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# Review

# The role of caspases in T cell development and the control of immune responses

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**Abstract.** Apoptosis is responsible for the removal of potentially autoreactive or useless T cells during thymic selection and activated T cells in the periphery. Specific families of receptors, kinases, transcription factors, and cysteine proteases, termed caspases, are involved in the apoptotic cascade leading to proteolysis of specific substrates and to morphological changes associated with programmed cell death. Although common members of the apoptotic cascade are shared between different cell types, it appears that cell-specific factors can influence the response to a given apoptotic stimuli. Characterization and understanding of the basic mechanisms involved in the different pathways protecting or leading to cell death may provide novel ways to control inappropriate apoptosis involved in several diseases.

**Key words.** Apoptosis; glucocorticoids; Nur77; SAPK; ceramide; Fas; substrates.

## Introduction

Programmed cell death is a suicide mechanism involved in normal cell turnover which can be induced by a variety of stimuli to eliminate damaged, infected or useless cells. Apoptosis is essential for the development of vertebrates, being involved in elimination of interdigital webs, palatogenesis, haematopoietic cell homeostasis, and development of intestinal mucosa and retina [1–3]. Inappropriate cell death plays a role in pathological conditions such as cancer, autoimmunity and neurodegenerative diseases [4]. Apoptosis is characterized morphologically by condensation of nuclear chromatin, internucleosomal cleavage of DNA, compaction of cytoplasmic organelles, decrease in cell volume and loss of plasma membrane asymmetry resulting in recognition and phagocytosis [1–3]. This review is focused on the role of caspases in the shaping and homeostasis of the immune system and the mechanisms involved in transducing the death signal.

## Caspases and apoptosis

Much information about programmed cell death has been gained from studies of the nematode *Caenorhabdi-*

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*tis elegans* in which 131 cells of the 1090 generated die by apoptosis during development [5]. Somatic mutants of *C. elegans* have allowed the identification of *ced-3* and *ced-4*, essential for cell death, while the *ced-9* gene antagonizes their function [5]. *Ced-9* is similar to mammalian *Bcl-2*, which acts to prevent apoptosis in mammals, and either one can functionally replace the other [6]. The first homologue of *ced-3* identified was interleukin-1$\beta$ (IL-1$\beta$) converting enzyme (ICE), responsible for processing of pro IL-1$\beta$ [7]. The finding that ICE overexpression in mammalian cells induced morphological changes associated with apoptosis [8] has led to the discovery of several family members, which are novel cysteine proteases that cleave after aspartic acid residues and can trigger apoptosis when activated [8–26]. Several groups have cloned the same protease and given it a particular name, thus leading to nomenclature confusion. A new designation is used for ICE-like proteases: caspases, for cysteine aspartases, and they are numbered according to their order of publication [27].

Phylogenic analysis of caspase family members (fig. 1) reveals that there are three subfamilies: an ICE subfamily comprising caspases 1, 4, 5, 11 and 12; a CPP32 subfamily including caspases 3, 6, 7, 8 and 10; and an ICH-1 subfamily comprising caspases 2 and 9 [27]. All apoptotic proteases are synthesized as inactive precursors that require internal cleavage after aspartic acid residues to generate the active enzyme. For instance, caspase-1 is synthesized as a protein of 45 kD that undergoes proteolytic conversion to remove an N-terminal pro-peptide and two intervening peptides to produce the active heterodimeric 20-kD and 10-kD enzyme [7], while caspase-3, the closest ced-3 homologue, is a pro-enzyme without intervening peptides [11,12]. Figure 2 shows the general structure of the pro-enzymes, with their internal cleavage sites and their optimal substrate specificity, based on combinatorial analysis [28]. While all caspases possess a pro-segment, it appears that the long pro-segment in certain caspases is involved in protein-protein interactions, which will be discussed later. The crystal structure of caspase-1 and caspase-3 revealed that both chains are involved in the formation of the active site, with fine substrate specificity mostly dictated by the small subunit [29–32]. The active-site motif QACXG (X = R, Q or G) is present in all caspases, and sequence comparison reveals that residues involved in binding and hydrolysis of the peptide P1-P1′ bond are conserved, while residues involved in substrate

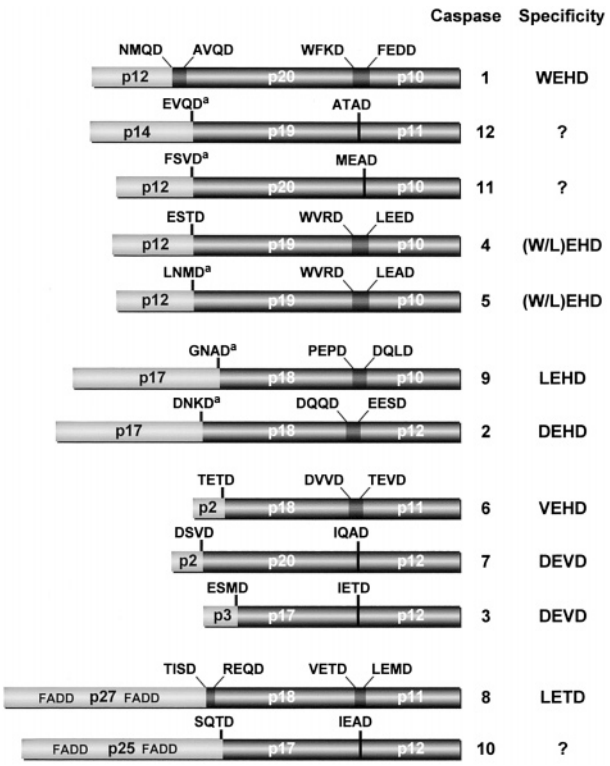


Figure 2. Structure of caspase pro-enzymes. Schematic view of caspase pro-enzymes with their internal processing sites. They are presented in the same order as in figure 1, and the computed molecular weight of fragments is shown in kD. The preferred substrate specificity has been determined based on combinatorial analysis [28]. [a]Exact cleavage site not known.

