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Review

Mechanisms controlling cellular suicide: role of Bcl-2 and caspases

R. T. Allen[a], M. W. Cluck[b] and D. K. Agrawal[a,b,c,*]

[a]Department of Medical Microbiology and Immunology, Creighton University School of Medicine, Omaha (Nebraska 68178, USA), Fax +1 402 280 1843, e-mail: dkagr@creighton.edu
[b]Department of Biomedical Sciences, Creighton University School of Medicine, Omaha (Nebraska 68178, USA)
[c]Department of Internal Medicine, Allergic Disease Center, Creighton University School of Medicine, Omaha (Nebraska 68178, USA)

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Abstract. Apoptosis is an essential and highly conserved mode of cell death that is important for normal development, host defense and suppression of oncogenesis. Faulty regulation of apoptosis has been implicated in degenerative conditions, vascular diseases, AIDS and cancer. Among the numerous proteins and genes involved, members of the Bcl-2 family play a central role to inhibit or promote apoptosis. In this article, we present up-to-date information and recent discoveries regarding biochemical functions of Bcl-2 family proteins, positive and negative interactions between these proteins, and their modification and regulation by either proteolytic cleavage or by cytosolic kinases, such as Raf-1 and stress-activated protein kinases. We have critically reviewed the functional role of caspases and the consequences of cleaving key substrates, including lamins, poly(ADP ribose) polymerase and the Rb protein. In addition, we have presented the latest Fas-induced signalling mechanism as a model for receptor-linked caspase regulation. Finally, the structural and functional interactions of Ced-4 and its partial mammalian homologue, apoptosis protease activating factor-1 (Apaf-1), are presented in a model which includes other Apafs. This model culminates in a caspase/Apaf regulatory cascade to activate the executioners of programmed cell death following cytochrome c release from the mitochondria of mammalian cells. The importance of these pathways in the treatment of disease is highly dependent on further characterization of genes and other regulatory molecules in mammals.

Key words. Apoptosis; Bcl-2; caspases; smooth muscle cell; Fas; apoptosis protease activating factor; *Caenorhabditis elegans*; retinoblastoma; interleukin-1β converting enzyme proteases.

Introduction

Apoptosis is a highly conserved and regulated 'programme' by which cells commit suicide under a variety of internal and external controls. It is the most common form of cell death, present physiologically from development through adult life, and pathologically in conditions such as cancer, autoimmune and neurodegenerative diseases, AIDS and vascular diseases [1–6]. The prevalence of apoptosis in human disease, and whether it is central to disease etiology or pathogenesis, has prompted researchers to investigate the molecular mechanisms by which it is induced and regulated. Since homeostasis within developing and adult multicellular

* Corresponding author.

Table 1. Bcl-2 family members that regulate apoptosis.

Category	Family members	Regulatory interactions
Bcl-2-related inhibitors of apoptosis	Bcl-2 [7, 8] Bcl-x_L [9] Bfl-1 [10, 11] Mcl-1 [12, 13] A1 [14] Ced-9 [15]	Bcl-2 binds Bax or Bak see text for Bcl-x_L; Bfl-1 suppresses p53-induced apoptosis
Viral homologues of Bcl-2 that inhibit apoptosis	E1B 19K [16–18] BHRF1 [19, 20] ASFV 5HL [21] HVS ORF 16 [22] HHV8-bcl-2 [23]	adenovirus E1B 19K can bind Bax, Bak and Nbk/Bik[16, 28]
Bcl-2-related promotors of apoptosis	Bax [24] Bak [25, 26, 17] Bad [27]	Bax, as well as Bak, binds to Bcl-2, Bclx_L and E1B 19K Bax induced during SST-dependent p53-mediated apoptosis Bak can also inhibit apoptosis (see text)
Promotors of apoptosis that contain primarily the BH3 region	Bcl-x_S [9] Nbk/Bik [28, 29] Hrk [30] Bid [31]	Nbk/Bik binds to Bcl-2, Bcl-x_L and E1B-19K Hrk binds selectively to Bcl-2 and Bcl-x_L

BHRF1, Epstein-Barr virus; ASFV 5HL, African swine fever virus; E1B 19K and ASFV 5HL have no obvious membrane-anchoring regions; HBS ORF16, herpes virus saimiri; HHV8, Kaposi sarcoma-associated human herpes virus 8. Modified from White et al. [53].

organisms is highly dependent on rates of cell proliferation to cell death, even slight alterations can likely result in dire clinical consequences. From high turnover haematopoietic progenitor cells to the low-level renewal of neural cells, either a lack of or too much apoptotic death may lead to neoplasia or degenerative conditions, respectively. Thus, it is hoped that understanding the molecular components of the death machinery may lead to the development of drugs which target these molecules to alter the fate of a cell. Although many are optimistic about the potential of this unexplored therapeutic avenue to remedy certain human diseases, it has been difficult to assess the precise functional characteristics of genes and signalling molecules in mammalian cells. Fortunately, recently accumulated evidence suggests that many aspects of the apoptotic pathway are evolutionarily conserved and that those molecules at the fulcrum of the life or death transition in the nematode may serve similar albeit more complex functions in mammalian cells.

