PROTEOLYTIC ACTIVITIES THAT MEDIATE APOPTOSIS

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ABSTRACT
Since the discovery that cells can activate their own suicide program, investigators have attempted to determine whether the events that are associated with this form of cell death are genetically determined. The discovery that the ced-3 gene of Caenorhabditis elegans encodes a cysteine protease essential for developmentally regulated apoptosis ignited interest in this area of research. As a result, we now know that cell death is specified by a number of genes and that this biologic process contributes significantly to development, tumorigenesis, and autoimmune disease. In this review I summarize what is currently known about signaling pathways involved in apoptosis, with particular emphasis on the function of the cysteine proteases known as caspases. However, there is also evidence that protease-independent cell death pathways exist. Is there a relationship between these two distinct mechanisms? If so, how do they communicate? Finally, even though the involvement of tumor necrosis factor/nerve growth factor family of receptors and cysteine proteases has been elegantly established as a component of many apoptotic signaling pathways, what happens downstream of these initial events? Why are only a selected group of cellular proteins—many nuclear—the targets of these proteases? Are nuclear events essential for apoptosis in vivo? Are the cellular genes that encode products involved in apoptotic signaling frequent targets of mutation/alteration during tumorigenesis? These are only a few questions that may be answered in the next ten years.

HISTORICAL INTRODUCTION
The death of individual cells from a larger population was first recognized during vertebrate development studies more than 40 years ago (1, 2). In the
early 1970s, Kerr, Wyllie & Currie found that cells undergo at least two distinct forms of death: the well-characterized, and usually rapid, necrotic tissue damage induced by trauma, and a more protracted and morphologically distinct form of cell death that they termed apoptosis (3, 4). Necrotic cell death is violent, characterized by cytoplasmic swelling, the rupturing of cellular membranes, and the disintegration of subcellular and nuclear components. Conversely, apoptosis is characterized by an ordered series of events that take place over a longer period of time. The length of time required for cells to undergo cell death is usually specified by the stimuli that trigger apoptosis (e.g. glucocorticoid, Fas ligand, growth factor withdrawal) (5–23), as well as the cell type. Although necrosis may be more analogous to random acts of violence that characterize murder, apoptosis is more appropriately referred to as cellular suicide. Apoptotic death is initiated by the cell when it senses that its environment or physical state has been compromised; this is, indeed, the ultimate self-sacrifice.

The criteria for determining whether a cell is undergoing apoptosis versus necrosis include distinct morphological changes in the appearance of the cell, as well as alterations in biochemical and molecular markers (3, 24, 25). For example, apoptotic cells often shrink and undergo cytoplasmic membrane blebbing, their chromosomes rapidly condense and aggregate around the nuclear periphery, and small apoptotic bodies are formed. In many, but not all, apoptotic cells, the condensed chromosomes are acted upon by specific nucleases that cleave the DNA to produce a characteristic ladder (5, 10). Conversely, necrotic cells swell and burst following physical trauma. The resulting cellular debris is quickly cleared by macrophages. Another distinction between necrotic and apoptotic cells is the requirement for new mRNA and protein expression during the early stages of some forms of apoptosis (restricted to specific cell types and stimuli). Apoptotic responses that help regulate immune defenses are most often associated with mRNA and protein synthesis (14, 16, 17, 26, 27). The nature of the stimuli that triggers cell death, e.g. T-cell receptor activation (14, 15, 28, 29), may be another important determinant of a requirement for de novo mRNA/protein synthesis during apoptosis. Such attributes of cell death remain controversial, particularly among those who strongly support the notion that the inherent apoptotic machinery (in the form of inactive precursors) is present in all living cells (30–35). This latter view of apoptosis is consistent with the process of neuronal cell death, as well as death mediated by some members of the tumor necrosis factor (TNF) receptor family, e.g. TNFR1 (36). In support of this view, it has been shown that a TNFR1-mediated proliferative response can be suppressed by the addition of protein synthesis inhibitors (37). Conversely, TNFR1-mediated apoptosis is not affected by the same agents. Thus the proper balance of intra- and/or extracellular signals (e.g. cellular response to withdrawal of a necessary growth factor, γ-irradiation, or chemical
mutagens of DNA) can determine whether a cell survives or is executed. Although there is evidence for rescue of certain forms of cell death (e.g. apoptosis induced by growth-factor withdrawal), once the death program passes a certain point (usually disruption of nuclear organization) there can be no reprieve.

Many of the early mechanistic studies of apoptosis were performed by biochemical and molecular analysis of cultured mammalian cells following their exposure to numerous physical, chemical, and biochemical agents that trigger cell death (38–40). These studies offered further proof that each cell had the inherent capacity to trigger its own death, given an appropriate signal. This early work also provided the first hints that the ability of tumor cells to undergo apoptosis, as compared with normal cells, was compromised. Inhibition of apoptosis might be fundamental for tumor progression. Independently, developmental biologists were identifying the genes responsible for determining cell fate (38, 41–46). In organisms such as the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*, large numbers of cells are eliminated by programmed cell death during development. Such cells can be readily identified by nuclear and cytoplasmic changes similar to those that accompany cell death in higher vertebrates. These organisms are more easily manipulated genetically than vertebrates, allowing the generation of mutants that disrupt the death of specific cells. These mutants can then be used to isolate the genes involved in these biologic processes. In the developing fly and nematode, there are many points where determination of cell fate is dependent on the action of cellular death genes. Therefore, the identification of mutant phenotypes that failed to eliminate specific cells by programmed cell death (PCD) during development facilitated the identification and characterization of the genes that regulate apoptotic pathways.

Studies of *C. elegans* development carried out by Horvitz and colleagues during the past decade have contributed significantly to our understanding of cell death (38, 41). Their genetic analyses resulted in the identification of three cellular genes required for PCD during development of *C. elegans*. A loss-of-function mutation in any of these genes prevents the death of specific cells during development; accordingly, all three genes were designated *C. elegans death* (ced) genes (47–49). The isolation and molecular characterization of these genes demonstrated that the *ced-3* gene was homologous to the mammalian interleukin-1β-converting enzyme (ICE) (47). ICE was originally isolated from mammalian cells as an enzyme essential for the proper processing and biologic activation of pro-interleukin-1β, a cytokine involved in mediating cellular inflammatory response (50). Furthermore, when the *C. elegans ced-3* gene was expressed in mammalian fibroblasts, apoptosis was rapidly induced (51). Independently, Steller and colleagues (44, 52) used genetic screens of *Drosophila* to identify the genes essential for apoptosis during fly development. These
discoveries have provided a solid foundation for the study of apoptosis, as well as demonstrating that defects in cell death contribute significantly to disease. Strong parallels can be seen when the mechanisms that regulate apoptosis are compared with those that regulate the cell cycle. Explosive growth in our understanding of the mechanisms regulating the cell cycle has taken place as a result of the realization that the mammalian cell cycle is governed by complex layers of control that respond to both external and internal factors, e.g. involving CDC2-related protein kinases, cyclins, and cyclin-dependent kinase inhibitors (CKIs) (53, 54). Such tight control of the cell cycle provides constant monitoring of progression through cell division, with important checks and balances to help ensure that genetic catastrophes occur infrequently. In a similar manner, the demonstration that the \textit{ced-3} gene encodes a cysteine protease essential for programmed cell death has acted as a catalyst for the recent rapid growth in this field.

PROTEASE SIGNALING OF CELL DEATH

The first clues suggesting that apoptosis often requires specific proteases came from studies of drug-induced death of tumor cells (22, 23, 42, 55). These early studies focused on the relationship between the action of protease inhibitors and chemotherapeutic drugs, as well as on their relationship to events that occur during specific intervals of the cell cycle. Even though these experiments were limited by the availability of reagents, they revealed the diverse nature of apoptotic signaling (e.g. selective inhibition of RNA synthesis, transcription, and/or translation). The serine protease inhibitors \textit{N}-tosyl-\textit{L}-phenylalanycarbonyl ketone (TPCK) and \textit{N}-tosyl-\textit{L}-lysylchloromethyl ketone (TLCK) were particularly effective in preventing certain forms of cell death, thereby establishing an important link between cellular proteases and apoptotic signaling.