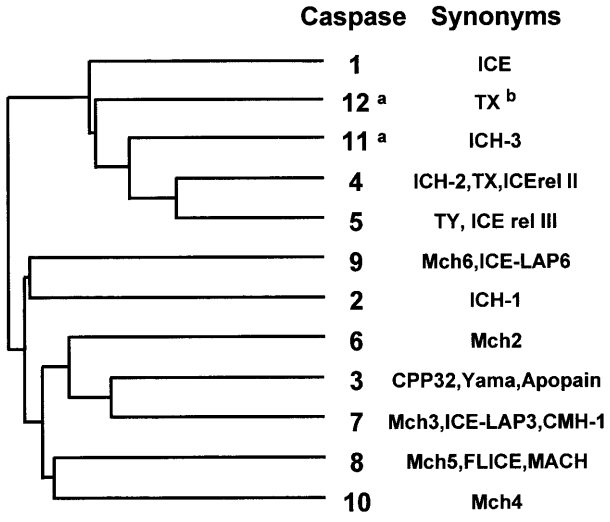


Figure 1. Phylogenic tree of caspases. The phylogenic tree was generated from the protein sequences of caspases using the PILEUP program from the Genetics Computer Group at its default settings. Also shown are the synonyms used to designate caspases. [a]Caspases 11 and 12 are of murine origin. [b]Caspase-12 was proposed to be the homologue of human caspase-4 [26], but the analysis shows that it is distantly related to caspase-4.

binding in the P4-P3-P2 pockets differ, suggesting different substrate specificities [29–32]. Certain caspases can cleave and activate other caspases, and this hierarchy of cleavage can be viewed as initiation, commitment and execution of apoptosis.

A detailed analysis of caspase expression in adult and embryonic tissues has yet to be performed. Caspase-1 and caspase-4 show similar patterns of expression being found in most tissues examined with the exception of the brain. However, appreciable levels of caspase-4 are found in lung, liver, ovary and placenta, where caspase-1 is barely detectable [13, 14]; caspase-5 follows the expression pattern of caspase-4, but at much lower levels [13, 17]. Caspase-2 is expressed at relatively high levels in various tissues, including the central nervous system, liver, kidneys and lungs [12, 13]. Caspase-2 is expressed in postmitotic neurons, and its level can be upregulated following brain ischaemia [33]. In adult tissues, caspase-3 is highly expressed in short-lived cells, such as haematopoietic cells, and expression is low in long lived cells, such as brain and spinal cord neurons [11, 12, 34]. While caspase-3 expression is low in the adult brain, caspase-3-deficient mice show severe embryonic brain abnormalities, confirming its role in morphogenetic cell death of the mammalian brain [35]. In addition, caspase-3 expression can be upregulated by thyroid hormone during tadpole tail resorption, implying an involvement in vertebrate morphogenesis [36]. Caspase-7 is expressed in many foetal and adult tissues, with lowest expression in the brain [18–20]. Low levels of caspase-8 transcripts can be detected in testis, skeletal muscle and brain tissues, while a higher level of expression is found in peripheral blood leukocytes, consistent with its role in Fas-mediated lymphocyte homeostasis [21, 22]. High levels of caspase-9 are found in the heart, testis and ovary [23, 24]. Caspase-10 can be detected in most tissues, with little expression in the brain, kidney, prostate, testis and colon and high levels in heart, liver and spleen [25]. While there is redundancy in substrate specificity between caspases, their different tissue distribution suggests that they are not entirely interchangeable and their expression might be regulated during development.

## Apoptosis and T cell development

During their development, T cells undergo a selection process in order to eliminate potentially harmful autoreactive cells. In negative selection, $CD4^+CD8^+$ immature thymocytes undergo a test of affinity towards antigen-presenting cells. Cells bearing T-cell receptors (TCR) with high avidity against major histocompatibility complex (MHC)-associated self-antigens undergo programmed cell death, whereas thymocytes with TCRs with moderate avidity towards MHC survive and develop into mature $CD4^+$ or $CD8^+$ single positive T cells [37–41]. This engagement of TCR with moderate strength appears to provide a life-giving signal and is called positive selection [40, 41]. On the other hand, T cells which failed to rearrange a TCR or bear a TCR having little avidity for MHC complexes cannot receive this life-giving signal and undergo death by neglect. A role for glucocorticoids (GC) has been suggested in thymic T-cell development, but their physiological role is still controversial. Thymocytes are extremely sensitive to GC-mediated cell death, yet GC and TCR signalling were originally found to be mutually antagonistic [42–44]. However, when dexamethasone, a GC agonist, was injected in combination with anti-CD3 antibody, this increased $CD4^+CD8^+$ T-cell depletion, whereas coinjection of RU-486, a GC antagonist, was shown to reduce cell death [45]. This suggested that GC might participate in TCR-induced apoptosis, but the effect of RU-486 might involve a nonspecific action of RU-486. Indeed, thymocytes derived from mice transgenic for an antisense GC receptor are abnormally sensitive to TCR-mediated cell death, this argues for the potential involvement of GC in T-cell development [46]. One model for thymic selection proposes that negative selection occurs through strong TCR signals, whereas intermediate-strength TCR engagement could be antagonized by endogenous corticosteroids allowing positive selection, and that no TCR engagement would allow GC to proceed unopposed to induce death by neglect [47, 48]. The mechanism by which these two signals oppose each other remains unclear, but GC could operate by transrepression, since a transcriptionnally inactive GC receptor can still induce apoptosis, presumably by blocking the induction of cell survival factors [48]. Also, a recently identified protein, glucocorticoid-induced leucine zipper (GILZ), was shown to be upregulated in thymocytes and peripheral T cells treated with dexamethasone, and overexpression of GILZ inhibits TCR-induced cell death through repression of Fas and Fas-ligand (FasL) expression [49].