The rapid increase in knowledge of the death machinery does not permit a complete review of the various avenues of its regulation. Therefore, in this article we have covered the molecules and their pathways thought to be most important in mammalian cells. First, the role of Bcl-2 family member proteins are introduced with emphasis on their discovery, survival function in response to numerous stimuli and importance in oncogenesis. We then briefly discuss structural Bcl-2 homology (BH) regions, including the pro-apoptotic BH3 region, emphasizing their importance in dimerization and effect on cell fate. The survival function of Bcl-2 is covered next, including its ability to form channels in mitochondrial membranes and regulate cytochrome c release. Post-translational regulatory mechanisms of Bcl-2, such as phosphorylation and proteolytic cleavage, are then detailed. We also discuss general caspase [interleukin-1 converting enzyme (ICE)-like protease] structure, function, key cleavage substrates including lamins, poly ADP-ribose polymerase (PARP), and the Rb protein, as well as the most recent Fas-induced signalling mechanism as a model for caspase regulatory events. In addition, we present the recent evidence defining the functional role of caspases, and their activation, in the apoptotic pathway of the nematode, and conclude with the mammalian cell death programme correlate of ced-4 called apoptosis inducing factors (Apafs) and how this may be integrated into the mammalian apoptotic programme, including its indirect regulation by Bcl-2 proteins.

Survival or death promoters: the Bcl-2 family

The bcl-2 family of related proteins constitute an important control mechanism in the regulation of apoptosis. There are over a dozen members in this family [7–31] (see table 1), some of which suppress apoptosis, including Bcl-2 and Bcl-x_L, and others which promote it, such as Bax and Bak. Bcl-2 is the prototypic family member and is homologous to the nematode *Caenorhabditis elegans* ced-9 product [15], suggesting not only that it is evolutionarily conserved, but that its

role in regulating a similar cell survival pathway may also be conserved. The bcl-2 gene was originally found in a proportion of B-cell follicular lymphomas at the t(14;18) chromosomal translocation breakpoint [32]. In interleukin (IL)-3-dependent myeloid and lymphoid cell lines, bcl-2 inhibited apoptosis induced by IL-3 withdrawal without stimulating proliferation [7]. Its oncogenicity, therefore, stems from the ability to promote cell survival rather than proliferation. Subsequently, it was found to be an outer mitochondrial membrane protein which specifically inhibits apoptosis [8], and has also been shown to be localized, through a putative carboxy terminus transmembrane domain, to endoplasmic reticular (ER) and outer nuclear membranes. Removal of this membrane-targeting region, however, does not entirely abrogate bcl-2 activity, suggesting that this association may not encompass every Bcl-2 function and that distinct actions within the cytosol may also exist [24]. Nearly every Bcl-2 family member containing a membrane-targeting domain, however, relies on this region to localize to mitochondrial, ER, or outer nuclear membranes during the inhibition of apoptosis [33].

The Bcl-2 protein inhibits apoptosis induced by a variety of stimuli and in many cell types, including haematopoietic and neural cell apoptosis induced by growth factor withdrawal [7, 34, 35]; and delay or prevention of apoptosis induced by glucocorticoids, γ-irradiation, heat shock and many chemotherapeutic drugs [36–38]. The ability to regulate death induced by such diverse stimuli suggests that bcl-2 is an integral, and possibly the central, player in cell survival programmes. Numerous examples of the in vivo function of Bcl-2 strongly supports this, as well as the essential physiologic role of Bcl-2. For example, during development bcl-2 is expressed widely throughout embryogenesis [39], and in adult life appears to be more topographically restricted to areas such as proliferating zones, postmitotic neurons and stem cells [40]. Selective overexpression of Bcl-2 in the lymphoid system produces B-cell lymphoma where progression to high-grade lymphoma often coincides with a c-myc translocation [41]. Indeed, simultaneous overexpression of Bcl-2 can abrogate c-myc-induced apoptosis, making their synergistic involvement in cellular transformation likely in the etiology of some human cancers [42–44]. In fact, bcl-2 is found in many types of tumours, including most breast carcinomas [1], and has been shown to cooperate with c-myc [7, 44, 45] and ras [46] family members during cellular transformation. Thus, deregulated Bcl-2 itself has the propensity to produce disease and to cooperate with growth-promoting proto-oncogenes [44]. Recent evidence also indicates that the Bax gene, a family member which promotes apoptosis to limit cell number in tissues, may be negatively selected for during progression of some human colon cancers, implying that it may function in a tumour suppressor role independent of p53 [47].

Understanding the mechanisms behind the regulation and survival functions of Bcl-2 are of critical importance, therefore, since therapeutic strategies aimed at inhibiting its modes of action may be useful in cancers such as breast, colon and lung where overexpression of bcl-2 most commonly occurs [1]. It should be noted that Bcl-2 cannot protect against every type of apoptosis-inducing stimulus. In particular, induction of programmed cell death (PCD) by cytotoxic T cells or via negative selection of thymocytes is not inhibited by Bcl-2 [48, 49].

Structure and biochemical function of Bcl-2 proteins

As the Bcl-2 gene family rapidly expands, so does our understanding of heterodimer and homodimer formation among family members and how these associations function to regulate apoptosis, or create a propensity to cause disease (see table 1). Most evidence suggests that each cell type has a set point for the ratio of Bcl-2 family dimers, such as levels of Bcl-2 to Bax, which gauges the sensitivity of a cell towards survival or apoptosis. In many cells, survival or death depends on the altered expression level of death inhibitor to death promoter, respectively. Furthermore, it appears that a range of molecular affinities exist which control the interactions between family members, such as Bcl-2 (or Bcl-x_L) for Bax to promote cell survival or Bax homodimer formation to promote cell death [50, 51]. The cumulative effect of dimerization on cell fate not only depends on which *bcl-2* family members are expressed in the respective cell type, but also on the level, regulation and context of that expression. For example, Bax and Bak can either promote or suppress apoptosis depending on cell type and tissue distribution. Thus, local external signals may alter a cell's internal regulation of certain Bcl-2 family members, likely for the benefit of the tissue and not the cell [26, 52, 53].