\textbf{The Death Receptors}

The activation of the caspase proteases has been linked to the aggregation of cell surface receptors related to the TNF and nerve growth factor (NGF) families (including TNFR1, TNFR2, CD95/APO-1/FasR, DR-2/APO-2/TRAIL, DR-3/Wsl-1/TRAMP/APO-3, CAR1, CD30, and CD40) (20, 36, 56–80). Caspase activation occurs when receptor-sensitive target cells are exposed to the appropriate ligand, or when the receptors self-aggregate in response to their high cell–surface density (62, 63, 72). These receptors are type-I membrane proteins, each containing an extracellular ligand-binding domain of two to six cysteine-rich repeated subdomains, a transmembrane (TM) domain, and a cytoplasmic domain that is essential for mediating either a proliferative or an apoptotic response (Figure 1) (62, 63, 71, 72). The ligand-binding domains of these TNF
Figure 1  Schematic representation of the prototype TNF receptors Fas and TNFR1 and their interactions with cytoplasmic proteins with death domains (DD) and/or death effector domains (DED). The *Drosophila reaper*, *hid*, and *grim* genes, which act in much the same way as vertebrate TNFRs are shown. The effects of the p35, crmA, XIAP, and FLIP inhibitors with a cellular caspase are shown.

receptors have structurally similar motifs, but they do not share significant sequence identity. Similarly, the intracellular domains of these receptors all contain an ≈80 amino acid region termed a death domain (DD) that is involved in transducing proliferative and/or apoptotic signals through its interaction with cytoplasmic proteins containing similar DD motifs (5, 10, 64, 67, 71, 72, 78, 81–85). Although it has been suggested that these DD motifs are involved in generating a death signal, molecular analysis of the *Drosophila reaper* gene has demonstrated that this motif is essential for protein-protein interactions and is not responsible for a death signal per se (82). The same DD motif is required for TNFR1-mediated NF-κB activation and suppression of apoptosis, which is consistent with its proposed role in mediating protein-protein interactions (36, 58, 86–88). Many of these receptors are expressed at constitutive low levels in a broad range of tissues and cell types, but the expression of some receptors (e.g. TNFR2) can be induced in response to different stimuli.

Ligands for many of the TNF receptors have also been identified (20, 60, 62, 63, 72, 76, 79, 80, 89, 90). They are synthesized as type-II membrane proteins that require proteolysis to generate a soluble cytokine. Unlike mitogenic
receptors that undergo dimerization, followed by the activation of either their own intrinsic tyrosine kinase or the recruitment of a cytoplasmic tyrosine protein kinase, TNFRs appear to function during cell death by recruiting novel proteins without intrinsic protein kinase activity. Ligand binding to the death receptor helps to facilitate oligomerization of these proteins, which is essential for a physiologic response (62, 63; see R Kolesnich & M Kronke, this volume, for a more detailed discussion). The various TNF receptors can also self-aggregate in the absence of ligand, mimicking what occurs when ligand is bound. The ability to self-aggregate appears to be dependent on the presence of a suitable DD and on the density of these receptors on the cell surface (62, 63, 72). This self-aggregation has been exploited to demonstrate the cytotoxic nature of newly isolated receptors because usually neither the ligand nor an appropriate bivalent receptor antibody is available (7, 73–75, 79).

Once the receptor oligomerizes, specific cytoplasmic proteins containing a similar DD are recruited to the aggregated receptor complex (e.g. TRADD, TRAF2, RIP, FADD/MORT1, RAIDD, CRADD, caspase 8/FLICE/MACH/Mch5, and caspase 10/FLICE2/Mch4) (36, 67, 71, 81, 83, 86, 91–98). Many of these cytoplasmic DD proteins function as adapters, linking the receptor to either a downstream protein kinase (e.g. the JNK protein kinase) or a caspase protease (Figure 2). The activation of the JNK protein kinase has been linked to the proliferative response of TNFR1, whereas the caspase proteases are the downstream targets of activation when cell death is specified by FasR, TNFRs, Wsl-1/DR-3/TRAMP, CAR1, or DR-4/TRAIL. It should be noted that a controversy regarding the activation of the JNK protein kinase during TNF-mediated apoptosis has arisen in the literature. Verheij et al (99) have suggested that JNK activation and c-jun phosphorylation occur following TNF- and ceramide-mediated apoptosis. Others have shown that the death effector functions of TNFR1 are not associated with these phenomena (100). Data from other laboratories demonstrating that JNK activity is associated with NF-κB activation and a proliferative response is most consistent with the conclusions of Liu et al (100). Furthermore, the FasR appears to mediate a much stronger apoptotic response than TNFR1 (7, 95, 101). Nevertheless, two separate studies have been published suggesting that NF-κB can be activated by stimulation of the FasR in certain cell types and with selected stimuli (95, 101). The associated factors (TRAFs) are responsible for mediating JNK protein kinase activity, NF-κB activation, and suppressing cell death (36, 100). Conversely, the Fas-associated protein with death domain (FADD/MORT1), FADD-like ICE (FLICE/MACH1), TNFR1-associated death domain protein (TRADD), and RIP-associated Ich-1/CED-3 homologous protein with death domain (RAIDD) are required for apoptotic signaling (66, 68, 97, 102).
A model depicting the differences between Daxx- and FADD-mediated responses to FasR activation is shown. The participation of protein kinases is still speculative, but a number of publications report that specific protein kinases are activated by caspase processing. The target of c-Jun is not known but several possibilities include the caspases, protein kinases, and NF-κB.

In a recent study, Baltimore and coworkers isolated a novel FasR-binding protein by two-hybrid interactive cloning (102a) (Figure 2). This protein, denoted Daxx for Fas death domain-associated protein, interacts with the DD of the FasR even though it does not have a DD sequence of its own. The FasR-binding domain was mapped to the carboxy-terminal region of this 120 kDa protein. This is the only region with significant homology to other proteins, but a 62 amino acid segment contains >70% glutamic acid and aspartic acid. Daxx co-expression can potentiate a FasR-mediated cell death signal, but its overexpression alone is not capable of inducing apoptosis. The most novel aspect of this study was the demonstration of JNK/SAPK pathway activation following transient transfection of Daxx. Intriguingly, a carboxyl-terminal deletion mutant of Daxx (removing the protein region important for FasR-binding) acted as a dominant-negative inhibitor of Fas-mediated cell death and JNK activation, providing further evidence for its involvement. Additionally, the authors
demonstrated that Daxx and FADD define two distinct FasR-mediated apoptotic signaling pathways (Figure 2). This might explain the conflicting results regarding JNK activation in response to FasR stimulation (99, 100). These results may also explain the FasR-mediated proliferative response observed in other studies (95, 101). It is possible, depending on cell type and possibly other factors, that stimulation of the FasR and recruitment of Daxx can lead to either apoptosis or proliferation, whereas the recruitment of FADD results exclusively in cell death (Figure 2). The activation of JNK in response to FasR stimulation might also be linked to the activation of several other protein kinases observed during FasR-mediated apoptosis (Figure 2) (102b–e, 139, 141, 142).

The TRAFs, TRADD, FADD, and possibly Daxx appear to function solely as adaptor proteins, whereas FLICE/MACH1 (recently renamed caspase 8) and FLICE2/Mch4 (recently renamed caspase 10) also have an associated protease function. These proteins contain a death effector domain (DED) that is highly related to a similar sequence found in the ICE/CPP32/caspase proteins (66, 68–70, 72). Caspase 8, caspase 10, and Daxx are unique because of their ability to directly interact with the FasR. Once these proteins associate with the oligomerized receptor, the resulting activation of its DED, or in the case of Daxx an undefined region, potentiates the death signal through the activation of caspases or JNK. Many of these adaptor proteins were isolated by two-hybrid interactive screening, using the DD from either FasR or TNFR1 as bait. In addition, when FADD or TRADD were used as the bait in a two-hybrid screen, they were re-isolated due to the strong affinity for protein-protein interactions in this motif. This is further evidence that both DDs and DEDs are essential for the protein-protein interactions required for receptor function.

Several new members of the TNF receptor family have recently been isolated by two-hybrid interactive cloning using the same DD bait, as well as by homology searches of the human EST database (73–75, 78, 79). Wsl-1/DR-3/TRAMP/APO-3, DR-4/TRAIL, and CAR1 are all capable of inducing apoptosis when overexpressed in mammalian or avian cells. In addition, Wsl-1/DR-3/TRAMP/APO-3 mediates NF-κB activation, suggesting that its function is more similar to TNFR1 than to FasR. Unlike some of the other TNF family receptors, the Wsl-1/DR-3/TRAMP/APO-3 receptor is preferentially expressed on thymocytes, lymphocytes, and some neuronal cells. When the Wsl-1/DR-3/TRAMP/APO-3 receptor signals apoptosis, FADD is recruited to the oligomerized receptor complex. This apoptotic response can be effectively inhibited by zVAD-fmk, a broad-spectrum polypeptide inhibitor of ICE-like proteases, as well as specific viral gene products (see section on Inhibitors of Caspases). However, expression of the apoptosis inhibitor gene bcl-2 does not affect the apoptotic response elicited by this receptor. These data suggest that a proteolytic cascade, similar to those associated with FasR and TNFR1, is
linked to other receptors in this family and that these protease cascades are an essential component of many apoptotic pathways.

