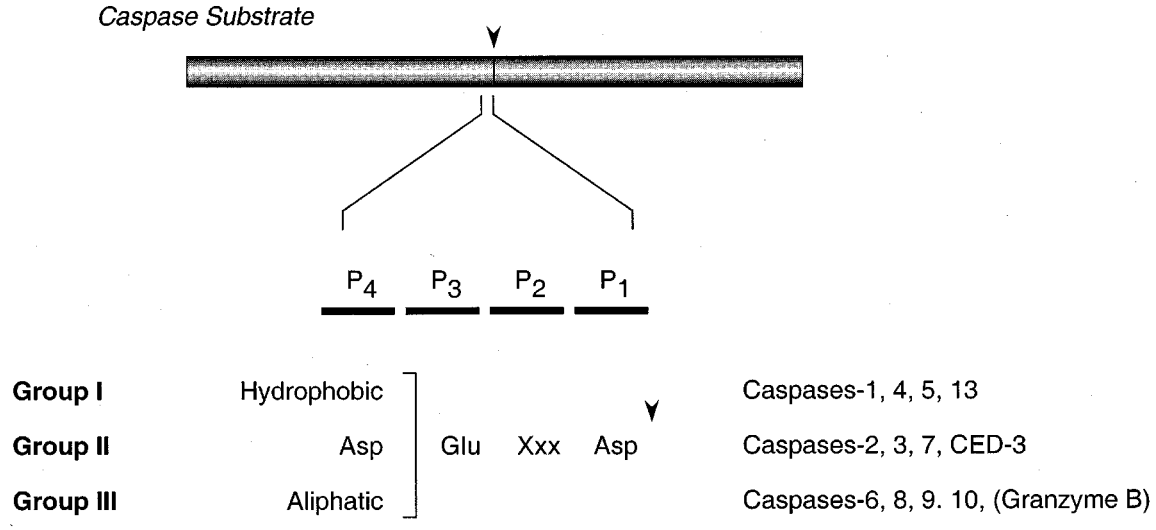

Figure 3 Caspase proteolytic specificity. The caspases recognize a core tetrapeptide motif corresponding to the four residues N-terminal to the scissile bond (P_4-P_3-P_2-P_1). A positional-scanning combinatorial substrate library, comprised of all 8000 possible P_1 Asp tetrapeptides, segregates the caspases into three specificity groups with the indicated sub-site preferences.[12,13] Caspase-2 also appears to have a P_5 requirement.[73]

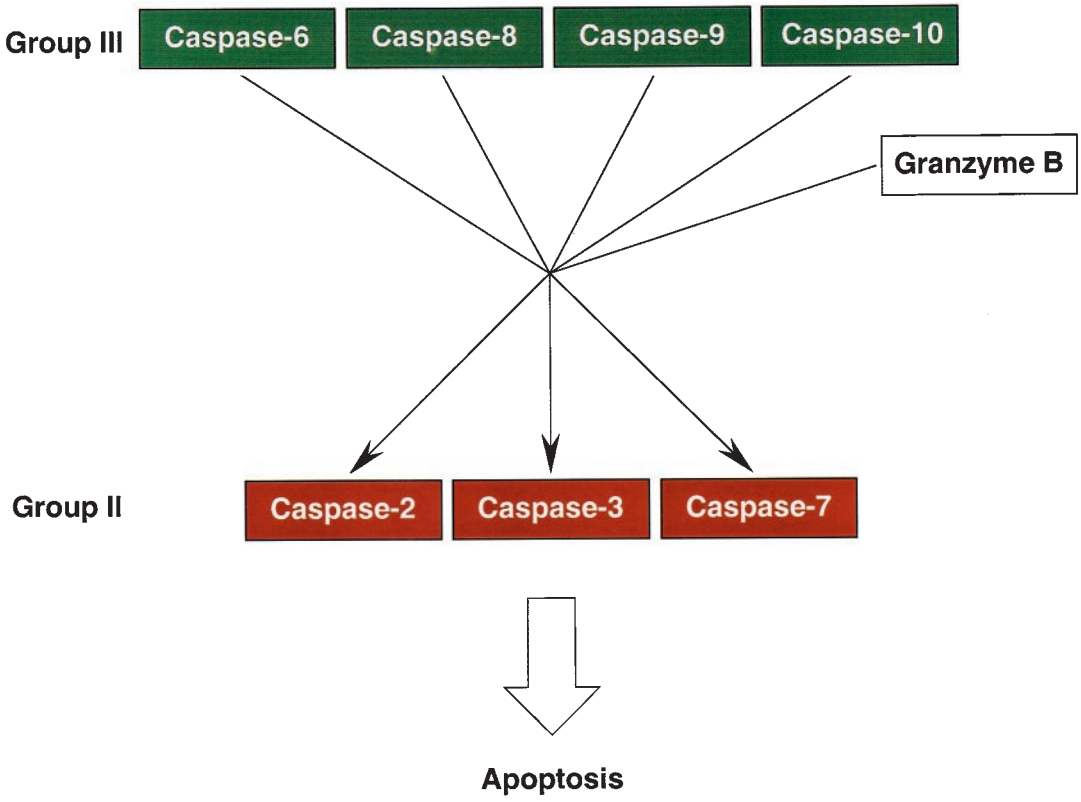

Figure 4 Molecular ordering of caspases. Exceptions to this general ordering may also exist. Caspase-2, for example, may be self activating. Caspase-6 may function as an effector protease. Amplification circuits also exist. The caspase complement varies considerably between different cell types, directly bearing on the pathways available and how they function within different cellular environments