Bcl-2 homology regions. Some of the special features of different family members may be attributed to certain aspects of their protein structure. Mutagenesis and sequence analyses have shown that three regions, termed Bcl-2 homology regions 1, 2 and 3 (BH1, BH2 and BH3), are relatively conserved among family members and are required both for protein-protein interactions and the regulation of apoptosis. Detailed studies of these structural components have implicated key regions which may function in dimerization and in the inhibition and/or induction of apoptosis. Although these are thoroughly reviewed elsewhere [53], one important emergent theme is the pro-apoptotic BH3 region of Bcl-2 family death promoters. For example, the

bcl-x gene transcript is alternatively spliced such that the bcl-x_L long form inhibits cell death, and the bcl-x_S short form, which has regions BH1 and BH2 spliced out, accelerates cell death [9]. This is also a characteristic of the *bak* gene product, which has Bcl-2 sequence homology within BH1 and BH2 regions, promotes apoptosis, and can bind to the apoptotic inhibitors Bcl-2, Bcl-x_L and the adenoviral early gene E1B 19K [17, 25, 26]. When a 50 amino-acid region of Bak encompassing BH3 but excluding BH1 and BH2 is expressed in mammalian cells, it is sufficient to induce apoptosis [54]. A similar case is observed with the death promoter Bax, the first Bcl-2-associated protein identified, which can interact with E1B 19K and Bcl-2 to antagonize their survival effects using a truncated sequence harbouring only the BH3 domain [18]. In fact, promoting cell death and binding to Bcl-2 require neither the BH1 nor the BH2 region of Bax [55, 56]. On the other hand, the pro-apoptotic BH3 region requires all three BH regions within Bcl-2-like proteins to bind and form a regulatory heterodimer. For example, structural recognition studies show that the BH3 domain of Bak binds in a hydrophobic cleft or 'pocket' formed by the BH1, BH2 and BH3 regions of Bcl-x_L [57]. Analysis of the structure and binding affinities of mutant Bak and BH3 peptides showed that Bak changes conformation to form an amphipathic α helix (unlike its random coil when free in solution) in order to complex with Bcl-x_L in this pocket domain. Consequently, a significant loss in affinity for Bcl-x_L is observed with mutations specific to this region.

Regulatory interactions between members of the Bcl-2 family. Interactions between different members may indirectly regulate each other without forming a dimer, such as the interaction between the death promoters Bad and Bax. Although Bad and Bax do not interact directly, Bax (which can suppress Bcl-2 function [24]), has a common target with Bad – the Bcl-x_L protein. Expression of Bcl-x_L often allows it to sequester Bax, thereby promoting cell survival. Unlike other family members, however, coexpression of Bad opposes Bcl-x_L-mediated survival and accelerates death by displacing Bax from Bcl-x_L in vivo [27]. This allows Bax homodimer formation and apoptotic cell death. Some controversy in the literature still exists concerning which Bcl-2-like proteins prevent or promote apoptosis in a dominant and active fashion. The evidence presented below, however, seems to support a dominant role for inhibitors of PCD in conjunction with their well-characterized ability to heterodimerize with potentially toxic death-inducing family members.

The exact manner in which Bcl-2 functions to inhibit apoptosis is not completely understood, although recent discoveries provide some possible explanations. It was initially thought that in response to cellular damage, and more specifically oxidative damage, Bcl-2 functioned to scavenge free radicals to promote cell survival [58]. In this role, it would protect from the generation of excessive reactive oxygen species induced by a variety of stimuli which would otherwise lead to cell death. However, the ability of Bcl-2 to inhibit apoptosis during nearly anaerobic conditions [59] and in cells which lack mitochondrial DNA [60] led to the suggestion that protection from reactive oxygen species may be a downstream consequence rather than a primary function of Bcl-2-mediated protection from apoptosis [53]. Moreover, it appears that Bcl-2 can modulate the pro-apoptotic influence of many stimuli commonly associated with death rather than directly triggering it. Calcium flux [61, 62] and mitochondrial membrane depolarization [63] are just two examples. Nonetheless, Bcl-2 family members still apparently function in close association with an antioxidant mechanism. It now appears that the structural identity of Bcl-2 is analogous to that of bacterial pore-forming toxins, and that this correlates strongly to its role in regulating apoptosis induced by cytochrome c release.