Subtractive hybridization has allowed the identification of Nur77 [50, 51], an immediate early gene induced by TCR cross-linking in T-cell hybridomas and thymocytes [52–54]. Nur77 is an orphan nuclear receptor belonging to the superfamily of steroid/thyroid receptors and is expressed in a variety of cell lines in response to signals for growth and differentiation [50, 51]. The involvement of Nur77 has been shown by constitutive expression of Nur77 in thymocytes of transgenic mice, leading to massive apoptosis of immature $CD4^+CD8^+$ cells and a substantial decrease in the number of thymocytes and peripheral T cells [55]. However, Nur77-deficient mice show normal negative selection and peripheral T-cell apoptosis [56]. The Nur77 homologue, Nor-1 [57, 58], is induced upon TCR stimulation of thymocytes with ki-

netics similar to Nur77, and constitutive expression of Nor-1 leads to massive thymocyte apoptosis, showing the functional redundancy between Nur77 and Nor-1 [59]. Since Nor-1 and Nur77 recognize a similar DNA element, overexpression of a dominant negative form of Nur77 can inhibit TCR-mediated apoptosis, presumably by blocking DNA binding of either proteins [60]. While the target genes regulated by Nur77/Nor-1 have not been characterized, it has been proposed that FasL expression might be regulated by Nur77 [61]. Mice overexpressing Nur77 crossed with *gld* mice, which have a defective FasL [62], show increased thymic cellularity and an almost normal distribution of thymocyte subpopulations [61]. It is noteworthy that GC can prevent FasL upregulation in hybridomas [63] and that the GC receptor can antagonize Nur77-dependent transcription in TCR-stimulated T cells [64]. This suggests a mechanism for the antagonism between TCR-mediated signals and GC.

In thymic organ culture, TCR triggering of immature thymocytes by peptide/MHC ligands leads to caspase-mediated apoptosis, since this can be blocked by the caspase inhibitor, zVAD-fmk [65]. Spontaneous and anti-CD3-mediated apoptosis of thymocytes can be blocked by inhibitors specific to the caspase-3 subfamily, but not to the caspase-1 subfamily [66], which is not surprising given that caspase-1-deficient mice undergo normal negative selection [67]. Since several proteases belong to the caspase-3 family, it is unclear which might be the relevant target of such inhibition studies. Thymocytes isolated from caspase-3-deficient mice apparently retain normal susceptibility to apoptosis induced by combined anti-CD3 and anti-CD28 treatment [35], suggesting that caspase-3 is not the only protease involved in negative selection. However, using TCR transgenic mice, it was shown that following antigenic challenge, caspase-3 was activated and the substrate poly [adenosine diphosphate(ADP)]-ribose polymerase (PARP) cleaved in isolated thymocytes [68]. Interestingly, in that model death by neglect was not accompanied by caspase-3 processing but PARP cleavage still occurred, suggesting the involvement of a distinct caspase-3-like protease in this phenomenon [68]. It would appear that several caspases might be involved in thymic selection and that a degree of redundancy exists between them.

## Apoptosis and the control of immune responses

Lymphocytes are among the few cells in the organism that can undergo massive clonal expansions. After clearance of the offender and establishment of memory T cells, the excess of expanded T cells is removed by apoptosis through activation induced cell death (AICD) [69]. Insights into this phenomenon came from studies of mice suffering from lymphoproliferative defects: peripheral clonal deletion and elimination of activated mature T cells is impaired in *lpr* and *gld* mice, resulting in accumulation of T cells in lymph nodes and spleen [62]. *Lpr* mice bear a mutation in the Fas molecule, while *gld* mice carry a mutation in the FasL molecule [70, 71]. Fas and FasL are members of the tumour necrosis factor (TNF) superfamily of receptors and ligands [70, 71], and the mechanisms by which TNF members transduce their death signal is discussed later. The Fas system also appears to be involved in preventing autoreactivity, as some *lpr* strains of mice develop autoimmune manifestations similar to systemic lupus erythematosus [62]. The human correlate of *lpr*, autoimmune lymphoproliferative syndrome, shows the same symptoms observed in *lpr* mice, demonstrating the importance of Fas in maintenance of self-tolerance [72, 73]. The Fas pathway is also used by cytotoxic T lymphocytes to reject tumours and grafts and appears to be involved in maintenance of immune privilege in certain tissues [62, 74, 75]. Upon TCR engagement by MHC-antigen complexes, T cells express both the Fas and FasL molecules [76, 77], and these trigger AICD through cell-to-cell interaction (fratricide) or in an autocrine manner (suicide), which can be blocked by FasL antagonists such as recombinant Fas-Fc protein [77–79]. However, naive $CD4^+$ cells can undergo apoptosis in a Fas-independent manner if cultured in the absence of antigen, and this can be prevented by CD28 costimulation, leading to secretion of growth factors and expression of survival genes such as Bcl-$X_L$ [80].