The Caspase Proteases

In the past two years, ten cysteine proteases related to the interleukin-1β-converting enzyme (ICE) have been identified as essential components of many apoptotic signaling pathways (Figure 3, Table 1) (66, 102–112). The *C. elegans* ced-3 gene was isolated, and its predicted protein sequence was found to be very similar to the mammalian ICE protein (47). Prior to the identification of ced-3 as a homologue of mammalian ICE, protease involvement in apoptotic signaling was demonstrated by the use of nonspecific protease inhibitors (113–117). These studies were often inconsistent because of the broad, nonspecific nature of the protease inhibitors used. Even so, these studies suggest that proteases are essential elements of apoptotic signaling pathways. Specific serine protease

![Schematic representation of a caspase](image)

*Figure 3* Schematic representation of a caspase. Shown are the locations of the pro-domain, the active site, and the cleavage sites involved in autocatalytic activation or used by another caspase for activation. For comparison a schematic is shown of the structures of caspases 8 and 10 (FLICE/MACH) and FLIP<sub>L</sub>/Casper/CASH, which are much like other caspases except for the DED found in the amino-terminal portion of these proteins. In the case of the FLIP<sub>L</sub>/Casper/CASH protein, the active site of the caspase has been extensively modified (as shown). The DED is lost once caspase 8 or 10 is activated (see 62, 66, 67, 70 for details). Also shown is the related structure of the adapter protein FADD.
Table 1  Caspase protease family

<table>
<thead>
<tr>
<th>Protease name</th>
<th>Other names for enzyme</th>
<th>Recognition site</th>
<th>Peptide, chemical inhibitors</th>
<th>Viral inhibitors</th>
<th>Substrates</th>
</tr>
</thead>
</table>
| caspase 1 ICE, CED-3 | YVAD ↓ G  
YVPD ↓ S | zVAD, YVAD, DEVD, NO | p35, CrmA, E8-FLIP | pIL-1β, pYama, PARP, pNEDD2, pICE, actin, PITSLRE |
| caspase 2 ICH-1, NEDD-2 |  | zVAD | p35 | pNEDD2, PARP |
| caspase 3 CPP32, Yama, apopain | DEVD ↓ G  
DMQD ↓ N  
YVPD ↓ S | zVAD-CHO, zVAD, TPCK, NO | p35, CrmA | pCPP32, PARP, PKCδ, PITSLRE, DNA-PK, SREBP, pRb, rho-GDI, fodrin? |
| caspase 4 ICErel-II, TX, ICH-2 |  | zVAD, YVAD, DEVD | p35 | pICE, pTX, PARP |
| caspase 5 ICErel-III, TY, ICH-3 |  |  | CrmA |  |
| caspase 6 Mch2 | VEID ↓ NG | zVAD, TLCK | p35, CrmA | PARP, lamins |
| caspase 7 Mch3, ICE-LAP3, CMH-1 | EVD, DEVD-CHO |  | CrmA | PARP, p caspase 6 |
| caspase 8 FLICE1, MACH1, Mch5 | zVAD, DEVD, IETD |  | CrmA, E8-FLIP | pICE-like, pCPP32-like |
| caspase 9 ICE-LAP6, Mch6 |  |  |  |  |
| caspase 10 FLICE2, Mch4 | DEVD |  | p35 | PARP, pICE-like, pCPP32-like |

A listing of many of the currently known mammalian caspases. Pertinent characteristics, such as their other names, cleavage sites (if known), inhibitors, and substrates, are shown. Details can be found in the text and references therein.
inhibitors can block the apoptotic activity of numerous ICE-related cysteine proteases (e.g. the ability of TPCK to block drug-induced and Fas-mediated cell death) (118, 119), which suggests that these enzymes represent a separate, and possibly distinct, subgroup of proteases that functions as mediators of death signals, much like the protein kinase cascades that are responsible for mitogenic signaling.

Many of the ICE-like proteases, referred to as caspases 1–10 (120), were isolated by molecular cloning using PCR-based methodologies (Table 1) (111, 112). The term caspase is based on a common nomenclature system adopted by investigators in the field; c reflects a cysteine protease mechanism, and aspase refers to the ability of these proteases to cleave a protein following an aspartic acid residue (50, 121). These caspases can induce apoptosis when they are ectopically expressed in mammalian cells. However, it should be pointed out that ectopic expression of several serine proteases with no known apoptotic function, including trypsin, chymotrypsin, and proteinase K (113), can also induce cell death. The caspases have been divided into three groups based on similarities in their structure: ICE-like, CPP32-like, and Ich1-like (39, 111, 112, 121). Many of the caspase enzymes contain a conserved sequence, QAC(R/Q)G, required for the catalytic activity of these enzymes (Figures 1 and 3) (50, 122, 123). However, both FLICE1/MACH1 (caspase 8) and FLICE2/Mch4 (caspase 10) contain the sequence QACQG at their active sites (66, 69), whereas a novel FADD- and caspase-related inducer of apoptosis (Casper) (123a–d) contains a sequence QNYVW (see Figures 1 and 3). All the caspases cleave their substrates following an aspartic acid (D) residue. The mechanism responsible for Casper-induced apoptosis in not known. The preferred recognition sequences for three of the caspases have been identified: Caspase 1 recognizes the sequence YVAD, caspase 3 recognizes the sequence DEVD, and caspase 6 recognizes the sequence VEID (121, 124, 125). One caveat to the assignment of recognition sequences to individual caspases is the very strong possibility that their specificity is flexible (see Cellular Protease Targets for further discussion).

A mammalian caspase polypeptide consists of a variable amino-terminal prodomain and two enzyme subunits (Figure 3) (50, 108, 121). These functional subunits include an ≈17–20-kDa protein species (Figures 1 and 3; p20), which contain the active site of the enzyme, and a ≈10-kDa subunit (Figure 3, p10). In a site-directed mutational analysis of murine caspase 2, amino acid residues necessary for activation and function were identified (122). Residues involved in generating the p20 subunit by autocatalysis were necessary for biologic activity. In addition, the specificity of aspartate residues involved in intramolecular cleavages was confirmed. Interestingly, mutations at these various sites had differential effects on the overall cleavage pattern of the protein. Once these subunits are generated by cleavage of the proenzyme, they often heterodimerize.
The activation of these caspases is directly linked to the aggregation of the TNF receptors (see above). In some cases (e.g., FLICE/MACH1/caspase 8), two DEDs occupy the carboxy-terminal domain of the protein, whereas the amino-terminal region contains a caspase-related domain (36, 67, 97, 128). A functional caspase enzyme can be generated following receptor oligomerization by autocatalysis, by the action of another caspase, or by granzyme B cleavage of the caspase precursor (zymogen) (116, 127–130). Thus a proteolytic cascade linked to these receptors is an integral part of the cellular apoptotic signaling apparatus associated with diverse stimuli. The activation of caspases can occur sequentially, as well as in parallel pathways, and some cellular targets are processed by multiple caspases/proteases (39, 68, 110, 111, 131). It should be noted that examples of caspase-independent apoptotic signaling have also been observed (132) (described in more detail below).

Where are these caspases located within a viable cell, and what happens once apoptosis is triggered? This is an important question, particularly because the morphologic changes of apoptosis can occur in the cytoplasm independently of nuclear events (30, 31, 133). One approach used to address this question involves the enucleation of cells prior to apoptotic stimuli (30, 31). Somewhat surprisingly, the enucleated cells exhibit many of the physical and biochemical characteristics, e.g., membrane blebbing, of cells containing a nucleus. Additional studies of cell death in neuronal cells have demonstrated that de novo synthesis of RNAs and/or proteins is not required for the induction or execution of cell death (134, 135). Such experiments are the basis of a popular model, which suggests that much of the cellular apoptotic machinery exists in a proform in viable cells and that apoptosis occurs independently of nuclear factors (35, 136). Although this is an appealing model with experimental evidence supporting this interpretation, it may not be indicative of all cells undergoing apoptosis. Furthermore, many of the known caspase substrates are nuclear proteins (e.g., PARP, lamins; protein kinases PKCδ, PCKθ, PITSLRE, MDM2, and MEKK1; topoisomerase U1–70K; and the retinoblastoma protein) (102b–e, 137–144, 144a). Considering the limited number of cellular proteins that are cleaved by caspases during apoptosis, the selection of predominantly nuclear targets as substrates suggests that the nucleus is an active participant in death.