Bcl-2 and cytochrome c release. Structural analysis of Bcl-x_L through nuclear magnetic resonance and x-ray crystallography has shown that this protein displays some similarities to the pore-forming domains of diptheria toxin and the colicins A and E1, suggesting that Bcl-x_L and other family members may regulate apoptosis as ion channels [64]. Furthermore, purified recombinant Bcl-x_L inserted into synthetic lipid membranes revealed that it indeed forms a cation-selective ion-conducting channel at physiologic pH [65]. Since the diptheria toxin pore-forming domain also allows passage of the 'A fragment' protein through the endosomal membrane [65, 66], Bcl-x_L-related channels are suspected to regulate the passage of proteins in vivo as well [65]. Thus, proteins which induce apoptosis through cytoplasmic redistribution (via channels) and subsequent ICE-like protease activation, such as cytochrome c [67, 68], may somehow be retained by Bcl-x_L (or Bcl-2) in vivo at membrane sites where this channel becomes localized. Two recent studies provide convincing evidence that this may be the case [69, 70]. Initial investigations indicated that release of cytochrome c from the mitochondria into the cytosol, in cooperation with other factor(s) present in the cytosol, was required for the activation of caspase-3 (CPP32) and DNA fragmentation during staurosporine-induced apoptosis [67]. Further mechanistic studies showed that only the holoenzyme could activate caspase-3, that cytochrome c release occurred very early in apoptosis and that it preceded changes in mitochondrial membrane depolarization. This suggests that mitochondrial depolarizations are not an immediate prerequisite for PCD, and implies that channels mediating those changes are pro-

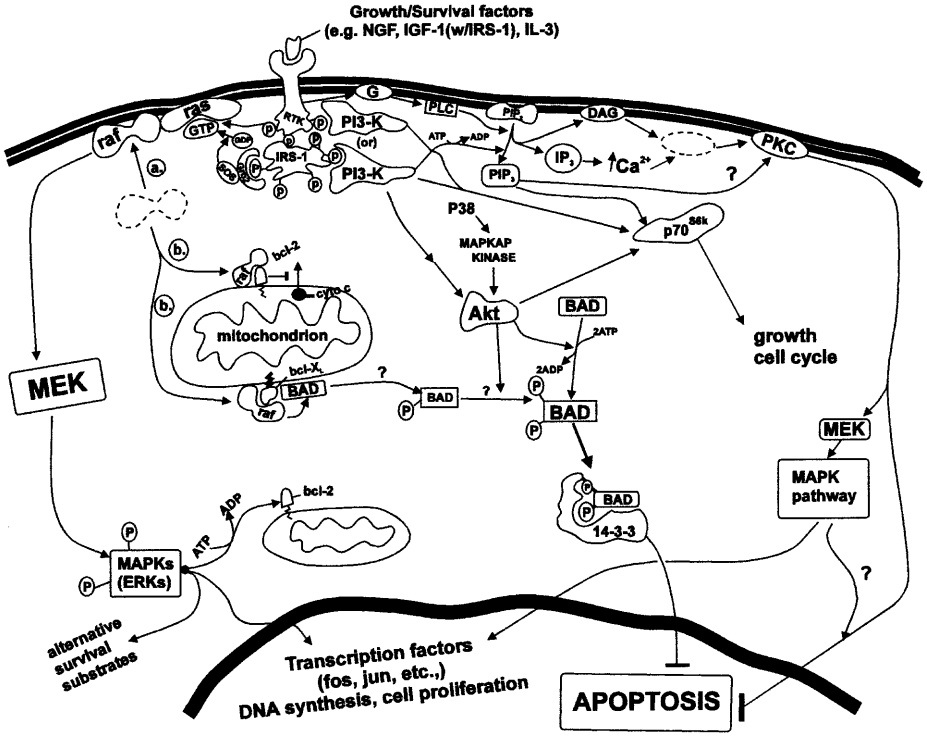

Figure 1. Schematic diagram showing antiapoptotic pathways regulating Bcl-2 proteins. PI3-K activation is via IRS-1 association following receptor binding of IGF-1. Association of Raf with the plasmalemma (a) activates ERKs downstream possibly for survival, or recruitment of Raf to mitochondrial membrane (b) may allow it to directly regulate Bcl-2 function(s).

bably not involved in the release or sequestration of cytochrome c [71]. In addition, using the cell-free PCD system of *Xenopus* egg extracts, overexpression of Bcl-2 (and Bcl-x_L) prevented cytochrome c release and the appearance of apoptosis, but only in the presence of mitochondria on which they were located. In contrast, when exogenous cytochrome c was added, or after its release into the cytosol, even the highest levels of Bcl-2 could not reverse or protect against apoptotic protease activation and cleavage events induced by cytochrome c, indicating that mitochondrial sequestration of cytochrome c is a key Bcl-2 function.

Although these data assign an important biochemical function to death-inhibiting family members, the exact mechanism of cytochrome c release is still undefined. For instance, does Bcl-2 directly alter mitochondrial and ER membrane permeability for cytochrome c and other proteins possibly involved in caspase activation? Or, does it sequester these proteins indirectly by altering ion flow to regulate nearby channels or membrane permeability [61, 62, 70]? Furthermore, the role of those family members which do not associate with membranes in vivo, and are therefore not targeted to mitochondria or to the nucleus, remains undefined. It is possible, however, that numerous protein-protein and protein-lipid interactions between Bcl-2 family members and other cytosolic regulatory proteins occur in vivo to modulate cell death inhibitors/promoters. The numerous post-translational events which regulate Bcl-2 proteins are now bringing into focus the integrated regulation of survival and death-promoting proteins.