The Fas pathway is not alone in AICD, since apoptosis still occurs in activated T cells derived from *lpr* mice; and this response appears to be mediated by TNFR1, since this can be blocked by TNFR-Fc fusion protein [81]. In mice lacking TNFR1, deletion of $CD8^+$ cytotoxic T lymphocytes is significantly impaired [82]. It would appear that Fas is essential for $CD4^+$ cell homeostasis, whereas TNF is involved in $CD8^+$ cell homeostasis. The suicide machinery can be suppressed by cytokines produced by helper cells such as other lymphocytes, and their continuous presence is required to keep 'death-primed' cells alive. Amongst life-giving cytokines is Interleukin-2 (IL-2), produced by activated T cells; IL-2 withdrawal can lead to apoptosis of activated T lymphocytes. IL-2 can also protect from radiation and GC-induced apoptosis through upregulation of Bcl-2 and of Bag-1, the latter being able to enhance Bcl-2 activity [83–85]. Thus, IL-2 is critical for T-cell survival by mediating proliferation and upregulation of anti-apoptotic Bcl-2 family members. Paradoxically, treatment of activated T lymphocytes with high doses of IL-2 seems to enhance their sensitivity to AICD in the

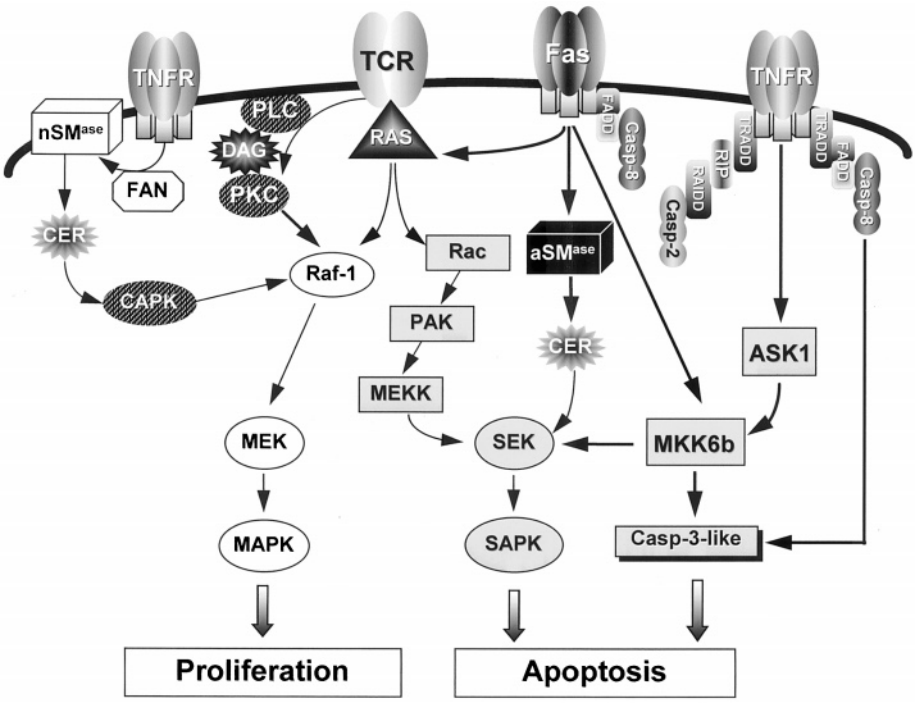


Figure 3. Signalling pathways in apoptosis. Schematic view of the MAPK/SAPK pathways (see text). Proteins that associate with the Fas and TNFR1 receptors are shown (see text).

presence of anti-TCR antibodies [86]. T-cell deletion mediated by TCR cross-linking can be inhibited by injection of neutralizing anti-IL-2 antibodies, and this killing by a self-produced cytokine has been termed propriocidal apoptosis and might be involved in peripheral tolerance to self-antigens. Indeed, mice with either the IL-2 gene or the IL-2 receptor gene disrupted develop autoimmune diseases [87, 88].

Cytotoxic T cells (CTLs) are key effectors for the elimination of virus-infected cells, and they deliver a lethal hit to their targets through a calcium-independent pathway involving Fas-FasL or through a calcium-dependent pathway involving granzymes and perforin [89, 90]. The majority of CTLs belong to the CD8 subset and recognize antigens presented by MHC class I molecules. MHC-I present peptides derived from proteins synthesized within the cell, and upon recognition of a foreign antigen, CTLs undergo granule exocytosis towards the target. These granules contain the pore-forming protein perforin and serine proteases known as granzymes; the calcium dependency comes from the fact that perforin requires calcium to polymerize into the channel-forming polyperforin, which allows the granzymes to penetrate the target cell [89, 90]. The importance of perforin and granzyme B in cytotoxic T-cell activity is illustrated by studies of mice in which these genes have been disrupted [91, 92]. Granzyme B is an aspartase able to directly activate most pro-caspases [23, 24, 93–95] and activate the suicide programme. The substrate specificity of granzyme B is similar to caspase-8 [28], a receptor-associated protease that can activate most caspases [25], and can bypass the steps required to activate caspase-8.

## Signalling events in apoptosis

The fate of a cell could be viewed as a balance between life and death signals, the strongest affecting the outcome. Two major signalling pathways can be distinguished, one for proliferation (MAPK/ERK) and one for stress response (SAPK/JNK) [96]. Both pathways initiate at Ras, located at the apex of the signalling pyramid, and activation of either branch ultimately leads to transcription factor activation. TCR stimulation can activate Ras during positive selection and in mature T cells [97]. Using dominant-negative forms of the MAPK cascade, the involvement of Ras, Raf1, MEK1 and MAPK in transduction of TCR signals has been shown [98] (fig. 3). A role for the MAPK pathway in protecting thymocytes from apoptosis during positive selection has been shown using mice transgenic for dominant-negative forms of Ras or MEK1 [99]; these mice have very few mature $\alpha/\beta$ T cells in the thymus

and in the periphery yet appear to undergo normal negative selection.