be activated by other caspases as part of an amplification cascade. Components of the proteolytic device, including the active site Cys and His residues, are harboured within the large subunit whereas residues which form the S_1 subsite that tethers the carboxylate side chain of the essential P_1 Asp are derived from both the large and small subunits (Figure 6). Similarly, both the large and the small subunit contribute residues to form the substrate binding cleft (S_4-S_1), although the major determinants for substrate specificity (e.g. S_4) are contained within the small subunit. Prodomain structures vary considerably between different caspase family members ranging from small peptides with unknown (if any) function (e.g. caspases-3, -6, -7) to large domains that are involved in recruitment-activation (e.g. caspases-2, -8, -9, -10). Two caspase X-ray crystal structures have been published (caspases-1 and -3)[13,24–27] and in both cases, the enzyme was found to be a tetramer containing two large and two small subunits (a $[p20:p10]_2$ (caspase-1) or $[p17:p12]_2$ (caspase-3) homodimer of the large subunit: small subunit heterodimer). The subunits of each heterodimer are folded into a compact cylinder that is dominated by a central six-stranded β-sheet and five helices which are distributed on opposing sides of the plane that is formed by the β-sheets (Figure 7). In the caspase tetramer, two of these cylinders align in a head-to-tail configuration, thereby positioning the two active sites at opposite ends of the molecule. Despite the presence of dual active sites, however, there is no evidence for cooperativity or allosteric modulation between these sites. The overall configuration of the tetramer and the orientation of the individual subunits within it suggests an attractive mechanism for protease activation. In this model, two proenzymes associate, for example by prodomain-facilitated dimerization, interdigitate and process to form a tetramer in which each of the two heterodimeric catalytic domains is composed of a subunit derived from each proenzyme. This model is supported by the proximity of the C-terminus of the large subunit from one heterodimer with the N-terminus of the small subunit in the opposing heterodimer, but it does not exclude other possibilities that will likely require a proenzyme X-ray structure to resolve.

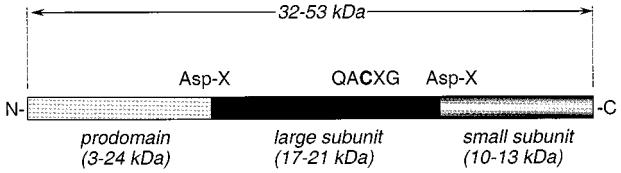

Figure 5 Caspase proenzyme organization

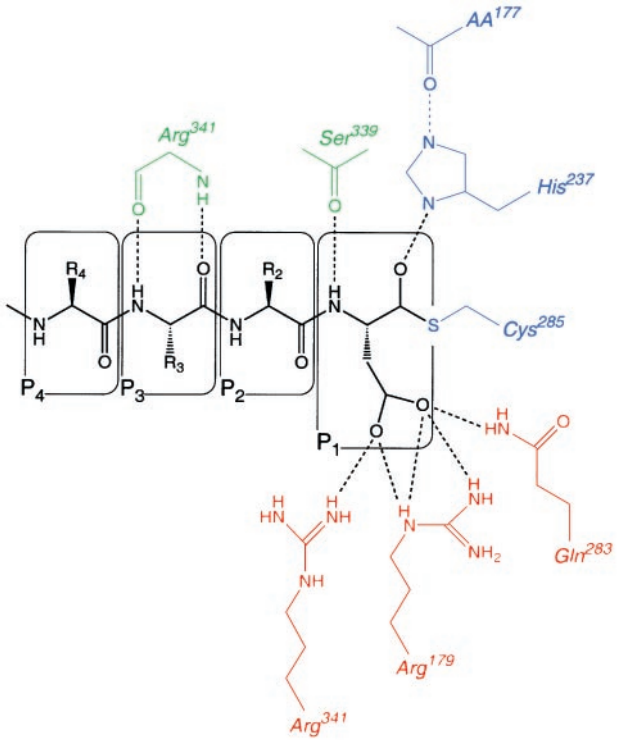

Figure 6 Major polar interactions within the caspase active site. The three-dimensional structure of two caspase : inhibitor complexes has been determined (caspase-1:Ac-YVAD-CHO, caspase-3:Ac-DEVD-CHO). Common polar interactions are shown. The inhibitor (shown in black) is tethered by a network of hydrogen bonds, including those which stabilize the carboxylate side chain of the P_1 Asp (red), the catalytic dyad (blue), and mainchain interactions (green). The P_4 network of interactions vary substantially between these two enzymes (not shown), accounting in part for the major differences in their respective substrate specificities. Numbering is based on caspase-1 residue positions. Also see Figure 9 for the conservation of these residues throughout the caspase gene family

The caspase active site (a fatal embrace)

Caspases recognize a very short tetrapeptide sequence within targeted substrate polypeptides and these motifs have