Regulation of Bcl-2 proteins

Current theories for the regulation of apoptosis by Bcl-2 family members rely heavily on the ratio of death promoter to death inhibitor [24, 28, 53]. This requires that the expressed Bcl-2 proteins retain those biochemical functions which actually promote survival. However, in response to certain death signals or a lack of survival signals, antiapoptotic protein function may not be retained, emphasizing the importance of mechanisms regulating Bcl-2 family members themselves. That Bcl-2 proteins cannot protect against every type of apoptosis-inducing stimulus implies that either the stimulus is too overpowering or that elements along these signalling pathways regulate Bcl-2 family mem-

bers in a distinct and possibly irreversible manner. Both may be true, but recent evidence supports the idea that the pro- and antiapoptotic extracellular signals encountered by a cell alter the function of Bcl-2 family proteins via post-translational regulatory mechanisms. As discussed below, this appears to occur either by phosphorylation or proteolytic cleavage.

Phosphorylation of Bcl-2 family proteins. Bcl-2 is phosphorylated on serine residues in response to a variety of stimuli, including chemotherapeutic agents and the phosphatase inhibitor okadaic acid [72]. This most often results in the inhibition of its survival effects and cell death, providing a possible explanation for how the induction of apoptosis in some cells cannot be overcome by any expression level of Bcl-2 proteins (reviewed in ref. 72) (fig. 1). It appears that a specific region within survival proteins may harbour the sites which become phosphorylated and thereby alter Bcl-2 member function. In particular, an unstructured 60-residue flexible loop domain exists within Bcl-x_L, and by structural modelling within Bcl-2 as well, which may act as a negative regulatory domain of their survival functions [73]. Since the ability of Bcl-x_L loop deletion mutants to bind Bax (or only the BH3 region of Bax) was unaffected, this regulatory site likely functions independent of its ability to form dimers. Bcl-x_L loop deletion mutants were also more effective at inhibiting apoptosis, retaining a similar pattern of protection at even lower expression levels. It is indeed possible, therefore, that post-translational modifications such as phosphorylation use this domain to inhibit the biochemical function of survival proteins [74].

The kinases implicated in the phosphorylation of Bcl-2 family proteins are thought to be cytosolic, prompting numerous studies on the possible connection between receptor-linked transduction molecules and the regulation of Bcl-2 by phosphorylation (see ref. 72 for discussion) (see fig. 1). Many survival factors such as IL-2, IL-4, insulin-like growth factor (IGF)-1 and IL-7 function independent of any change in Bcl-2 protein levels, suggesting that post-translational effects are mediating their actions. For example, the ability of IGF-1 to protect fibroblasts from c-myc-induced apoptosis was independent of new RNA and protein synthesis or any change in Bcl-2 protein levels [75]. Bcl-2 protein levels also remain unchanged during the survival effects of ciliary neurotrophic factor on ciliary neurons [76]. In addition, survival factors such as IL-3, IGF-1 and granulocyte macrophage colony stimulating factor (GM-CSF) can stimulate antiapoptotic signals through different but overlapping signals from which they promote growth, suggesting distinct modes of signalling are involved which likely depend on cellular context. For example, GM-CSF acting through a truncated GM-CSF receptor unable to activate Ras and Raf-1 could not maintain cell survival, yet retained the ability to promote DNA synthesis [77]. Since Ras (a guanosine triphosphate (GTP)-binding protein) and its downstream effector Raf-1 (a serine-threonine kinase) are activated in response to a number of growth factors, it appears they may function as survival factors under certain cellular conditions. Exactly how this may occur is unclear, but localizing Raf-1 kinase to a site different than under the plasma membrane may contribute to this effect. Raf-1, including a mutant form lacking kinase activity, coimmunoprecipitates with Bcl-2, indicating that it associates with but does not phosphorylate Bcl-2 directly [78]. Furthermore, Wang et al. [79] showed that fusion of Raf-1 to the transmembrane domain of a yeast outer mitochondrial membrane protein, Mas70p, localized Raf-1 to the mitochondria and protected IL-3-dependent cells from apoptosis during IL-3 withdrawal. This occurred in the absence of Bcl-2 overexpression. Raf-1 was also targeted to the mitochondria by Bcl-2 overexpression where the active form, but not a mutant lacking kinase activity, was found to phoshphorylate the death agonist Bad. In contrast, plasma membrane-bound Raf-1 did not protect cells from apoptosis, but resulted in the phosphorylation of growth-associated extracellular signal-regulated kinase (ERK)-1 and ERK2 members of the mitogen activated protein (MAP) kinase family. Consistent with this, the prevention of apoptosis in nerve cells has been shown to be independent of the Ras-Raf-MAPK pathways, requiring phophatidylinositol 3(PI3)-kinase instead [80]. The serine-threonine kinase Akt, which acts downstream of PI3-kinase in response to survival signals such as IGF-1 (see fig. 1), has recently been shown to directly phosphorylate Bad, linking the ability of growth factors which function as survival factors with the direct regulation of key Bcl-2 family members [81]. Thus, Bcl-2 appears to specifically recruit Raf-1 to the outer mitochondrial membrane, possibly allowing it to phosphorylate and thus negatively regulate Bad. On the other hand, Zha et al. [82] provide evidence to suggest that Raf-1 may not phosphorylate Bad directly, leading to the possibility that local mitochondrial events may enforce this regulatory step after recruitment of Raf-1 [72, 82]. This group has also shown that in the presence of IL-3, Bad is phosphorylated on two serine residues embedded in a site which binds to a rather ubiquitous cytosolic protein called 14-3-3. Consequently, only hyperphosphorylated Bad was found sequestered in the cytosol bound to 14-3-3. In fact, Bad phosphorylation occurred as early as 15 min after addition of IL-3. In contrast, only nonphosphorylated Bad was able to heterodimerize with Bcl-x_L at membrane sites and act as a death agonist. Thus, in response to a survival signal, Bad is phosphorylated and thereby sequestered in the cytosol by binding to 14-3-3.