On the other hand, pro-apoptotic receptors can activate the SAPK signalling pathway. Fas engagement leads to activation of Ras, which in turn leads to activation of Rac1 and Rac2, required for Fas-mediated apoptosis [100]. Rac proteins are small GTPases that regulate the activity of PAKs (P21-activated kinases) Ser/Thr kinases; PAK2 becomes activated by caspase cleavage during Fas-mediated apoptosis, and a PAK2 dominant negative can block cell death [101] (fig. 3 and table 1). Apoptotic signals delivered by TNF-$\alpha$ can be blocked by SEK1 and c-Jun dominant-negative forms or an inactive ASK1 kinase [102, 103]. ASK1 is a recently cloned kinase able to activate the SAPK pathway through SEK1 or MKK6, but it is still unknown whether ASK1 can regulate other SAPK members [103]. Surprisingly, thymocytes from mice deficient for SEK1 (on a $Rag2^{-/-}$ background) are more sensitive to Fas- and CD3-mediated apoptosis [104]. These mice show a reduced proportion of $CD4^{+}CD8^{+}$ thymocytes, suggesting that SEK1 is either involved in protection from negative selection or is required for positive selection of immature T cells [104]. This paradox might be explained by the involvement of costimulatory molecules (such as CD30 or CD28) during positive selection, which might require SEK1 for signal transduction.

The delicate balance between death and survival pathways can be altered in favour of apoptosis through activation/inactivation of substrates by caspase cleavage. For example, a number of kinases (PAK2, PKC$\delta$ and $\theta$, MEKK1 and PITSLRE kinase) become activated by caspase cleavage and were shown to enhance the apoptotic response (see 'Substrates cleaved by caspases' and table 1). On the other hand, inactivation of kinases involved in survival pathways (such as Raf1 and Akt-1) by caspase cleavage was also shown to occur [105]; this coordinate cleavage shifts the balance towards commitment to apoptosis.

Ceramide (Cer) is a lipid second messenger involved in triggering apoptosis in a variety of cells. Within minutes after TNFR1-TNF-$\alpha$ or Fas-FasL engagement, Cer is generated by hydrolysis of sphingomyelin by acidic sphingomyelinase (aSMase), and the apoptotic effects of TNFR1 or Fas engagement can be mimicked by exogenous Cer [106, 107]. Ceramide production can lead to different biological outcomes, as it can induce proliferation, cell-cycle arrest or apoptosis [106, 108]. TNFR engagement can activate the production of mitogenic Cer via FAN, a protein that activates neutral SMase [109, 110], while Cer generated by aSMase is pro-apoptotic, suggesting that the location of Cer production is critical for the subsequent chain of events (fig. 3). In support of this, transfection of bacterial SMase induced apoptosis, whereas externally applied SMase did not, even though the total levels of Cer were comparable, arguing that the location of Cer production distinguishes the biological outcome [111]. Interestingly, ceramide can lead to upregulation of FasL, suggesting that a potential amplification loop may exist, since Fas-FasL interaction leads to Cer production [112]. Apoptotic proteases appear to play a direct role in ceramide generation, as caspase inhibitors can prevent ceramide accumulation during Reaper-mediated apoptosis and after Fas engagement or TNF-$\alpha$ treatment [113–115] (fig. 3).

Mitogenic Cer can activate a Ser/Thr kinase (CAPK), which phosphorylates Raf-1, a key mediator of the MAPK pathway [116], while apoptotic Cer can activate the SAPK pathway [117, 118]. The decision between one pathway or the other depends in part on other kinases such as protein kinase C (PKC), which activates the MAPK branch. Conventional PKCs require diacylglycerol (DAG) for activity, and hydrolysis of lipids by the action of phopholipases generates DAG that can activate PKCs and suppress apoptosis [119] (fig. 3). Death receptor-mediated activation of SAPK does not necessarily require transcription factor activation, since AP-1 activity is not always correlated with apoptosis [120]. As such, inhibition of apoptosis by dominant-negative forms of SAPK suggests that kinase activity plays a role in commitment towards cell death, possibly through phosphorylation of substrates unrelated to transcription factor activation.

Besides kinase activation, ceramide appears to participate in the disruption of mitochondrial function. Fas cross-linking leads to ceramide production, which in turn leads to GD3 synthase activation and GD3 ganglioside production; inhibition of GD3 production can prevent Fas-mediated apoptosis, and overexpression of GD3 synthase leads to cell death [121, 122]. These lipids affect the mitochondrial transmembrane potential ($\Delta\Psi_m$) following Fas stimulation and participate in caspase activation, presumably leading to a self-amplification loop [121, 122].