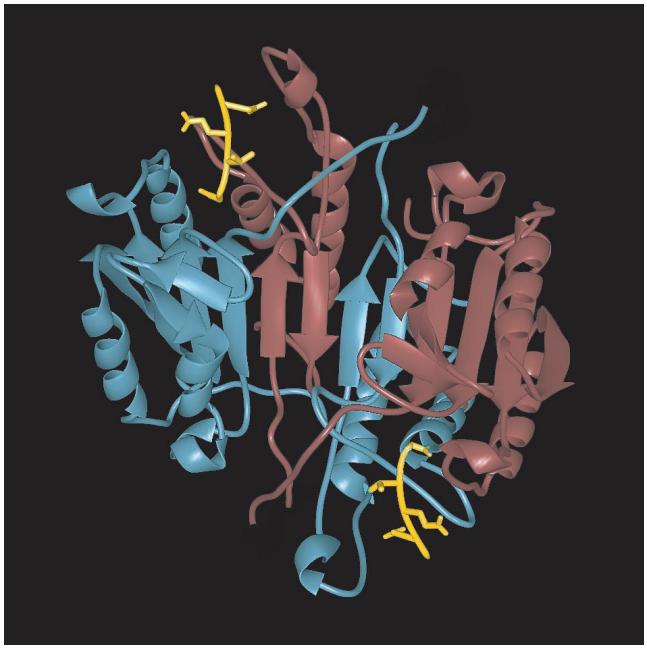

Figure 7 Caspase X-ray crystal structure. The caspase tetramer is comprised of two large subunits (outermost left (blue) and right (bronze) subunits) and two small subunits (inner left (bronze) and right (blue)). The caspase-3 structure is shown with its inhibitor, Ac-DEVD-CHO (yellow), in each of the two resulting active sites.[26]

formed the basis for inhibitor and synthetic substrate design. As described above, these proteases have an absolute requirement for Asp in P_1, are promiscuous in P_2, prefer Glu in P_3, but have varying preferences in P_4 that enable their assignment to one of three generic subgroups (I, II, III). Despite these apparently simple requirements, however, caspases are extremely stringent, indicating that three-dimensional context and the appropriate surface presentation are key factors in determining whether the presence of an appropriate motif also makes it eligible for caspase proteolysis within a polypeptide. The molecular basis of high affinity substrate binding and the specificity determinants at S_4 have been demonstrated for caspases-1 and -3 and can be inferred, as a consequence, for the other caspases. The active site groove is well defined and is extended along the surface of the enzyme (Figure 8). The carboxylate side chain of the P_1 Asp fits into a highly restrictive 'socket' and is tethered by hydrogen bond interactions with three residues that are conserved in all caspases (Arg^{179}, Gln^{283}, Arg^{341}) (Figure 9). The tight physical dimensions of S_1 accounts for the lack of tolerance for anything other than Asp in this position. The P_2 and P_3 binding sites (S_2 and S_3) are reasonably distinctive, although tolerant of broad substitutions. The peptide backbone of the bound substrate (or peptidyl inhibitor) forms main-chain hydrogen bonds with Ser^{339} (conserved in most caspases) and Arg^{341} (conserved in all caspases) as it extends along the binding cleft through these sites. The P_4 binding site (S_4), which is the key determinant of substrate specificity, varies markedly between the different caspase family members. This is best exemplified in a comparison of caspase-1 *versus* caspase-3 where the S_4 subsites vary radically in both geometry and chemical nature (see Figure 8). In the case of caspase-1, S_4 is a large shallow depression on the protease surface that readily accommodates bulky hydrophobic residues, such as the preferred Tyr or Trp. This site accommodates other residues as well; hence the relative promiscuity of this enzyme. Caspase-3, on the other hand, has a well defined and narrow S_4 pocket that envelops the side chain of the P_4 Asp. The intricate network of polar interactions and the physical geometry of the site accounts for the strong Asp preference and the enzymes overall specificity profile. The physical shape of the pocket is conferred in part by Trp^{348} (conserved in all groups II and III but not group I caspases) and by a small subunit-derived surface loop that makes an irregular reverse turn over the active site and contributes to the formation of S_4. Overall, this information affords a molecular understanding of the features which dictate the specificity of these enzymes and their relative promiscuity or stringency. Following substrate binding, catalysis employs a typical cysteine protease mechanism involving a catalytic dyad that is composed of Cys^{285} and His^{237}, plus an 'oxyanion hole' involving Gly^{238} and Cys^{285} (all of which are conserved in all caspases). Interestingly, inhibitors bind in an unexpected non-transition state configuration with the oxyanion of the thiohemiacetal being stabilized by the active site His^{237}.