In addition, some stimuli (such as the negative selection of autoreactive T cells) that trigger apoptosis are dependent on active gene transcription and translation (16, 17). Finally, in a study of etoposide-induced apoptosis in HL-60 human acute myelomonocytic leukemia cells, multiple caspase homologues were found in both the cytoplasm and nucleus (145). The mechanism responsible for the appearance of active nuclear caspases has not been definitively established; however, the data suggest (a) that there is transport of the active caspase into the nucleus and (b) that specific, inactive caspases are already in the nucleus awaiting activation promoted by a factor or another caspase.
The identification of caspase substrates has contributed significantly to determining the sites of cleavage. Many of the caspases cleave at highly related, possibly even identical, sites in vitro and in vivo (68, 121, 110, 131, 141, 142, 145–154). Thus it is not clear whether each caspase has a specific recognition site that directs its cleavage or if the caspases are functionally redundant. Possibly both assumptions are true. These observations are quite similar to the developments that have taken place in cell cycle research, where a number of cellular proteins were mis-identified as substrates of the cyclin-dependent p34\textsuperscript{cdc2} protein kinase (CDK1) (156–158), prior to the identification of additional CDK family members. During the past six years, many of these substrates have been identified as bona fide targets of other cyclin/CDK complexes, or even multiple cyclin/CDK complexes (159). Furthermore, only one caspase (Ced-3) has been isolated from the nematode \textit{C. elegans} thus far, whereas at least ten distinct caspases have been found in mammals. This discrepancy could reflect signal and/or cell-type specificity in mammals that is not required in \textit{C. elegans}. The apparent functional redundancy of these proteases may be necessary to prevent the catastrophic effects that might occur if all caspase function were lost. The large family of mammalian caspase proteases may have evolved to protect cells from the potentially lethal effects of environmental stress. Such stress could result in mutations and/or loss of normal proliferative controls. In fact, there is experimental evidence to support this hypothesis. Disruption of the caspase 1 gene in mice is not lethal and, in fact, may have no significant effect on the development or viability of the mouse (160, 161). However, thymocytes from these caspase 1\textsuperscript{−/−} mice no longer respond properly to UV-irradiation or glucocorticoid. In contrast, elimination of the caspase 3 gene from mice primarily affects the development of certain neuronal cells (see Regulation of Development by Caspases) (162).

Although the identification of caspase targets will undoubtedly provide important new information regarding caspase function, and ultimately the mechanism(s) responsible for apoptosis, it may be just as important to understand why many cellular proteins are not targeted for cleavage by these proteases. Of particular interest is the mechanism(s) responsible for the selection of specific cellular proteins for caspase cleavage. Is caspase processing linked to phosphorylation and/or dephosphorylation of these cleavage sites? More importantly, do caspase activities regulate additional cellular processes such as progression through the cell cycle? The answers could have significant impact on how the process of cell death is viewed, as well as the possible relationship(s) between death and other cell functions.

\textit{Granzymes and Cathepsin D}

There is substantial evidence supporting the role of additional proteases, other than the caspases, in various forms of apoptosis (128–130, 163–166). These
proteases include the granzymes and cathepsin D. Granzyme B is a serine esterase that can activate several members of the caspase family (see 167 for a detailed review). Studies of granzyme B are extremely relevant to any discussion of programmed cell death because cytotoxic T lymphocytes (CTL) induce apoptosis by releasing granzyme B and perforin. Perforin is essential for this process because its activity is required to form pores in the membrane of the cell under attack. These pores then allow granzyme B to enter the cell and initiate apoptosis. The in vivo targets of granzyme B remain obscure. Recently, granzyme B was shown to activate caspase 3 and caspase 9 directly in vitro (128–130, 165, 168, 169). Treatment of mouse embryonic fibroblasts deficient in caspase 1 (the caspase 1 gene has been knocked out) with perforin and granzyme B does not result in apoptosis (130), which suggests that caspase 1 is downstream of caspase 3. However, if thymocytes from the same caspase 1−/− mouse are used in the same experiment, caspase 3 is processed and activated, and the nuclear enzyme poly(ADP-ribose) polymerase (PARP; a nuclear marker for apoptosis in many cell types) is processed appropriately. Granzyme B is also capable of cleaving and activating caspase 3, and although caspase 1 is also cleaved by granzyme B, it is not activated (165, 168, 170). One interpretation of these results is that a caspase 1-dependent pathway is necessary for caspase 3 activation in fibroblasts, whereas a caspase 1-independent pathway is intrinsic to the thymic cells from the same mouse. Even though this may be correct, it is important to consider the possibility that multiple, parallel apoptotic signaling pathways could coordinately regulate cell death in a number of different cell types. Such parallel signaling pathways could provide functional redundancy, or they could represent another level of cellular control in response to different stimuli.

Granzyme B may help activate the nuclear events associated with CTL-induced apoptosis, consistent with the observations indicating that it can be passively transported into the nucleus (164). Once the granzyme is inside the nucleus, it apparently binds to nuclear/nucleolar proteins. The translocation of granzyme B into the nucleus, however, is not accompanied by alterations in either the active or passive nuclear transport properties of the cell. It will be of interest to determine whether granzyme B targets nuclear proteins or components of the nuclear envelope. If granzyme B does affect nuclear proteins, it might suggest that the nucleus is required for the proper execution of cell death in certain cell types. Although enucleated cells have been found to be fully competent to execute certain aspects of a cell death program, this situation is rarely encountered in vivo. The apoptotic machinery required for certain death signals may be contained entirely within the cytoplasm; however, both nuclear and cytoplasmic factors may be necessary for a response to other death signals.
Besides the caspases and granzymes, the aspartic protease cathepsin D and the serine protease AP24 (apoptosis protease 24) may also be involved in interferon-\(\gamma\) (IFN-\(\gamma\)-), TNF-, and Fas-mediated cell death (40, 42, 166, 171). Cathepsin D is best known for its proteolysis of endocytosed proteins in the lysosome, proteolytic activation of secreted proteins, and an extracellular activity associated with the migration of metastatic cells (166). Its function during cytokine-mediated programmed cell death was revealed by its isolation in a genetic screen for positive mediators of IFN-\(\gamma\)-induced apoptosis of HeLa cells. Expression of cathepsin D antisense constructs effectively inhibited IFN-\(\gamma\)-, TNF-, and Fas-mediated apoptosis. In addition, when these cells were treated with pepstatin A, a specific inhibitor of aspartic proteases, a significant reduction in the level of apoptotic cells was observed. Thus cathepsin D and AP24 may have previously unrecognized roles in certain forms of apoptosis.

**Receptor/Protease Complexes**

How do death receptors activate an intracellular death program? What prevents the inadvertent activation of this apoptotic signaling pathway? Two of the most-studied receptor complexes are TNFR1 and FasR (7, 36, 56–60, 64, 65, 69, 71). TNFR1 presents its own particular challenge because it can mediate both proliferative and death responses (60, 63, 88, 100). The FasR complex, denoted as the death-inducing signaling complex (DISC) by Krammer and colleagues, has provided an excellent model for determining how these complexes assemble once the ligand is bound, as well as the subsequent intracellular changes that follow (60, 62, 64, 67, 70, 172, 173). As mentioned above, the DD has been implicated in the recruitment of specific proteins that interact with the TNF-related receptors and, depending on which cytoplasmic DD-containing proteins are recruited, potentiate either a proliferative or death signal (36, 67, 81, 83, 86, 91, 93–98). For example, once the TNF and Fas receptors are activated by binding their respective ligands, cytoplasmic proteins containing similar DDs (e.g. TRADD and FADD) are recruited to interact with the DD of these receptors, as well as with each other (Figure 1). FADD, but not TRADD, also contains a motif at its amino terminus, the DED, which is responsible for signaling downstream apoptotic targets (66, 67, 92, 174). Thus although the DD is associated with a number of TNF receptors and adaptor proteins, the DED is found only in FADD, caspase 8, and caspase 10 (Figure 3). Most of these proteins are involved in signaling cell death, but the related TRAFs are not. This appears to be an important distinction, with the presence or absence of these DED domains determining whether these receptor complexes stimulate cell proliferation or trigger cell death. For example, the TNF-related receptors (particularly TNFR1) can function in an anti-apoptotic capacity by recruiting
cytoplasmic proteins (e.g. TRAF) that contain DDs but not DEDs. Conversely, the FasR is primarily associated with apoptotic signals, which is consistent with its interactions with FADD, caspase 8, and caspase 10, all of which have DEDs.

The dynamics of receptor/complex interactions and their effect on caspase activity have been best defined for the FasR and its corresponding DISC (60, 62, 64, 67, 70, 172, 173). Molecular analysis of the DISC following persistent activation by agonistic FasR antibodies has revealed that several novel peptides generated for the DISC correspond to cleavage products of the caspase 8 prodomain (70). Furthermore, the release of the active subunits of caspase 8, namely p20 and p10, into the cytoplasm is associated with the activation of a protease cascade in these cells. All the cytosolic caspase 8 appears to be recruited to the DISC and converted to the active p10 and p20 subunits, which eventually depletes all of the caspase 8 precursor from the cytoplasm. DISC-associated caspase 8 activation was blocked by several peptide inhibitors (e.g. zVAD-fmk, zDEVD-fmk, and zIETD-fmk), but not by the peptide inhibitor Ac-YVAD-CHO or CrmA overexpression. CrmA is well known for its ability to inhibit Fas-mediated apoptosis (see Inhibitors of Caspases), so its inability to block caspase 8 processing and activation suggests that a CrmA-inhibitable caspase is blocked downstream of caspase 8. When fluorescent substrates were used to monitor the caspase activities following FasR activation, a caspase 1-like activity was rapidly and transiently activated, whereas a caspase 3-like activity gradually increased with time (64, 68, 125, 175). Similar sequential activation of caspases was also seen when cell-free systems were used to examine lysates from Fas-activated and nonactivated cells (32). It will be of interest to determine whether the activation of additional FasR (DISC)-associated caspases (e.g. caspase 10) results in the same sequential activation of downstream caspases and substrate proteolysis.