## Membrane receptors and apoptosis

Fas and the TNF receptors (TNFR1, TNFR2) are members of the TNF/nerve growth factor (NGF) receptor family. TNF-$\alpha$ is representative of a superfamily of ligands which includes TNF-$\alpha$, TNF-$\beta$, NGF, FasL, OX40L, CD27L, CD30L, CD40L, 4–1BBL and TRAIL [71], which are type-II proteins having a receptor belonging to the TNF receptor family (TNFR). The TNFR members are type-I proteins and possess conserved cysteine-rich repeats in their extracellular domains [123]. The cytoplasmic tails of Fas and TNFR1

possess a region of homology of about 80 aa, essential for transmission of the death signal, which was named the death domain (DD) [124–126] (fig. 3). Using the Fas DD as a bait in the yeast two-hybrid system, the DD-containing protein FADD was identified [127]. Deletion analysis of FADD showed that the DD-containing C-terminus is needed for Fas association, whereas the N-terminus is required for cell death, this region being named the death effector domain (DED) [127]. Transfection of FADD lacking its DED can block both Fas- and TNFR1-mediated apoptosis, showing the importance of the DED [127]. Immunoprecipitation of oligomerized Fas revealed that phosphorylated forms of FADD were associated with Fas and that caspase-8, having two DEDs in its long pro-domain, was associated with the Fas-FADD complex [128]. The ligand-induced aggregation of these molecules to Fas has been termed the DISC [128], and transfection of a FADD lacking a DED blocks recruitment of caspase-8 to the DISC [127]. Thus, FasL-induced trimerization of Fas results in Fas-FADD interaction through their DDs, and FADD recruits caspase-8 to the DISC through their DEDs, leading to caspase-8 auto-catalysis [129] (fig. 3).

TRADD is a DD-containing protein that associates with TNFR1 and does not possess a DED [130]. Overexpression of TRADD or its C-terminus containing the DD is sufficient to trigger apoptosis, indicating that TRADD acts as an adapter molecule [130]. TRADD can associate with FADD to bridge it to TNFR1, leading to caspase-8 recruitment to the complex (fig. 3). TRADD can also interact with RIP, a DD-containing Ser/Thr kinase which binds to TRADD through their respective DDs [131, 132]. Since RIP can induce apoptosis when overexpressed and its DD is responsible for transduction of the death signal, this suggested that another adapter molecule might be recruited [131]. Indeed, the adapter RAIDD (or CRADD) has been identified [133, 134]. RAIDD has a C-terminus DD that binds to RIP and an N-terminal domain that binds to the pro-domain of caspase-2 (and possibly caspase-9), leading to their activation [133, 134] (fig. 3). However, RIP-deficient mice show normal Fas-mediated apoptosis, and it would appear that RIP might be involved in an anti-apoptotic pathway, since these mice display massive apoptosis in lymphoid and adipose tissues [135]. In support for this hypothesis, RIP was shown to be involved in TNF-$\alpha$-induced NF-$\kappa$B activation, which plays a role in preventing cell death.

The complexity of the death machinery has recently been increased by the discovery of new death receptors: DR3, DR4 and DR5. The broadly distributed DR4 and DR5 also have a DD that can transduce an apoptotic signal [136–139] and are the receptors for TRAIL, a cytokine that can induce cell death in Fas-resistant lines. These receptors were originally described as FADD-independent [140, 141], but in certain cell lines, DR4 and DR5 were subsequently shown to associate with FADD, and TRAIL-mediated apoptosis can be blocked by cotransfection of a dominant-negative FADD [142, 143]. These receptors also appear to be linked to caspase-10 through an adapter molecule which has not been identified yet [140, 141]. Finally, a new receptor called TRID (or DcR1) has homology to the extracellular domains of DR4 and DR5, but acts as a decoy receptor for TRAIL, since it lacks a DD. The sensitivity of several tumour cells lines to TRAIL-induced apoptosis can be explained by their lack of TRID expression [140, 141].

While the long pro-domains of certain caspases are involved in death receptor interaction (at least for caspases 2, 8, 9 and 10), the other long pro-domain-containing caspases (1, 4, 5, 11 and 12; members of the ICE subfamily) have not been linked to surface molecules. In the case of caspase-1, its pro-domain is involved in dimerization and autoprocessing [144]. Caspase-3 can associate with the DISC through CASPER, a FADD-, caspase-8- and caspase-3 interacting protein [145]. Transfection of a truncated form of CASPER can inhibit both TNFR1- and Fas-mediated apoptosis, showing that caspase-3 may be involved in proximal signalling events [145]. Thus, it appears unlikely that a single caspase is located at the apex of the death-signalling complex, since various adapter molecules can recruit several caspases to death receptors. Moreover, the signalling pathways appear to differ between cell lines as in some lymphoid cell lines, (SKW6.4 and H9), caspase-8 and -3 are activated very early after Fas cross-linking, and Bcl-2 or Bcl-$X_L$ are unable to inhibit their activation, while in Jurkat and CEM cells, this event occurs later and can be blocked by overexpression of Bcl-2 or Bcl-$X_L$ [146]. Although it is clear that caspases play an essential role in apoptosis, much has still to be discovered about the molecular ordering of events leading to their activation.

## Substrates cleaved by caspases

The number of caspase substrates is still limited, and they can be subdivided according to their function in maintenance of DNA integrity, cell cycle progression, cellular structure, DNA fragmentation, loss of plasma membrane asymmetry and tissue disembedding, functions that are altered during programmed cell death. Although cleavage of some caspase substrates appears to be directly linked to cell death mechanisms, proteolysis of the majority of substrates does not seem to be essential for apoptosis. The first caspase substrate identified was the DNA-repair enzyme PARP, which is cleaved by caspase-3 at a DEVD-G motif [147], al-

Table 1. Substrates cleaved by caspases.