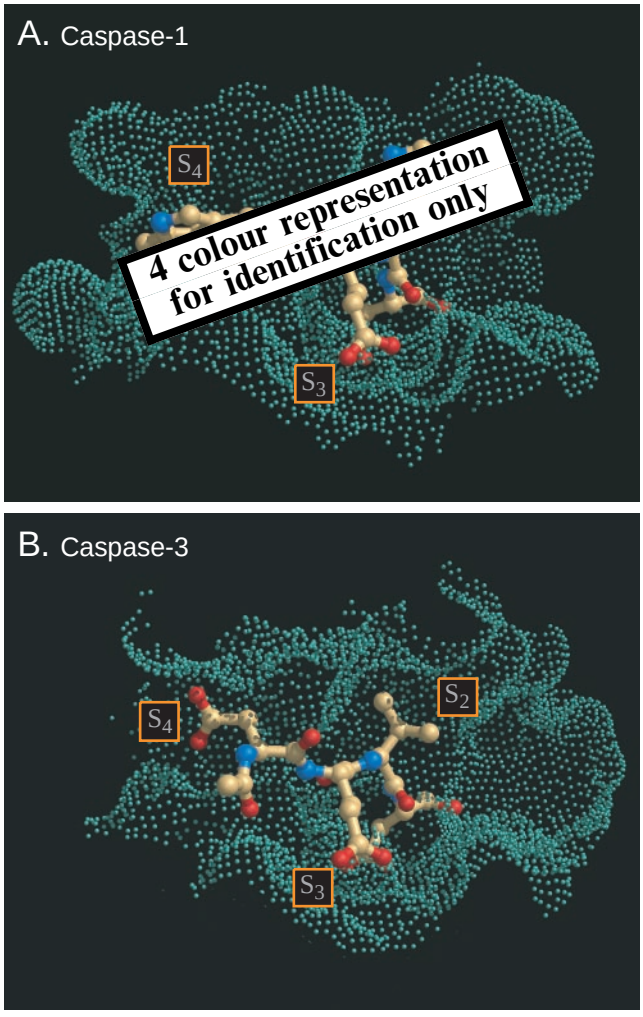

Figure 8 Topology of caspase-1 (**A**) and caspase-3 (**B**) active sites. The solvent-accessible surface is shown in green. Bound inhibitors are shown in yellow (nitrogens are coloured blue, oxygens are red). Caspase-1 (**A**) is shown with Ac-WEHD-CHO[13] whereas caspase-3 (**B**) is shown with Ac-DEVD-CHO[26] (aldehyde inhibitor versions of the optimal substrate for each enzyme). The major subsites (S_4, S_3, S_2, which bind their respective P_4, P_3, P_2 residues) are indicated. The P_1 Asp penetrates into the plane of the figure and is not visible. Note the major difference in S_4 subsite which is a large, open depression in caspase-1 versus a smaller, tighter pocket in caspase-3

Caspase inhibition

The sufficiency of a P_4-P_1 tetrapeptide for caspase recognition and high-affinity binding has been the platform for most of the currently available caspase inhibitors (as well as fluorogenic and colorimetric substrates). Suitable electrophiles that can interact reversibly with the active site Cys^{285} include aldehydes, nitriles and ketones. Because of the anticipated stability of ketones *in vivo*, this class of inhibitor is well suited for further development. Irreversible caspase inhibitors, which form covalent adducts with the active site Cys^{285}, are of the general structure [tetrapeptide]-CO-CH_2-X, including ketones where X is $-$Cl or $-$F (chloro- or fluoromethylketones), -N_2 (diazomeylketones) or $-$OCOR ((acyloxy)methylketones). Inhibitors of the latter class ((acyloxy)methylketones) are the most promising of the irreversible inhibitors owing to their very high potency against caspase enzymes and low intrinsic reactivity with other biological nucleophiles. The major challenge in developing

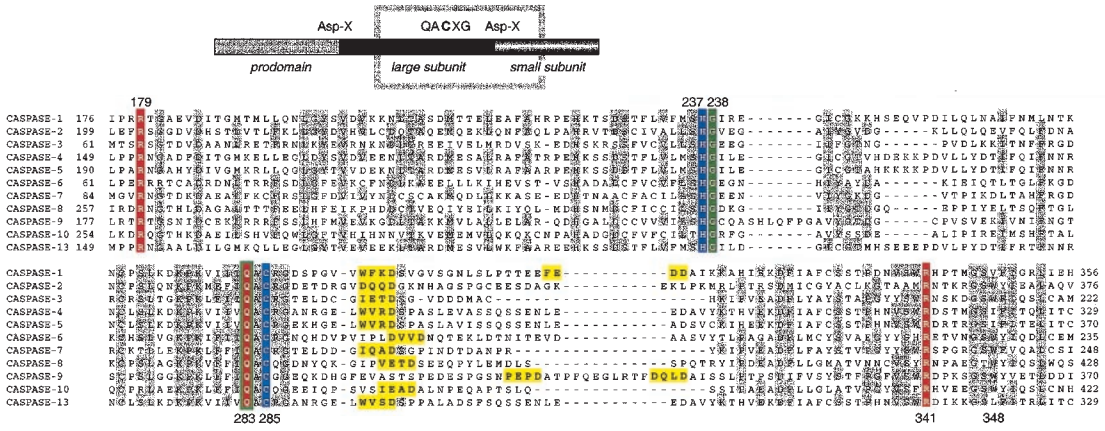

Figure 9 Conservation of residues critical for substrate binding and catalysis. The area shown bridges most of the large subunit and part of the small subunit (as indicated in upper panel by grey frame). The catalytic dyad (blue), residues which participate in stabilization of the carboxylate side chain of the P_1 Asp (red) and residues that contribute to the 'oxyanion hole' (green) are indicated. Known or predicted maturation sites between the large and small subunits are highlighted in yellow. Caspase-1, for example, undergoes two cleavage events to liberate a linker peptide that separates the large and small subunits. In caspase-3, however, the large subunit is contiguous with the small subunit. Caspase-9 can be activated by either autolytic cleavage (left site) or by a caspase-3-mediated amplification event (right site). Numbering is based on caspase-1 residue positions

inhibitors that are suitable for the current state of apoptosis research or for therapeutic usage is replacement of the tetrapeptide with non-peptide moieties. Peptide-based inhibitors have severely limited utility in cell-based and *in vivo* models owing to their very poor membrane permeability and other substantial disadvantages including poor metabolic stability. Despite these obstacles, interesting experiments have been performed *in vivo* with the esterified non-selective tripeptide-fluoromethylketone, Z-VAD(OMe)-CH_2F. The non-esterified counterpart of this inhibitor is modestly potent against most caspases, with the exception of caspase-2, although drawbacks of this compound include its lack of chemical stability ($t_{1/2}$ of free acid <50 min)[28] and electrophilic promiscuity which enables it to attack other biological nucleophiles, including cathepsins.[29] Several macromolecular caspase inhibitors have also been identified, including baculovirus p35[30–32] (a broad-spectrum caspase inhibitor), the cowpox serpin CrmA (selective for group I and III caspases, but not group II enzymes),[28] and members of the IAP superfamily (which appear to be largely selective for group II caspases).[33–36]