Table 2. Caspase superfamily.

Protease	Substrate	Substrate function
Caspase-1 (ICE)	pro-IL1B, procaspase-3/4	cytokine production, apoptosis
Caspase-2 (Nedd2, ICH-1)	PARP	DNA repair enzyme
Caspase-3 (CPP32, Yama, apopain)	PARP, Rb, SREBP1/2	cell cycle, sterol regulation
	U1-70 kDa, DNA-PKcs	RNA splicing, dsDNA repair
	ribonuclear proteins C1/C2	processing pre-mRNA
	D4GDP dissociation inhibitor	regulate Rho GTPases
Caspase-4 (ICErel-II, ICH-2, TX)		
Caspase-5 (ICErel-III, TY)		
Caspase-6 (Mch2)	lamin A	nuclear shape
Caspase-7 (Mch3, ICE-LAP3, CMH-1)	PARP, SREBP 1/2	
	ribonuclear proteins C1/C2	
Caspase-8 (MACH, FLICE, Mch5)		
Caspase-9 (ICE-LAP6, Mch6)	PARP, procaspase-3	
Caspase-10 (Mch4, FLICE2)		

Modified from Cohen [200]. SREBP, sterol regulatory element binding protein.

It is still speculative as to which kinase(s) phosphorylate Bad in this system, especially since protein kinase C (PKC) did not phosphorylate Bad at the appropriate serine sites in vitro even though kinase activity in this system is phorbolester-inducible and inhibited by staurosporine. Heart muscle kinase (HMK), however, a form of the cyclic-adenosine monophosphate (AMP)-dependent protein kinase (PKA), did phosphorylate at the appropriate sites of Bad in vitro; but as suggested, the fact that Bad remains cytosolic following survival factor addition may imply another kinase is involved. It would be interesting to see whether Akt is active in this system.

Stress-activated protein kinases and phosphorylation of Bcl-2. Signalling molecules further dowstream of receptor-linked events have recently been shown to modify Bcl-2 family proteins (see fig. 2). Initially, it was shown that in response to nerve growth factor withdrawal activation of c-jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) together with inhibition of ERK are critical for induction of rat PC12 nerve cell apoptosis [83]. The onset of PCD in Jurkat T cells and B104 lymphoma cells was also shown to correlate with sustained activation of JNK/SAPK [84, 85]. Moreover, expression of dominant inhibitory mutants of JNK/SAPK SEK1 or of the Fas-interacting protein Daax actually blocked JNK/SAPK activation while conferring cellular resistance to apoptosis induced by Fas and other PCD stimuli [86, 87]. It now appears that JNK/SAPK specifically phosphorylates Bcl-2 in vitro as well as in COS-7 cells when JNK/SAPK is coexpressed with a constitutively active Rac-1 (G12V) GTP-binding protein [88]. ERK1 and p38 MAP kinases catalysed only weak modification of Bcl-2 both in vitro and in the COS-7 cells, while cotransfection with the MAP kinase-specific phosphatase MKP3/PYST1 blocked both p54-SAPK activation and Bcl-2 phosphorylation. In addition, expression of a mutant Bcl-2 containing four altered residues within the flexible loop domain almost entirely abrogated Bcl-2 phosphorylation in COS 7 cells, suggesting that p54-SAPK negatively regulates Bcl-2 via phosphorylation within this domain. This was verified in vitro by analysis of tryptic digests of p54-SAPK-phosphorylated Bcl-2 in the presence of radiolabelled adenosine triphosphate (ATP). In fact, expression of a Bcl-2 deletion mutant lacking the flexible loop region remained unphosphorylated in WEHI B cells in response to a death stimulus and actually retained its antiapoptotic activity [89]. Alternatively, Horiuchi et al. [90] have shown that angiotensin type 2 receptor signalling activates MAP kinase phosphatase-1 (MKP-1) and inhibits the phosphorylation of Bcl-2, resulting in the induction of apoptosis. This effect opposed nerve growth factor-mediated survival of PC12 cells, which are normally dependent on the activity of ERK1 and ERK2 MAP kinases for survival. Apparently, inactivation of these kinases by MKP-1 results in the dephosphorylation of Bcl-2 and apoptosis. Taken together, phosphorylation of Bcl-2 within the flexible loop region likely inhibits its survival function. However, the findings that ERK1/ERK2 MAP kinases phosphorylate Bcl-2 for survival in one system while p54-SAPK phosphorylates Bcl-2 in another to inhibit survival function may indicate that multiple factors, such as cell type, stimulus, local environment and differences in phosphorylation site(s) are important determinants of Bcl-2 function.