Many important questions regarding the changes that occur in these receptor/protease complexes once ligand binds have not been answered. Obviously, these complexes are dynamic entities, assembled and physically altered during apoptotic signaling. Are there other proteins involved in this signaling process? In all likelihood there are important downstream effectors that are not part of the DISC. Of particular interest are the PKCδ, PKCθ, MEKK1, and PITSLRE protein kinases, all of which are activated by caspase processing during apoptosis (119, 139, 141, 142). Are these protein kinases linked to the caspase cleavage of other substrates? Such crosstalk between the caspases and protein kinases during cell death would suggest yet another level of cellular control. Perhaps the most important and exciting questions concerning the regulation of cell death involve the elucidation of a link between receptor/caspase activation and the many physical changes (e.g. changes in cellular organelles, nuclear morphology, chromatin condensation, and DNA cleavage) that occur as a result of apoptosis.
Cellular Protease Targets

Clearly, the early apoptotic events associated with the TNF- and Fas-related receptors (e.g. receptor interaction with TRADD, FADD, and caspases 8 and 10) have been characterized more extensively than the events occurring in the later stages of cell death (e.g. dissolution of the nuclear envelope, disruption of the transcription, processing and translation of RNAs, chromatin condensation and DNA cleavage by nucleases). Our understanding of how specific TNF receptor/caspase complexes are formed and activated can now be exploited to identify the relevant cellular target(s) of these proteases, as well as their downstream targets (71). What are the downstream effectors? A downstream effector of a receptor-caspase complex should be a protein whose function is altered in a meaningful manner by the action of the caspases (e.g. the activation of additional caspase proteases) (112, 112, 154, 176). It should be emphasized, however, that a number of important components in apoptotic pathways may not be linked directly to caspases.

As mentioned above, all the known caspases cleave their substrates after an aspartic acid residue. Relatively few of these substrates have been identified. Those that have been fall into three categories: (a) other caspase proteases, (b) cellular proteins that need to be inactivated for cell death to occur, and (c) cellular proteins whose activation is required for execution of cell death. Examples of proteins in the first group include many of the pro-enzyme forms of the caspases: pro-caspase 1, pro-caspase 3, pro-caspase 8, and pro-caspase 10 (66, 68–70, 110, 111, 129, 175, 177). This list will undoubtedly grow as new caspases are identified and as the nature of receptor-caspase complexes associated with various apoptotic stimuli is elucidated.

The second group of substrates includes poly(ADP-ribose) polymerase (PARP), the 70-kDa protein component of the U1-ribonucleoprotein (U1–70K), the catalytic subunit of the DNA-dependent protein kinase (DNA-PK), the GDP dissociation inhibitor for the Rho family GTPases (D4-GDI), the retinoblastoma protein (pRb), fodrin, actin, and lamins (137–139, 143, 144, 146, 148, 154, 178, 179). Cleavage of these proteins by caspases results in their functional inactivation, which may be critical for progression of cell death. PARP, U1–70K, and DNA-PK function in the splicing of mRNA and the repair of double-strand DNA breaks, whereas D4-GDI is required for Rho GTPase signaling and cytoskeletal events; their functional inactivation would be useful in the context of cell death (137, 138, 146, 148, 154, 179). The pRb protein participates in the regulation of the cell cycle, and it exhibits an anti-apoptotic effect (143, 144). Obviously, neither this effect nor a vigorous cell cycle is desirable during apoptosis (143, 144). Finally, fodrin, actin, and the lamins are important structural components necessary to maintain cell shape and integrity. The disruption of the cytoskeleton and nuclear envelope are events that have been associated with most forms of apoptosis (110, 150–153).
The third group of proteins, all of which could serve as downstream effectors of caspase-mediated apoptosis, include the protein kinases PKCδ, PKCθ, MEKK1, and PITSLRE; the sterol regulatory element-binding proteins 1 and 2 (SREBP-1 and SREBP-2); and the DNA fragmentation factor (DFF) (102b–e, 119, 139, 141, 142, 180, 181). Activation or alteration of specific protein kinase activities by caspase processing raises the intriguing possibility that crosstalk between these proteases and protein kinases contributes to cell death. Overexpression of PKCδ, PKCθ < MEKK1, and PITSLRE cDNAs that mimic their apoptotic cleavage products results in cell death (119, 141). If caspase processing of these protein kinases alters their substrate specificity significantly, it would suggest that they are active participants, and not merely by-standers, in apoptotic signaling. It is possible that these protein kinases regulate caspase activity by phosphorylating important cleavage sites or nearby residues in substrates; considering the number of cellular proteins that have likely caspase cleavage sites and the actual number of proteins cleaved, this hypothesis is attractive. The cleavage of the SREBP-1 and SREBP-2 proteins releases an amino-terminal fragment that can activate transcription of specific genes (180). Activation of a transcription factor could facilitate the expression of specific genes during apoptosis, quite similar to the induction of the steroid orphan receptor Nurr-77 that occurs during T-cell receptor-mediated apoptosis (16, 17).

Protease-Independent Signaling

Many of the recent studies of apoptotic signaling pathways have focused on the role of the caspase proteases and their cellular targets. However, caspase-independent pathways that contribute significantly to cell death may also exist. Evidence for this comes from a study of the Bcl-2 antagonists Bax and Bak, as well as the C. elegans ced-4 gene (132, 182–185). Bax is a member of the Bcl-2 protein family whose expression can induce cell death, presumably by forming heterodimers with Bcl-2 (188). The ratio of Bcl-2 antagonists to Bcl-2 agonists in these heterodimers helps to determine the sensitivity of a cell to death signals (184, 187–191). Bax overexpression results in cell death, as determined by characteristic morphological changes, nuclear condensation, and DNA fragmentation (132, 183, 186, 187, 192). A concomitant increase in the proteolytic cleavage of DEVD-AFC, a specific fluorogenic peptide substrate of caspase 1, was also observed, but caspase 3 activity was not increased when measured in a similar manner. Suppression of caspase activity in these cells by zVAD-fmk, an effective inhibitor of caspase 1 and all other tested caspases, prevented chromatin condensation and DNA fragmentation (132). On the other hand, changes in membrane permeability, cell membrane blebbing, and cytoplasmic vacuolation were not inhibited. Similarly, apoptosis induced by specific oncogenes,
radiation-induced DNA damage, or overexpression of Bak did not require caspase activity (182, 193, 194). Ectopic expression of the C. elegans ced-4 gene in the yeast Schizosaccharomyces pombe induced rapid chromatin condensation and cell death, which was inhibited by expression of the ced-9 gene (185). The possibility that either an undefined caspase activity or another protease participates in Bax- and Bak-mediated death cannot be ruled out. However, these results suggest that Bax activates two separate apoptotic pathways, one of which is caspase-dependent and the other caspase-independent. Whether a similar situation exists for other mediators of cell death is not known.

INHIBITORS OF CASPASES

Normal, nontransformed cells have an inherent capacity to initiate and block apoptotic signals. In the proper context, these functions allow a cell to defend itself against viral invasion or potentially deleterious alterations in its genome. As an example of the former, virally infected cells can undergo apoptosis mediated by activated cytotoxic T cells to defend themselves against viral infection (195). Cell-autonomous mechanisms of apoptosis, which do not rely on the delivery of apoptotic-inducing proteins from another cell, can also contribute to cell death. However, many of these cellular suicide programs are subverted by oncogenes and viruses to promote their own survival. As we have learned more about the mechanisms involved in signaling cell death, it has become apparent that these agents recruit cellular inhibitors of apoptosis to promote their own survival in an otherwise unhealthy cell.

The Bcl-2 Family of Genes

The bcl-2 gene was first isolated as the proto-oncogene involved in a t(14;18) translocation found in human follicular lymphomas (196–198). Numerous studies have shown that Bcl-2 can protect cells from (or at least delay) apoptosis induced by a number of stimuli, including growth factor withdrawal (e.g. from IL-3-dependent 32D myeloid cells) (199, 200); trophic factor withdrawal (e.g. neurons) (201); c-myc overexpression (9, 202–204); p53-regulated (205); and Fas- (125, 206, 207) and glucocorticoid-induced cell death (208). The Bcl-2 family of proteins have been localized to cytoplasmic and nuclear membranes, where they may function to generate membrane pores (209–215). X-ray and NMR analysis of Bcl-xL indicates that its tertiary structure is very similar to pore-forming bacterial toxins, suggesting that it too may function by forming similar pores (215). Thus far the C. elegans ced-9 gene is the only bcl-2 homologue in the nematode, and it effectively prevents programmed cell death in this organism (49, 216–218); however, a single amino acid substitution in Ced-9 protein transforms it into a death agonist (192). This ability of Bcl-2 to
protect cells from apoptosis suggests that its normal function is to negatively regulate essential apoptotic gene products. Of course, other interpretations of these observations are compatible. Additional evidence supporting Bcl-2 function as a pore-forming agent has come from studies using cell-free apoptotic systems (211–213). The release of cytochrome c from mitochondria, which is essential for apoptosis to occur (219), can be prevented by Bcl-2. This effect is circumvented when sufficient cytochrome c is added back into the system. Thus Bcl-2 appears to inhibit cytochrome c translocation from mitochondria, which is consistent with its possible role in forming membrane pores.