Caspase activation mechanisms

At least three distinct pathways for caspase activation exist in mammalian cells; (1) recruitment-activation, (2) trans-activation, and (3) autoactivation. In the first case, two examples of caspase activation following recruitment of multiple homologous proenzymes to a common site have been demonstrated. Ligation of the CD95 (Fas, APO-1) receptor, for example, recruits procaspase-8 to an oligomeric activation complex using the adapter protein FADD/MORT1.[14,15] This mechanism appears to be common to other 'death domain'-containing receptors (e.g. TNF-R1) and probably to caspase-10 as well. Similarly, oligomerization of procaspase-9 is mediated by APAF-1 following its release from Bcl-X_L and a cytochrome *c*-dependent conformational change.[16] In this case, recruitment is mediated by homophilic CARD-domain interactions. Other recruitment-activation mechanisms appear to exist (e.g. RAIDD-mediated activation of caspase-2[22,23] and CARDIAK-mediated activation of caspase-1),[37] but are less well characterized. Following recruitment of multiple caspase proenzymes to a common oligomerization site, the low level of endogenous catalytic activity that the proenzymes harbour is sufficient to initiate full catalytic activation by proteolysis of the Asp-X site at the junction between the large and small subunits.[38–41] Trans-activation of one caspase by another is a second well established mechanism for caspase proenzyme maturation and activation. In general, upstream group III activator caspases (e.g. caspases-8 or -9 once they have undergone recruitment-activation) cleave and activate downstream group II effector caspases (e.g. caspases-3 or -7) by proteolysis of the Asp-X site between the large and small subunits. CTL-derived granzyme B can also mediate this event and thereby 'trick' the target cell into launching its endogenous apoptotic response.[7–9] These trans-activation events are assisted by the activity of Hsp60, suggesting that the vulnerability of group II effector caspases to activation by upstream group III caspases is modulated by Hsp-driven folding and/or unfolding of the proenzyme.[42] Finally, caspases can, in principle, undergo autocatalytic activation, although definitive proof for a non-recruitment type of autoactivation has not been established. One indication that such a mechanism may exist comes from the observation that RGD peptides can directly stimulate the autoactivation of procaspase-3.[43]

Substrates for caspases during apoptosis

During apoptotic cell death, only a fraction of the cellular proteome is cleaved by caspases. Current estimates based on comparative 2-D gel analysis between healthy and apoptotic cells place the number at fewer than 200 polypeptides.[44] Thus far, about 70 of these caspase