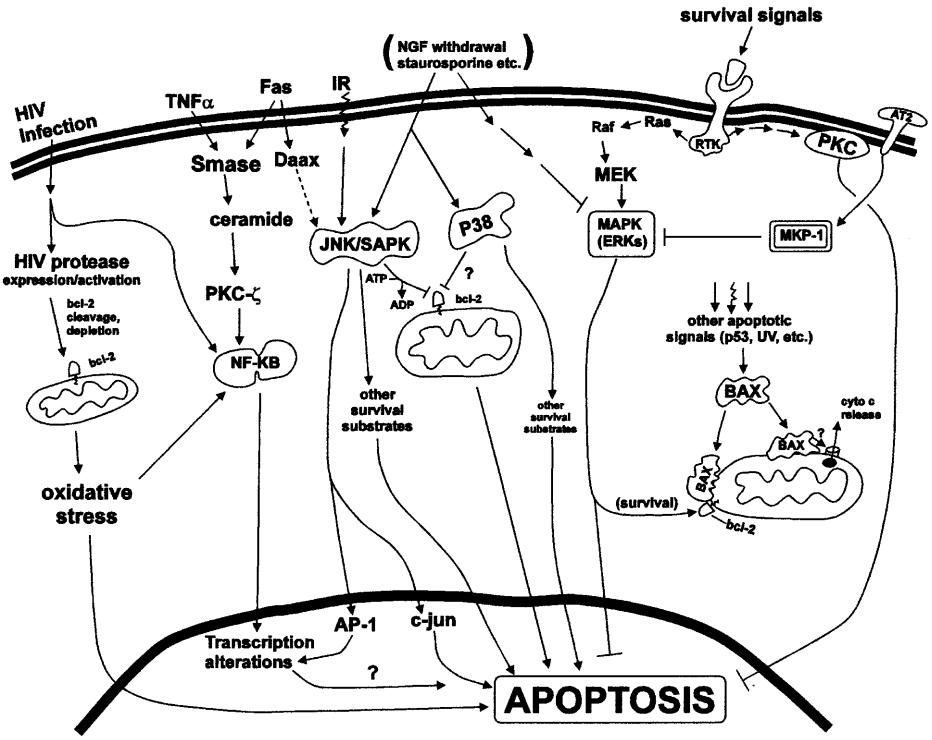

Figure 2. Schematic diagram showing proapoptotic pathways regulating Bcl-2 proteins. Fas or TNFα receptor promotes sphingomyelinase (Smase) activity and ceramide accumulation/signalling. Subsequent NF-κB activation via PKC-ζ leads to transcriptional alterations.

Proteolytic cleavage of Bcl-2. An alternative mechanism for the regulation of Bcl-2 may be through proteolytic cleavage. Human immunodeficiency virus (HIV) protease expression in cultured fibroblast, epithelial and lymphocytic cells leads to the cleavage of Bcl-2 and subsequent apoptotic cell death [91]. In this study, depletion of Bcl-2 by the viral protease was followed by activation of the oxidative stress-dependent nuclear factor-kappa beta (NF-κB) transcription factor. Overexpression of Bcl-2 protected cells from PCD, also reducing viral proteins and infectivity. The model proposed in this study suggests that HIV-protease degradation of Bcl-2 is an integral part of HIV-induced apoptosis, and leads to oxidative stress, NF-κB activation (a key constituent of HIV replication) and ultimately to cell death. On the other hand, lymphocytes overexpressing Bcl-2 downregulate NF-κB-containing sequences such as the HIV long-terminal repeat and tumour necrosis factor (TNF)-α, protecting cells from apoptosis as well as decreasing HIV infectivity. (see fig. 2)

Taken together, phosphorylation of Bcl-2 in the flexible loop region appears to inhibit its function, as does the phosphorylation of Bad and its subsequent sequestration by 14-3-3. In addition, viral infection, which increases the susceptibility of a cell to die, may proteolytically cleave the primary cell death inhibitors to gain other advantages such as increasing replication, even though it ultimately means giving up their own home. A number of scenarios have been presented for exactly how these regulatory steps interplay with signals generated by the surface binding of extracellular survival factors [72]. However, further investigation is needed to identify which kinases and phosphatases are responsible for post-translational modifications of Bcl-2 family members. Since the death signals and apoptotic programme cover such a diverse arena, it is likely that these signalling molecules will integrate into a more complex and multifunctional pathway.

ICE proteases: the caspases

In 1986, Ellis and Horvitz [92] demonstrated that loss of function mutations in *ced-3* and *ced-4* prevented normal developmental apoptosis in the nematode *C. elegans*. Subsequently, *ced-3* was cloned and shown to be similar to the mammalian ICE, which encodes a cysteine protease [93]. Because of this homology, it was

postulated that ICE may be the vertebrate cysteine protease necessary for PCD. Indeed, Miura et al. [94] showed that overexpression of ICE induced apoptosis in Rat-1 cells and that crmA, a specific inhibitor of ICE [95], could inhibit ICE-dependent PCD. Fernandes-Alnemri et al. [96] then identified the actual mammalian homologue of *ced-3*, CPP32 (caspase-3), which later was shown to induce apoptosis [97]. Caspase-3, like Ced-3 and ICE, exists in the cytosol as a proenzyme that is protolytically activated during apoptosis [98]. In 1996, Alnemri et al. [99] proposed the term 'caspase' to represent this new family of pro-apoptotic proteases. The 'c' denotes the cysteine protease mechanism of action, while 'aspase' reflects their ability to cleave after an Asp residue (table 2).

Structurally, active ICE exists as a homodimer $(p20/p10)^2$ derived from two 45-kDa proenzyme molecules. During processing of proICE, the active site is formed when a p10 subunit from one p45 joins with the p20 subunit from a separate p45 dimer, generating a unique orientation for specific autocatalysis [100–102]. Thus, each p45 is autocatalysed to produce p20 and p10, the mature ICE subunits. The essential cysteine residue is part of the p20 fragment, and charge interactions with an aspartic acid residue of the substrate confer the unique specificity requirement for Asp in the P1 position.