At least 11 distinct bcl-2-related genes have been identified in mammals; they include bcl-2, bcl-xL, bcl-w, bfl-1, BRAG-1, and mcl-1 (antagonists of apoptosis); and bax, bik, bad, bax, and bcl-xs (agonists of apoptosis) (184, 186, 187, 189, 220–222a, 231c). All these family members can either homo- or heterodimerize with one another, depending on their abundance in a particular cell (187, 222). In fact, the ratio of Bcl-2 agonists to antagonists may help determine cellular sensitivity to various apoptotic stimuli. Bax-induced cell death in mammalian cells is caspase independent (132). Intriguingly, expression of mammalian bax or bak in yeast is lethal, but this cell death is not accompanied by the activation of caspase-like activities or internucleosomal DNA fragmentation, and it cannot be rescued by the baculovirus p35 protein (182, 183, 193). Further studies are certainly warranted to determine whether this response defines a conserved, caspase-independent apoptotic signaling pathway in eukaryotes.

The phosphorylation status of several Bcl-2 family members, including Bcl-2 and Bad, may also influence their ability to bind to other Bcl-2 family members (223–225). Furthermore, Bcl-2 may target protein kinases and phosphatases to cytoplasmic membranes (223, 224, 226–228). The Raf-1 protein kinase is targeted to mitochondria by Bcl-2, whereas a constitutively active form of Raf-1 enhances Bcl-2-mediated resistance to apoptosis and hyperphosphorylation of Bad, a death agonist (224, 225). In separate studies, Bcl-2 was found in association with the calcium-regulated phosphatase calcineurin on cytoplasmic membranes, suggesting that it might affect gene expression in these cells (227, 228). Calcineurin associated with Bcl-2 was active, but it was incapable of facilitating the nuclear translocation of NF-AT, one of calcineurin’s proposed functions. NF-AT is a transcription factor essential for activation-induced T-cell death. When Bax, a Bcl-2 family agonist, was co-expressed in these same cells, the interaction between Bcl-2 and calcineurin was disrupted, and NF-AT was translocated into the nucleus.

Overexpression of bcl-2 or other related death antagonists will effectively block apoptosis caused by a variety of stimuli in vitro and in vivo (125, 207, 229–231). Normally, Fas-mediated cell death is accompanied by mitochondrial damage. This Fas-induced damage can be effectively blocked by either
overexpression of bcl-2 or caspase inhibitors, suggesting that Bcl-2 functions upstream of the caspases (207). Genetic studies in C. elegans are also consistent with this interpretation, with the Bcl-2 gene homologue ced-9 functioning upstream of the caspase gene homologue ced-3 (49, 216–217). Thus the endogenous levels of these proteins in a given cell should influence the cell’s sensitivity to apoptotic signals. In support of this hypothesis, the C. elegans CED-9 protein was recently found in association with CED-4, a death agonist (218). This association inhibited the ability of CED-4 to induce apoptosis, presumably by blocking the ability of CED-4 to activate the CED-3 caspase-like protein.

In the process of biochemically defining mammalian cytosolic proteins that activate caspase 3, Wang and colleagues identified three distinct apoptosis activating factors (Apaf-1–3) (231a,b). Apaf-2 was shown to be the electron transfer protein cytochrome c, which when added to an in vitro apoptotic system facilitates apoptosis (231a). Bcl-2 may protect cells from death, in part, by preventing cytochrome c release from mitochondria and/or binding to and inhibiting CED-4. Recently, Apaf-1 was isolated and shown to be a mammalian CED-4 homologue that can bind to cytochrome c, possibly resulting in the activation of caspase 3 (CED-3 in C. elegans) (231b). Activation of caspase 3 also requires a third factor, Apaf-3, a 45-kDa protein not yet identified. Thus, as suggested by genetic studies in C. elegans, CED-4/ Apaf-1 may function as an adaptor protein to facilitate the interaction of CED-9/Bcl-2 and CED-3/caspase 3. Clearly, mammalian and nematode death pathways are highly conserved. Finally, an intriguing question concerns the possibility that one or more of the Bcl antagonists are cleaved by caspases to generate Bcl agonists. Examination of the human Bcl-2 and Bcl-xL protein sequences reveals that both contain a possible caspase recognition sequence, LFRD↓G, at a location that almost mimics the region removed from Bcl-xS (231c). Thus caspase activation could lead to the subsequent cleavage of Bcl-2 and/or Bcl-x L, inactivating their antagonist function(s) and/or creating agonists. If this occurs, it could further define the nature of CED-3/CED-4/CED-9 (caspase/Apaf-1/Bcl-2 in humans) interactions and their consequences.

CrmA, p35, and the Cellular IAPs

The identification of the caspase proteases as downstream effectors of TNF receptor-related apoptotic pathways has facilitated the identification of cellular, viral, and synthetic inhibitors of cell death. The caspase inhibitors initially identified in these experiments correspond to viral genes whose function is to prevent host cell death (137, 138, 232–240). Viruses inhibit host cell death by blocking caspase activity, by activating a Bcl-2 antagonist, or by influencing cellular transcription factors that regulate apoptotic or anti-apoptotic gene
functions. One novel class of viral genes whose product effectively inhibits apoptosis corresponds to the \textit{crmA} gene from the cowpox virus and to the p35-kDa inhibitor of apoptosis (IAP) from baculovirus (137, 138, 232--237). The cowpox virus-encoded cytokine response modifier \textit{A} (crmA) is a 38-kDa serine protease inhibitor (serpin) that can effectively block both TNF- and Fas-mediated apoptosis, apparently by forming a stable complex with the caspase (232, 234, 241). CrmA preferentially inhibits caspase 1-like proteases versus caspase 3-like proteases, as well as granzyme B (233, 234).

The p35 protein from baculovirus is another viral protein inhibitor of cell death, which was discovered as a viral gene mutation that causes premature death of the host (242). The substrate specificity of p35 is much broader than that of crmA because it can inhibit both caspase 1-like proteases and caspase 3-like proteases with similar effectiveness (236, 237, 243). Another baculovirus gene product that can inhibit apoptosis is the IAP protein, which has no apparent sequence identity with p35, even though both proteins function equally well to block apoptosis in insect cells (193, 235, 244--247). Mammalian \textit{iap} gene homologues, \textit{c-iap1} and \textit{c-iap2}, have been found to interact with TNFR2 via the TRAF1 and TRAF2 adaptor proteins and effectively suppress apoptosis in selected cell lines (245, 248). The baculovirus IAP protein has also been shown to inhibit \textit{reaper}-mediated apoptosis in \textit{Drosophila}, possibly via its interaction with the DCP-1/\textit{drICE} protease (244). In an effort to identify additional cellular targets of the IAPs, two-hybrid screens have been performed using a \textit{Drosophila} embryonic cDNA library (247). An alternatively spliced product of the \textit{Drosophila mod} gene, \textit{mdg4} (whose product has been named doom), was identified by its interaction with IAP. This alternatively spliced \textit{mdg4} gene transcript does not contain a leucine-zipper motif (BTB) found in normal \textit{mod} gene transcripts. The \textit{mod} gene encodes a protein that regulates chromatin structure and gene expression. The \textit{mdg4} gene transcript contains a unique sequence, designated DSD in place of the BTB, and its ectopic expression in insect SF-21 cells causes apoptosis. Deletion/mutation analysis of the \textit{mdg4} gene transcript demonstrated that the DSD motif was necessary and sufficient to induce apoptosis. The mechanism of the DSD-induced apoptosis is not clear, but it may involve sequestering and/or altering the normal cellular localization of the \textit{iap} gene products (247).

Recent studies of the human X-chromosome-linked \textit{iap} gene product (XIAP) have shown that it functions by directly inhibiting at least two members of the caspase family of proteases (247a). XIAP was found to effectively inhibit many cell death signals, including cytochrome \textit{c}-mediated nuclear destruction that is not affected by exogenously added \textit{bcl-2}. Further examination of this inhibitory activity demonstrated that XIAP functions by directly binding to the partially processed products of the caspase 3 and 7 zymogens; in the presence...
of XIAP, the caspase 3 zymogen precursor was cleaved at a site between the large and small subunits without the subsequent removal of the pro-domain (Figure 1). Thus while caspase 8 can be activated in the presence of XIAP, the subsequent activation of caspase 3 is inhibited, as are the morphologic changes (e.g. nuclear envelope destruction, chromatin condensation) normally associated with caspase 8-mediated apoptosis. In addition to the ability of XIAP to inhibit caspase-mediated death in vivo, XIAP was shown to efficiently bind and inhibit caspase 3 and 7 in vitro (247a). Interestingly, although XIAP could bind and inhibit a partially processed caspase in vitro, it could not bind the unprocessed pro-zymogen form of the caspase, suggesting that XIAP directly binds active caspase 3 and caspase 7 but not their inactive zymogen precursors. Clearly, further elucidation of IAP function may provide significant insights into the apoptotic process. In addition, it would be of interest to determine whether the processing and/or activity of caspases is affected by phosphorylation/dephosphorylation. Such post-translational modifications could greatly enhance the control of cell death processes.