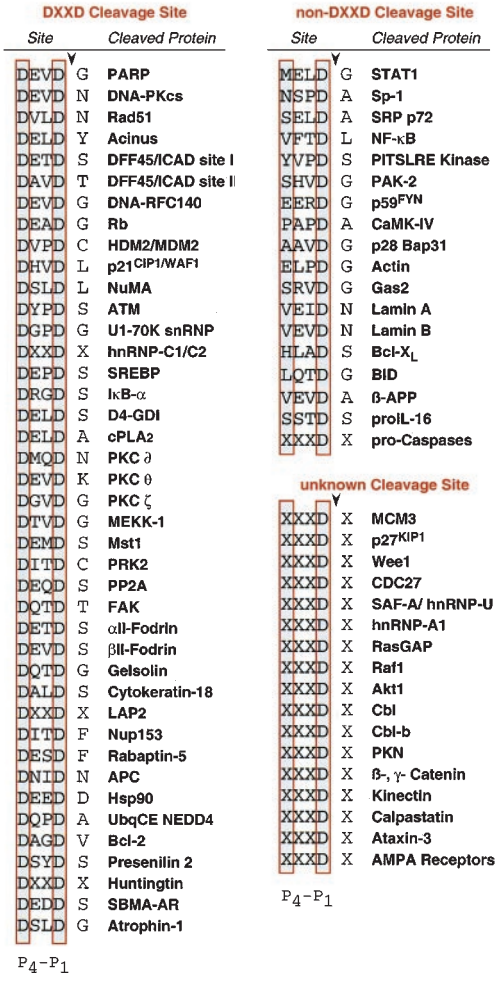

Figure 10 Substrates cleaved by caspases during apoptosis. PARP (poly(ADP-ribose) polymerase),[74,75] DNA-PK$_{cs}$ (catalytic subunit of DNA-dependent protein kinase),[76–82] Rad51 (mammalian RecA recombinational repair homologue),[83,84] Acinus (apoptotic chromatin condensation inducer in the nucleus)[85] DFF45/ICAD (45 kDa component of DNA fragmentation factor; inhibitor of the caspase-activated deoxyribonuclease),[86–89] DNA-RFC140 (140 kDa subunit of DNA replication factor C),[90–92] Rb (retinoblastoma gene product),[93–97] MDM2 (murine double-minute chromosome *mdm2* oncogene),[98,99] p21$^{CIP1/WAF1}$ (21 kDa inhibitor of cyclin-dependent kinases),[100–103] NuMA (nuclear-mitotic apparatus protein),[104–106] ATM (ataxia telangiectasia mutated gene product),[107] U1-70 kDa (70 kDa component of U1 small nuclear ribonucleoprotein),[108,109] hnRNP-C1/C2 (heteronuclear ribonucleoproteins C1 and C2),[110] SREBP (sterol responsive element binding protein),[111,112] IκB-α (α isoform of Rel/NF-κB inhibitors),[113] D4-GDI (Rho GDP-dissociation inhibitor, D4),[114,115] cPLA2 (cytosolic phospholipase A$_2$),[116–118] PKC (protein kinase C),[119–126] MEKK-1 (MEK kinase-1),[127,128] Mst1 (aka Krs2, mammalian homologue of yeast Ste20 kinase),[129–131] PRK2 (protein kinase C-related kinase 2),[132] PP2A (protein phosphatase 2A),[133] FAK (focal adhesion kinase),[134–137] fodrin (aka non-erythroid spectrin; note that a second cleavage site was also reported within αII-fodrin),[138–142] gelsolin,[143,144] cytokeratin-18,[145–148] LAP2 (lamin associated protein 2),[149] Nup153 (153 kDa nucleoporin),[149] rabaptin-5 (Rab5 GTPase effector protein),[150] APC (adenomatous polyposis coli oncosuppressor protein),[93,151] Hsp90 (90 kDa heat shock protein),[148] UbqCE NEDD4 (ubiquitin conjugating enzyme, neural-expressed developmentally downregulated gene 4 protein),[152] Bcl-2 (B-cell lymphoma gene 2 product),[153–155] presenilin 2,[156–159] Huntingtin (Huntington's disease gene product),[45,160] SBMA-AR (androgen receptor defective in spinal bulbar muscular atrophy (Kennedy's disease)),[160,161] atrophin-1 (DRPLA gene product),[160] STAT1 (signal transducer and activator of transcription factor),[162] Sp1 (transcription factor Sp1),[163,164] SRP p72 (72 kDa protein of signal recognition particle),[165] NF-κB (nuclear factor-κB transcription factor),[166,167] PITSLRE kinase (p34^{CDC2}-related protein kinases; note that additional cleavage sites have been identified in addition to this major site (TEGD/Y and DDRD/S)),[168–170] PAK2 (aka PAK65, γ-PAK, PAKI; p21-activated protein kinase),[171–173] p59^{FYN} (src-like tyrosine kinase p59^{FYN}),[174] CaMK-IV (Ca/calmodulin-dependent protein kinase IV),[175] p28 Bap31 (BCR-associated protein, 28 kDa Bcl2-interacting protein),[176,177] actin,[178–183] Gas2 (growth arrest-specific gene product 2),[184] lamins,[21,185–189] Bcl-X$_L$ (long version of Bcl-2-related gene product X),[190,191] BID (BH3 interacting domain death agonist),[192–194] β-APP (amyloid-β precursor protein),[47–49,195,196] proIL-16 (pro interleukin-16),[197] pro-caspases,[12] MCM3 (minichromosome maintenance protein 3, nuclear replication factor),[198] p27^{KIP1} (27 kDa cyclin dependent kinase inhibitor),[102] Wee1 (Wee1 kinase, inhibitor of cdc2 and cdk2),[199] CDC27 (CDC27 component of anaphase-promoting complex)),[199] SAF-A/hnRNP-U (scaffold attachment factor A, heteronuclear ribonucleoprotein U),[200] hnRNP-A1 (heteronuclear ribonucleoprotein A1),[44] RasGAP (Ras GTPase-activating protein),[201] Raf1 (Ras activated/associated factor 1),[201] Akt1 (aka protein kinase B),[201] Cbl (Cbl protooncogene product, negative regulator of receptor tyrosine kinase signaling),[201] PKN (protein kinase N),[202] catenin (aka plakoglobin),[203–207] kinectin,[208] calpastatin (calpain inhibitor),[209,210] ataxin-3 (gene product defective in spinocerebellar ataxia type 3 (SCA3)),[160] AMPA receptors[211]

'victims' have been identified (Figure 10) and in most of these cases the cellular rationale for proteolysis during cell death can be reasonably predicted. For example, one of the hallmark events of apoptotic cell death is genomic disassembly and breakdown into oligonucleosomal fragments. Caspases disable normal DNA repair processes, in order to prevent counterproductive events from occurring simultaneously, by inactivating at least two key proteins involved in the homeostatic maintenance of genomic integrity; PARP and DNA-PK. At the same time, an apoptosis-dedicated endonuclease (CAD) is activated by caspase-mediated crippling of its cognate inhibitor (ICAD/DFF45). Together these cleavages contribute to the changes in the genomic DNA that accompany apoptotic cell suicide. Comparable scenarios can be envisioned for most of the polypeptides that are cleaved by caspases during cell death and the biological consequences that are associated with the apoptotic phenotype. The cumulative effects of these cleavage events are to (a) disable homeostatic and repair processes, (b) halt cell cycle progression, (c) inactivate inhibitors of apoptosis, (d) mediate structural disassembly and morphological changes, and (e) mark the dying cell for engulfment and disposal. In order to mediate these events, caspases can modify the function of their target polypeptides in one of four generic ways. For example, they can either inactivate the normal biochemical function of their substrates (e.g. ICAD, PARP, DNA-PK$_{cs}$) or activate them by removal of regulatory domains (e.g. cPLA$_2$, PKCs, SREBP). Alternatively, caspases can alter or invert the function of their target proteins (e.g. BID, which converts from mildly to strongly apoptotic following caspase proteolysis; Bcl$_2$ and Bcl-X$_L$, which switch from anti- to pro-apoptotic following cleavage). Finally, the structural components of the cytoskeleton and nuclear scaffold require disassembly during apoptosis and caspases play a key proteolytic role in these steps as well (e.g. lamins, fodrin, gelsolin). Caspase cleavage normally occurs at a single, discrete site within the target polypeptide, although examples of multiplicity, redundancy and nesting also exist (Figure 11).