| Substrate | Site | Caspase(s) | Function | Ref |
|---|---|---|---|---|
| PARP | DEVD G | 3 > 7 > 1 > 4 | DNA repair | [149] |
| RFC140 (site I) | DEVD G | 3 | DNA replication | [153] |
| RFC140 (site II) | DLVD S | 3 | DNA replication | [153] |
| RFC140 (site III) | IETD A | 3 | DNA replication | [153] |
| MCM3 | nd | DEVD-inh | DNA replication | [154] |
| DNA-PKcs (site I) | DEVD N | 3 | DNA repair | [155] |
| DNA-PKcs (site II) | DWVD G | 3 | DNA repair | [155] |
| Topoisomerase I | nd | zVAD-inh | DNA topology | [156] |
| U1-70K snRNP | DGPD G | 3 | pre- Messenger RNA (mRNA) splicing | [157] |
| hnRNP C | nd | 7 > 3 > 6 | pre-mRNA splicing | [158] |
| Actin | ELPD G | 1 > 3 | cytoskeletal microfilaments | [159, 160] |
| Lamin A | VEID N | 6 | nuclear envelope mesh | [161, 162] |
| $\alpha$-fodrin | nd | 3 | cortical cytoskeleton | [163] |
| Keratin 18 | VEVD A | 6 > 3 > 7 | intermediate filaments | [164] |
| Gelsolin | DQTD G | 3 | actin assembly | [165] |
| NuMA | nd | DEVD-inh | nuclear structure integrity | [166] |
| Gas2 | SRVD G | ? | cytoskeletal microfilaments | [167] |
| MDM2 | DVPD G | 3 > 7 > 6 | p53 inhibition | [168, 169] |
| Rb | DEAD G | DEVD-inh | cell-cycle progression | [170, 171] |
| PITSLRE kinase | YVPD S | 1 > 3 | cell-cycle progression | [172] |
| Cyclin A2 | DEPD C | DEVD-inh | cell-cycle progression | [173] |
| $p21^{Cip1/Waf1}$ | DHVD L | 3 > 7 | cell-cycle progression | [174] |
| DFF-45 (site I) | DETD S | 3 | DNA fragmentation | [181] |
| DFF-45 (site II) | DAVD T | 3 | DNA fragmentation | [181] |
| SREBP-1a | SEPD S | 3 | sterol biosynthesis | [182] |
| SREBP-2 | DEPD S | 7 > 3 | sterol biosynthesis | [182, 183] |
| PKC$\delta$ | DMQD N | 3 | cell-cycle progression | [184, 185] |
| PKC$\theta$ | DEVD K | 3 | cell-cycle progression | [186] |
| MKK6b | nd | YVAD-inh | stress signalling | [187] |
| MEKK1 | DTVD G | 7 > 3 > 8 | stress signalling | [188] |
| PAK2 | SHVD G | 3 | stress signalling | [101] |
| Bcl-2 | DADG A | 3 | inhibits caspase activation | [189] |
| p28 Bap31 | nd | 8 > 1 | Bcl-2/Bcl-$X_L$-binding protein | [190] |
| D4-GDI | DELD S | 3 | sustain Rho-GTPase | [191] |
| Rabaptin-5 | nd | DEVD-inh | endosome fusion | [192] |
| FAK | nd | 7 > 3 > 6 | regulate cell adhesion | [193] |
| IL-1$\beta$ | YVHD A | 1 | inflammation | [7] |
| IL-18 (site I) | LESD Y | 1 | induction of INF-$\gamma$ | [194] |
| IL-18 (site II) | DMTD S | 3 | induction of INF-$\gamma$ | [194] |
| IL-18 (site III) | DCRD N | 3 | induction of INF-$\gamma$ | [194] |
| I$\kappa$B-$\alpha$ | DRHD S | 3 | inhibits Rel/NF$\kappa$B | [195] |
| Huntingtin | nd | 3 | (unknown) | [197] |
| Presenilin 1 | nd | DEVD-inh | (unknown) | [198] |
| Presenilin 2 | DSYD S | 3 | (unknown) | [198, 200] |
| DRPLA protein | DSLD G | 3 | (unknown) | [199] |
| PRK2 (site I) | DITD C | 3 | actin cytoskeleton | [201] |
| PRK2 (site II) | DEVD S | 3 | actin cytoskeleton | [201] |

though the significance of PARP cleavage in the apoptotic process remains obscure [148, 149]. While other caspases can cleave PARP, caspase-3 remains the most active towards PARP due to its 100 to 1000 times higher catalytic efficiency compared with other caspases [147]. Since caspase-3 is broadly distributed and is homologous to *C. elegans* Ced-3 [150], most substrate studies have involved caspase-3, and among 40 known substrates, 28 were shown to be cleaved by caspase-3 (table 1).

The specificity of caspases has been broadly determined using peptides [28, 151, 152]. Factors besides amino acid composition appear to play a role in cleavage, as the DEVD-G site present in RFC140 is cleaved at a much lower efficiency in PARP [153], suggesting that conformation might affect cleavage site accessibility. Nevertheless, the caspase families have been subdivided based on their apparent substrate specificity [28, 151, 152]. Group I includes caspases 1, 4 and 5 (and probably caspases 11 and 12) and have a (W/L)EHD optimal recognition sequence (fig. 2), while group II includes caspases 2, 3 and 7, which preferentially recognize the sequence DEXD present in several proteins essential for cell survival and are likely to be involved in the execu-

tion phase of apoptosis. Several substrates cleaved by caspases are proteins involved in maintaining genome integrity, such as PARP [147], the large subunit of replication factor C (RFC140) [153], MCM3 [154], DNA-dependent protein kinase [155], topoisomerase I [156] and components of the spliceosome [157, 158]. Group III includes caspases 6, 8 and 9, which recognize the sequence (L/V)EXD, similar to the motif recognized by granzyme B. The similarity between the motif recognized by group III caspases and internal sites present in pro-caspases (fig. 2) suggests that they are involved in activating other caspases and serve to amplify the death signal.