**Viral and Cellular FLICE-Inhibitory Proteins (FLIPs)**

The isolation and characterization of cellular genes responsible for mediating apoptotic signals (e.g. the caspase-mediated death pathways involving receptors, adaptors, and proteases) has resulted in the identification of numerous viral genes that encode death inhibitors (238–240). Many of the protein-protein interactive domains, such as the DD and DED, are excellent potential targets of these viral inhibitors. When death receptors or adaptors containing mutations in their DD and/or DED are expressed in cells, they function in a dominant-negative fashion to inhibit cell death (67, 249). Based on these observations, genomic databases were screened in an attempt to identify novel genes with homology to the DED (238–240). Several viral genes encoding proteins with DED-related motifs were identified: the equine herpesvirus type 2 E8 protein, bovine herpesvirus-4, herpesvirus saimiri (HVS), human herpesvirus-8 (HHV-8), and the human molluscipox virus (molluscum contagiosum virus, MCV). These viral proteins interact with FADD and/or the caspase 8 (FLICE1) pro-domain when they are cotransfected into mammalian cells and have been termed FLIPs (FLICE-inhibitory proteins). Based on the similarities between the caspase 8 and caspase 10 sequences, including the pro-domain, it is likely that these viral FLIPs will also effectively inhibit apoptotic signals mediated by caspase 10.

In addition, various TNFRs are able to act as in vivo partners of numerous FLIPs, resulting in inhibition of their receptors’ death activity, although not to the same extent observed when they are associated with FADD (238, 240). The expression of viral transcripts corresponding to the FLIPs during the viral
replication cycle appear well before cellular lysis, suggesting that they may protect the virally infected cells from premature apoptosis by viral overload (240). Others have observed that loss-of-function mutations in the p35, iap, and E1B19K viral genes lead to a decrease in viral productivity following infection (242, 244, 250). This suggests that a general mechanism for inhibition of cell death may be utilized by a large number of viruses and that there are potentially many more of these viral death-inhibitory proteins. By blocking the DD or DED in the caspase system of proteins, these viral proteins can effectively inhibit apoptosis mediated by caspases (195, 242, 244).

As anticipated, cellular FLIPs have now been identified (123a–d). FLIP₅, one form of the human cellular FLIPs, is short and contains two DEDS, much like the viral FLIPs. FLIPs can also effectively block cellular death induced by all known death receptors. However, in contrast to the viral FLIPs, a much longer human protein, FLIP₇, has been identified. Three groups have also identified this protein: Casper (for caspase-eight-related protein) (123b); I-FLICE (for an inhibitor of FLICE) (123c); and CASH (for caspase homologue) (123d). FLIP₇/Casper/CASH/I-FLICE is apparently generated by alternative splicing and contains the same two DEDs found in FLIP₅ and a caspase-like domain in which a tyrosine has been substituted for the cysteine normally found in the active site (Figures 1 and 3). The exact function of FLIP₇ is controversial, with two groups (123b, 123c) reporting that FLIP₇ overexpression induces or potentiates apoptosis, whereas other groups (123a,d) report that it effectively inhibits apoptosis. These differences are unlikely to be due to cell-type specificity because it was shown that FLIP₇/Casper/CASH/I-FLICE induced apoptosis in HeLa cells (123b), and others reported that it did not have cytotoxic effects when expressed in the same cells (123c). A more likely explanation is that the discrepancy reflects differences in the level of expression of the protein. Tschopp and colleagues reported that while a very high, nonphysiological level of FLIP₇ was capable of inducing apoptosis in 293T cells, a lower, more physiological level was not (123a).

Synthetic Peptide Inhibitors and Substrates of Caspases

In an attempt to understand the mechanisms underlying various apoptotic signaling pathways, synthetic peptide inhibitors that mimic cleavage sites of the caspases (usually at an Asp-X bond) have been chemically derived and used for in vitro and in vivo analysis of enzyme activity (32, 124, 172, 251). These peptides are generally very small, 3 to 4 amino acids in length, and include zVAD-fmk (benzyloxy carbonyl-Val-Ala-Asp-fluoromethylketone), DEVD-fmk (acetyl-Asp-Glu-Val-Ala-Asp-fluoromethylketone), YVAD-fmk (acyetyl-Tyr-Val-Ala-Asp-fluoromethylketone), and VEID-fmk (acyetyl-Val-Glu-Ile-Asp-fluoromethylketone). The sequences of these various peptides are based on the caspase
recognition sites of substrates. YVAD-fmk is based on the caspase 1 recognition site in pro-interleukin-1, DEVD-fmk is based on the caspase 3 recognition site found in PARP, and VEID-fmk is based on the caspase 6 recognition site in lamin. These inhibitors are not highly specific, but they do appear to effect caspases identified within a particular group (e.g. caspase 3-like). Even though these peptides are related to one another, their subtle sequence differences can dramatically influence the specificity of their substrate inhibition. The zVAD-fmk and YVAD-fmk peptides are capable of effectively blocking most caspase-mediated apoptosis (e.g. caspase 1 and 3), whereas DEVD-fmk effectively blocks only caspase 3 (CPP32-like) protease activity, and VEID-fmk primarily blocks caspase 6 activity (32, 125, 172). These peptide inhibitors are soluble, relatively stable (a half-life of 2–4 h), and show dose-dependent inhibition of cell death after several different stimuli (e.g. Fas-induced death is inhibited best by 10 \( \mu \)M zVAD-fmk) (32, 172). In fact, the rate of enzyme inactivation by these peptide inhibitors can be determined by addition of excess inhibitor to the enzyme reactions and observing the nature of the time-dependent inhibition of substrate hydrolysis (125).

Peptide substrates for these caspases are also available [e.g. YVAD-AMC (acetyl-Tyr-Val-Ala-Asp-aminomethylcoumarin) and DEVD-AMC (acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin)]. The hydrolysis of these substrates by caspase activities associated with cytosolic or nuclear proteins can be measured by determining the amount of fluorescent AMC product formation (125, 145). In addition, the molecular weight of a caspase or caspases responsible for a particular biologic response can be determined by affinity labeling with \( N-(N\text{-benzyloxycarbonylglutamyl}-N\epsilon\text{-biotinyllysyl})\text{-aspartic acid [(2, 6-dimethylbenzoyl)oxy]methyl ketone followed by one- or two-dimensional gel analysis of the proteins. This approach was used effectively by Kaufmann and colleagues (145) to demonstrate that multiple caspase isoforms (i.e. two species that comigrated with active caspase 6 and three species that comigrated with active caspase 3) were associated with etoposide-induced apoptosis in HL-60 cells. This affinity labeling of caspases also helped these investigators to demonstrate that a unique set of nuclear pro-caspases (i.e. pro-caspase 2) exists in these cells. Considerable effort is currently being focused on the development of caspase-specific inhibitors, which would have potentially important clinical and pharmacological uses, as well as important research applications. Caspase-specific inhibitors could be used to target selected apoptotic pathways, such as the Fas-mediated pathways recently associated with several autoimmune disorders (252). These inhibitors could also be used to block individual caspase proteases, allowing the definitive identification of each protease involved in the processing of specific substrates, as well as helping to order the upstream and downstream events within a particular apoptotic signaling pathway.
REGULATION OF DEVELOPMENT BY CASPASES

Much of the early work demonstrating that PCD was mediated by a specific set of genes came from developmental studies of *C. elegans* and *Drosophila* (44, 47, 216). Loss-of-function mutations in genes such as *reaper* (*rpr*) in *Drosophila* and *ced-3* in *C. elegans* prevent the normal death of specific cells during development (44, 47). Thus much like the conserved cyclin/CDK regulatory pathway in eukaryotes, which facilitated the isolation of the human CDK1 homologue by complementation of a yeast *ts* mutant (253), the conserved nature of apoptotic signaling in *Drosophila* and *C. elegans* has resulted in the identification of the important genes.

*Drosophila* and *Reaper*

In contrast to the vertebrate TNF-related receptors described above, *Drosophila* expresses a group of unique DED-containing proteins corresponding to the *rpr*, *head involution defective* (*hid*), and *grim* genes (43–46) (Figure 1). These proteins act as apoptotic activators that mediate their function through one or more caspases, and all are essential for triggering PCD (157). Apoptotic signals generated by activation of reaper, hid, and grim can be effectively blocked by the baculovirus p35 protein (244), suggesting that the general mechanism of PCD in *Drosophila* is similar to that of vertebrates.