Pathogenic exacerbation by caspase-mediated cleavage of disease-associated substrates

Apoptotic cell death is dependent on caspase activity and inappropriate apoptosis contributes to or accounts for several disease pathogeneses. In some cases, however, caspases also appear to play a role in aberrant processing events that culminate in an increased propensity or vulnerability to cell death. Recent examples include the role of caspases in polyglutamine-repeat disorders and Alzheimer's disease (Figure 12). Huntington's disease, for example, is a progressive neurodegenerative disorder in which the molecular defect is attributable to an expanded polyglutamine stretch in the amino terminus of the Huntingtin protein. Caspase-3 (a group II effector caspase) liberates the amino-terminal fragment, containing the polyglutamine expansion, by cleavage within a cluster of DXXD sites.[45] These truncated fragments then appear to aggregate within the cell and recruit the proenzyme of caspase-8 (a group III activator caspase) to the polyglutamine aggregates.[46] The ensuing recruitment-activation of caspase-8 presumably initiates an apoptotic cascade similar to the CD95 (Fas, APO-1) pathway, including further activation of caspase-3. This cycle may begin at a low level that is below the threshold necessary for cell death; however, with the cumulative cycle of polyglutamine fragment generation and caspase activation, a point may be reached in vulnerable neurons where this threshold is breached and the cells die prematurely. In Alzheimer's disease, caspase-3 adulterates the normal processing of the amyloid-β precursor protein (APP) by removal of the carboxy-terminal cytosolic domain.[47–49] The resulting truncated APP, now deprived of key re-internalization signals, appears to be shunted to a degradative pathway that results in the generation of the cytotoxic amyloid-β peptide (Aβ) as one of the peptide derivatives of the full length APP polypeptide. Although the mechanism by which Aβ mediates its cytotoxicity is not understood, it leads to an increased propensity for apoptosis, including caspase-3 activation. Increased caspase-3 activity (again below the apoptotic threshold) may result in accelerated formation of Aβ and further neuronal stress, eventually resulting in exacerbation of the cycle to a point which leads to cell death and neurodegeneration. In both of these examples, a vicious cycle appears to exist, although how the cycle is initiated or emerges is unclear. Vulnerability of the target polypeptides to caspases may arise as a consequence of genetic mutations (e.g. polyglutamine expansion in the case of Huntington's; APP or presenilin mutations in the case of Alzheimer's). Alternatively, the seed of caspase activity may arise as a consequence of other trauma or from the endogenous low-level activity of the caspase proenzymes themselves.

Prospects for caspase-directed therapeutics

Inappropriate apoptosis clearly underlies the etiology of several human diseases.[50–53] The control of caspases, as a key and central component of the biochemical pathway that mediates apoptotic cell death, is an attractive first step in modulating this process. Caspase activation for the treatment of disorders where insufficient apoptosis occurs (e.g. cancer)

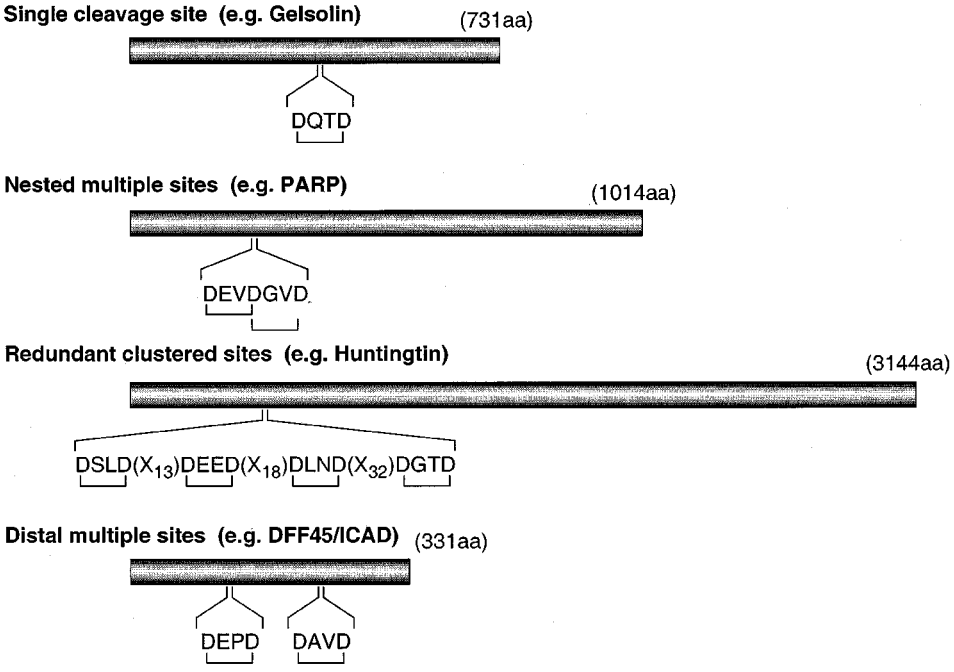

Figure 11 Caspase cleavage strategies. The majority of substrates that are proteolyzed by caspases during apoptosis are cleaved at a single site, although some polypeptide substrates contain multiple sites that are either nested, redundant within a short stretch, or spread out across the molecule

represents a substantial challenge. 'Trojan horse' gene therapy approaches may be viable, such as that described for HIV infection in which a TAT-caspase-3 construct containing a HIV-protease recognition motif selectively induces apoptosis in HIV infected cells only.[54] Alternatively, the molecular constraints on caspase activation is poorly