Several structural proteins are cleaved by caspases, such as actin, lamin, fodrin, keratin, gelsolin, NuMA and Gas2, consistent with the morphological events seen during apoptosis [159–167]. Some proteins involved in the cell cycle are caspase substrates, such as Mdm2 [168, 169], the retinoblastoma protein (pRB) [170, 171], PITSLRE kinase [172], cyclin A2 [173] and $p21^{Cip1/Waf1}$ [174]. Mdm2 is both a transcriptional target for p53 and an inhibitor of p53, blocking its transcriptional activity and targeting it for destruction, being part of a feedback loop to terminate the p53 apoptotic signal [175, 176]. In addition, Mdm2 can interact with pRB and block its growth inhibition function [177]. The consequence of caspase-mediated cleavage of Mdm2 would be to abolish p53 and pRB inhibition and allow p53 to induce pro-apoptotic Bax and pRB to cause cell-cycle arrest. p53-dependent cell cycle arrest occurs through induction of p21, which prevents cyclin-mediated phosphorylation of pRB; unphosphorylated pRB interacts with E2F1 and prevents transcription of several genes involved in S-phase progression [178]. Cleavage of pRB by caspases would prevent interaction with Mdm2, while retaining its ability to block E2F1 activity. PITSLRE kinases are related to cdc2 kinases and have been implicated in apoptotic signalling [179], but the significance of caspase cleavage is unclear. Apoptosis is often associated with cells being in the G1 phase of the cell cycle [180]. Progression through G1 and entry into the S phase is regulated by CDK2 activation, itself regulated by $p21^{Cip1/Waf1}$. The recent demonstration that p21 is cleaved by caspases and that overexpression of a p21 mutant resistant to caspase cleavage can partially suppress apoptosis demonstrates the close relation between cell cycle and apoptosis [174].

While most substrates become inactivated by caspases, some become activated, such as DNA fragmentation factor (DFF) [181], sterol regulatory element-binding proteins (SREBPs) [182, 183], PKC$\delta$ and $\theta$ [184–186], MKK6b [187], MEKK1 [188] and PAK2 [145]. DFF is responsible for internucleosomal DNA fragmentation after activation by caspase-3. SREBPs are transcription factors regulating cholesterol biosynthesis, and transient caspase-3 activity during proliferation might lead to their cleavage and activation [183]. Some kinases involved in transduction of the stress signal also become activated by cleavage (fig. 3). Cleavage of PKC$\delta$ by caspase-3 releases the kinase domain, and transfection of this fragment leads to chromatin condensation and nuclear fragmentation [184, 185]. The kinase MKK6b can become activated by caspase cleavage, in turn leading to caspase-3 activation, indicative of a potential amplification loop [187]. Following loss of contact with the extracellular matrix, MEKK1 is cleaved to an active fragment by a caspase, and this fragment can induce caspase-7 processing [188]. Cleavage of PAK2 by caspases activates it, and transfection of a truncated PAK2 leads to membrane blebbing and formation of apoptotic bodies [145]. While the anti-apoptotic role played by Bcl-2 is well known, it was recently shown that Bcl-2 can be a caspase substrate, generating a Bax-like death molecule which serves to amplify the apoptotic cascade [189]. p28Bap31 can associate with Bcl-2 and caspase-8; in the absence of Bcl-2, it becomes a target for caspase-8, and this cleavage product can induce apoptosis after ectopic expression [190].

Other substrates are associated with membrane changes that occur during apoptosis, like D4-GDI, a regulator of the Rho GTPases [191], rabaptin-5 [192] and focal adhesion kinase (FAK) [193]. Inactivation of rabaptin-5 inhibits endocytosis and possibly alters surface expression of receptors like integrins. Interaction between integrins and the extracellular matrix activates FAK, and this can suppress apoptosis in different cell types. Cleavage of FAK might facilitate cell detachment and recognition by macrophages. Beside the well-characterized role of caspase-1 in IL-1$\beta$ processing [7], it can also process IL-18 to its active form, while caspase-3 cleavage generates an inactive product [194]. Cleavage of I$\kappa$B-$\alpha$ by caspase-3 could potentially increase apoptosis by generating a constitutive inhibitor of Rel/NF$\kappa$B transcription complexes [195]. Inhibition of NF$\kappa$B activation by such a repressor would render cells more susceptible to TNF-induced apoptosis [196].

Disease-associated proteins like huntingtin (associated with Huntington's) [197], the presenilins (associated with Alzheimer's disease) [198] and dentatorubral pallidoluysian atrophy (DRPLA) protein [199] were shown to be cleaved by caspases. While the function of these proteins is largely unknown, their possible implication in apoptosis may provide clues to their function. Interestingly, presenilin-2 cleavage generates a fragment that is anti-apoptotic and could be an example of caspase-mediated retrocontrol of apoptosis [200].

## Concluding remarks

The identification of caspase substrates has helped to explain some of the morphological events associated with apoptosis. While the signalling events that mediate

life and death signals are becoming clearer, much remains to be discovered, such as the role of caspase-mediated activation of kinases and identification of their targets. A better understanding of caspase regulation should help to develop strategies to control the apoptotic response for therapeutic purposes. Influencing the fate of T cells through programmed cell death could provide novel ways to treat autoimmune diseases, leukaemic disorders or apoptosis associated with human immunodeficiency virus (HIV) infection.

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