Much like the FADD, TRADD, and FLICE/MACH1 (caspase 8) and FLICE2/Mch4 (caspase 10) proteins in vertebrates, these proteins contain death domains. However, none of these proteins contains the pro-caspase-like domains associated with caspase 8 and 10, and in the case of the 65-amino acid *rpr* gene product, encode little more than a DED. DED function in these proteins may be linked to promoting protein-protein interactions involving these activators and their corresponding caspases. In a recent study of *rpr*, it was shown that mutations in its DED, mimicking mutations in the DEDs of TRADD, FADD, and FLICE1/MACH1 known to affect their binding to the TNF and Fas receptors, did not influence the ability of reaper to induce cell death per se (82). When all the relevant *Drosophila* caspases, as well as other possible death effectors, are identified, the similarities and differences between PCD pathways and apoptotic signaling in vertebrates should be clear.

*C. elegans* and the *ced* Genes

Horvitz and colleagues were the first to use genetic analysis of *C. elegans* to determine whether genes are responsible for the PCD that occurs during development (38, 47, 49, 216). They identified three genes essential for cell death to occur: *ced-3*, *ced-4*, and *ced-9*. The *ced-3* gene encoded a pro-apoptotic product that could be directly inhibited by the product encoded by the *ced-9* gene (47, 256). Similarities between the *ced-3* gene product and the interleukin-1β-
converting enzyme (ICE) suggest that a cellular protease is responsible for these PCDs, and its deletion or inactivation in *C. elegans* completely prevents the death of cells that normally occur during development (38). Furthermore, the *ced-3* gene product can induce apoptosis in transfected mammalian cells (51). Conversely, the *ced-9* gene product can inhibit the pro-apoptotic function of the *ced-3* gene, and it was shown that this gene is a *C. elegans* homologue of the mammalian bcl-2 gene (38, 49, 216). The exact function of the final *ced* gene, *ced-4*, was not elucidated until recently when it was demonstrated that CED-4 acts as an adaptor that allows interaction between CED-3 and CED-9 (48, 217, 218). In addition, ectopic expression of the *ced-4* gene in *S. pombe* leads to rapid chromatin condensation and cell death (185).

The CED-9 protein was isolated as a binding-partner of CED-4 in an interactive genetic screen (218). Loss-of-function mutants in the *ced-9* gene prevented the corresponding protein from associating with CED-4. Normally, CED-4 is localized in the cytosol, but when CED-9 is expressed in mammalian cells, it targets CED-4 from the cytosol to intracellular membranes, suggesting that CED-9 plays an important role in the subcellular localization of CED-4 (218). It will be of interest to determine whether the relationship between CED-4 and CED-9 is associated with the phenotype of CED-4 overexpression in *S. pombe* (185, 218). The rather striking ability of ectopic *ced-4* gene expression to induce chromatin condensation and death in yeast may indicate that this interaction is, indeed, one of the functions of CED-9 and that its sequestering of CED-4 inhibits apoptosis. In a separate study, the product of the *ced-4* gene was shown to facilitate the interaction of CED-3 with CED-9 (217). Furthermore, CED-4 also facilitated physical interactions between the mammalian ICE and FLICE proteins. The exact relationship between these three proteins is not clear but will undoubtedly be the focus of intense research.

**Effects of Caspase Gene Elimination on Mouse Development**

In mammals, the elimination of either the ICE (caspase 1) or CPP32 (caspase 3) genes in mice by homologous recombination produces two very distinctive phenotypes (160–162). Deletion of ICE did not affect the normal development of the caspase 1−/− mice or the ability of their thymocytes to respond to exposure to glucocorticoid or UV. However, the thymic cells from these caspase 1−/− mice were insensitive to apoptosis mediated by the Fas receptor (161). Conversely, deletion of the CPP32 (caspase 3) gene in mice resulted in developmental defects primarily affecting neuronal cells and the brain, which resulted in premature embryonic lethality (162). This phenotype is of some interest because the developmental defects exhibited by these caspase 3−/− mice are very similar to the neuronal hyperplasia that occurs in loss-of-function *ced-3*
gene mutants in *C. elegans* (41, 48). It will be of interest to determine whether there are phenotypes associated with other caspase gene knockouts, or if their functions are conserved well enough to act in a redundant fashion.

**CASPASE INVOLVEMENT IN DISEASE**

The relationship between Fas/TNF receptor function and the targeting of certain immune cells for elimination by apoptosis has been of interest for some time. Clearly, defects in the ability of these cells to be eliminated from their host could contribute significantly to the pathogenesis of many autoimmune-type disorders (25, 255). In addition, many tumors, particularly those that have become refractory to chemotherapeutic drugs, have circumvented the apoptotic signaling pathways that would normally lead to their elimination (22, 256). By doing so, they enhance their chances for survival, as well as increase the frequency of genetic mutations within that particular cell’s genome (255, 257). The identification of specific genes required for normal apoptotic signaling should facilitate this analysis and possibly demonstrate that the normal function of these genes is required to avoid these diseases (see below).

**Cancer**

As mentioned above, several early cell death experiments were based on the ability of certain tumor cells to become insensitive to the chemotherapeutic drugs being used for treatment (22, 173, 256). A number of studies have shown that the in vivo effects of these drugs are to induce tumor cell apoptosis, which suggests that the acquired insensitivity of these cells to cytotoxic drugs and radiation-treatment is the result of inactivation of one or more genes involved in apoptotic signaling (258, 259). In several studies it was elegantly demonstrated that the ability of tumor cells to undergo apoptosis was linked to the tumor suppressor p53. Even so, there is little proof of a direct relationship between the function of pro-apoptotic proteins and the elimination of tumor cells.

The isolation of the *bcl-2* gene, which is responsible for certain follicular lymphomas, was a milestone for investigators studying tumorigenesis and programmed cell death (196–198). This was the first example of an oncogene whose function was linked to the regulation of apoptosis, demonstrating that alterations in apoptotic signaling pathways and mitogenic pathways could contribute to tumorigenesis (255, 257, 260, 261). The *bcl-2* gene was isolated almost 12 years ago, and yet its exact function is not known. But the pioneering studies of many laboratories have demonstrated that defects in pro- and/or anti-apoptotic genes are as significantly linked to tumorigenesis, as are the defects that occur in the well-studied mitogenic and cell cycle pathways
involved in normal cell proliferation. In fact, recent experiments have suggested that significant crosstalk between Bcl-2 and the regulators of cell cycle occurs and that Bcl-2 likely contributes to life and death decisions made at cell cycle checkpoints (222, 227, 228, 262). Further evidence of the involvement of Bcl-2 family members in cancer came from a recent study that identified frameshift mutations in the \textit{bax} gene in a subset of colon tumors (263). This particular subset of colon cancers, the microsatellite mutator phenotype (MMP), exhibits exaggerated genomic instability at simple nucleotide repeat sequences scattered throughout the genome as a result of dysfunctional DNA mismatch-repair enzymes. In this study, more than 50% of the MMP colon tumors examined contained frameshift mutations in a tract of eight deoxyguanosines [(G)\textsubscript{8}] within their \textit{bax} genes. By eliminating Bax, a Bcl-2 antagonist, these cells can promote their own survival in much the same way as do tumors that overexpress Bcl-2, e.g., follicular lymphomas described above. This is further, direct evidence that the ability to properly signal cell death may be just as crucial as the control of the cell cycle in tumor cells.

Recently, several studies have linked a heterozygous loss-of-function allele of the \textit{FasR} gene to increased frequency of Hodgkin’s lymphoma (264). One interpretation of this data is that the enhanced survival capabilities of the \textit{FasR}\textsuperscript{+/-} cells allow them to accumulate genetic insults that can ultimately result in malignancy. In this sense, these genes function as tumor suppressors. However, unlike other traditional tumor suppressors, activating mutations in these genes result in the complete destruction of tissues and organs, leading to death.

\textbf{Autoimmune Disease}

The dysfunction of many cellular immune responses contributes significantly to the development of autoimmune disorders (72, 252). The resulting inflammatory response and cellular damage that occur can result in the loss of organ function or even death. The nature of these diseases suggests that many are linked to defects in apoptotic signaling. In fact, mutations in both the Fas receptor and Fas ligand have been associated with autoimmune-type disorders (252). Targeted mutations in the \textit{FasR} gene of mice result in cellular hyperplasia in the peripheral lymphatic organs and liver (265). Another possible interpretation of how Fas-mediated cell death could contribute to autoimmune disease has been recently suggested by two groups (89, 266) who suggest that the Fas ligand may normally function to eliminate activated T cells as soon as they enter sites of “immune privilege.” This might explain why the inflammatory response in many organs associated with immune privilege, such as the endothelium, eye, and Sertoli cells of the testis, is so dramatic in \textit{gld} mice, which harbor a defective \textit{FasL} gene (252).
The Fas/TNF receptors are clearly important participants in immune surveillance because even partial loss-of-function within one of the signaling components of these apoptotic pathways could significantly alter the course of life. If this surveillance mechanism is altered even minimally, there are obvious consequences as a result. Furthermore, the fact that altering these pathways either up or down can result in autoimmune disorders or cancer demonstrates that their influence and importance persist long after development and birth.

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