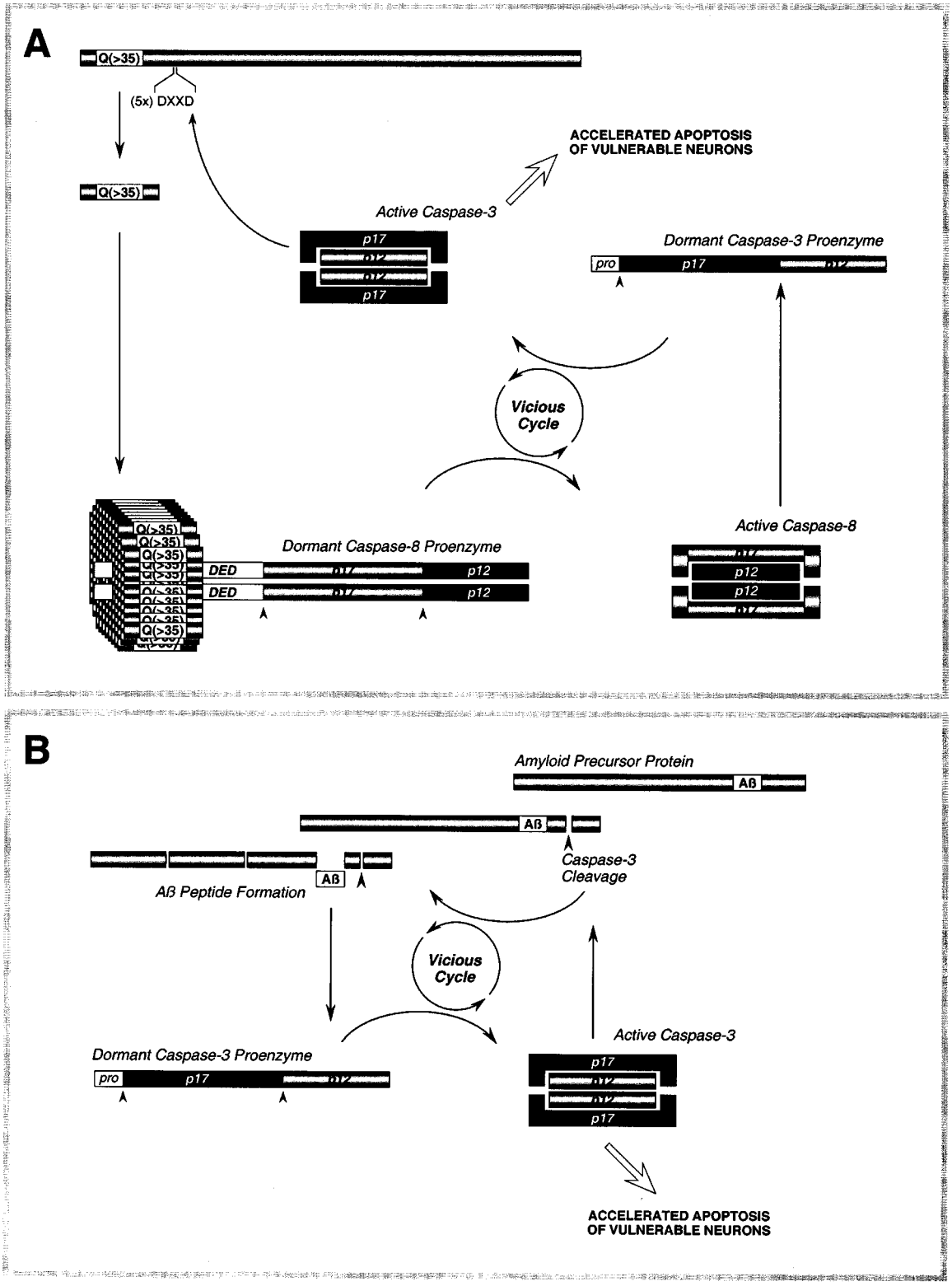

Figure 12 Potential involvement of caspases in 'vicious cycles' leading to pathogenic exacerbation in Huntington's disease (**A**) and Alzheimer's disease (**B**)

understood and my harbour opportunities for selective caspase activation once they are resolved. On the other hand, caspase inhibition for the treatment of disorders where excessive apoptosis occurs (e.g. neurodegeneration) appears to be more amenable to therapeutic intervention with classical small-molecule inhibitors. Preliminary experiments in animal models using non-selective caspase inhibitors such as Z-VAD(OMe)-CH_2F, for example, have shown *in vivo* efficacy in ischemic and hypoxic brain injury, traumatic and excitotoxic brain damage, neuronal transplantation, acute bacterial meningitis as well as in cardiac and kidney ischemia/reperfusion injury and models of acute liver failure.[55–65] In addition, transgenic mice expressing dominant-negative caspase-1 show resistance to CNS injury in models of ALS, focal ischemia, excitotoxic injury and Huntington's disease.[66–70] (It is not yet clear whether this protection is afforded by the attenuation of a secondary inflammatory response, or whether the dominant-negative caspase-1 affects other caspase family members as well.) The clinical utility of caspase inhibitors will depend on several key issues that require further resolution. For example, will cells that are saved from apoptotic death remain functional and survive without perpetual caspase inhibition? Will the delayed-administration 'window of opportunity' be sufficient for practical use in a clinical setting? Can highly selective caspase-3 inhibitors be used for chronic administration without unacceptable adverse events occurring? Early work in all of these areas is highly encouraging.

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