Although cell death occurs naturally in the development of the nervous system and immune system [103, 104], apoptosis may be a pathological event. For example, virus-infected host cells undergo PCD to prevent viral propagation. p35 is an antiapoptotic viral gene expressed by the baculovirus to elude PCD [105, 106]. p35 irreversibly binds ICE and prevents cleavage of ICE substrates. Caspases-2, -3 and -4 are inhibited by p35 as well [105, 107, 108]. Other viral proteins from the inhibitor of apoptosis gene, iap, block the activation of host caspase-3 and caspase-7 but do not inhibit previously active caspases [109, 110]. Caspases are intimately involved in regulating apoptosis, and the substrates cleaved by these enzymes are undoubtedly important for successful execution of PCD.

The highly conserved process of apoptosis unfolds through distinct intracellular events where caspases function as the final executioner. Many of the morphological features evident during apoptosis occur through biochemical reactions some of which involve caspases and their substrates. Not only are they critical for correct morphologic changes during apoptosis, but targeting substrates such as Rb, which control the cell cycle, proliferation and apoptosis, implies that they can effectively oppose any cellular attempt to redirect the death programme.

Activity and function of caspases

Substrate specificity varies among the caspase family (table 2), and not all substrates are closely linked to apoptosis. Lamins A, B and C are proteins that form intermediate filaments, constitute the main structural component of the nuclear membrane and function in chromatin organization [111]. Oberhammer el al. [112] showed that chromatin condensation during apoptosis occurred in parallel with lamin degradation but without activation of p34 cdc2 kinase, an enzyme involved in lamin turnover during mitosis [113, 114]. Previously, Lazebnik [115] showed that cell extracts devoid of p34 cdc2 kinase induced chromatin condensation and DNA fragmentation in isolated nuclei. Since protease inhibitors prevent lamin cleavage, it is possible that an ICE-like enzyme is responsible for lamin degradation [116]. Both lamin A and C contain an aspartate residue at position 230 that is cleaved during apoptosis [117].

While lamin degradation may contribute to the membrane changes during apoptosis, DNA condensation and fragmentation can be explained, at least in part, by the action of caspases on other substrates. PARP is a nuclear enzyme involved in DNA repair [118, 119] that is cleaved and inactivated by caspases [97, 117, 120]. PARP negatively regulates the Ca^{2+}/Mg^{2+}-dependent endonuclease activated during apoptosis [121, 122]. Cleavage of PARP leads to increased DNA damage resulting in DNA fragmentation through uncontrolled endonuclease activity. Other DNA enzymes, such as the large subunit of the DNA replication complex C, are also important targets of caspase cleavage [123]. In addition, DNA fragmentation factor, DFF, is a cytosolic protein capable of inducing DNA fragmentation after activation by caspase-3 [124]. Recently, Rudel et al. [125] demonstrated that caspase-3 cleaves PAK2 (p21 activated kinase-2) at Asp212 in T cells resulting in apoptosis. PAKs are serine-threonine kinases that regulate the actin cytoskeleton [126]. Proteolysis of PAK2 separates the catalytic domain from the regulatory domain, which constitutively activates the enzyme to promote apoptosis. Inevitably, DNA damage without proper repair mechanisms results in PCD.

Rb protein and caspases. It is known that underphosphorylated(active) $p105^{rb}$ acts at the G1 phase restriction point to limit cell cycle progression. Much of this attribute of $p105^{rb}$ stems from its ability to bind to and suppress the action of E2F family transcription factors [127–129] which themselves coordinate expression of many genes responsible for the mitotic G1-S phase transition in mammalian cells. Growth-promoting stimuli cause hyperphosphorylation of $p105^{rb}$ by the action G1 phase-specific cyclin-dependent kinases (CDKs) [130, 131]. Unbound E2F now acts with its partner DP-1 to increase cyclin A, E and CDK2 expression and activity leading to further pRb hyperphosphorylation

(inactivation) during G1 to S phase transition [131]. Rb is also an important inhibitor of apoptosis in numerous cell types [132]. Much evidence suggests that the initial step in cell cycle progression, entry into S phase, may be a key regulatory point in the pathway of cell death [130]. Thus, targeted inhibition of Rb not only deregulates cell cycle entry, but it increases susceptibility to PCD. In fact, deregulated E2F-1 expression, or lack of inhibition by Rb, causes aberrant S-phase entry (often seen in cells undergoing apoptosis) and promotes p53-dependent apoptosis [130, 131]. It is also possible that p53-mediated cell death and caspase-3 activation [133] are dependent on cell cycle progression. This may indeed expose cells to direct protease-activating apoptotic factors present during cell cycling.

Recent reports provide convincing evidence that a key function of certain caspases is the cleavage of the retinoblastoma protein [134–138]. Treatment of Jurkat (human leukaemic) T cells with anti-fas antibody induces pRb protein dephosphorylation and subsequent pRb proteolysis, both preceding internucleosomal DNA fragmentation [134]. This is apparently due to the action of ICE-like proteases following Fas-induced apoptosis, since it was inhibited by a specific ICE-like protease tetrapeptide inhibitor, by CrmA expression or by Bcl-2 protein expression. In addition, a consensus caspase cleavage site at the C-terminus of pRb was shown to be cleaved in vitro and in vivo by proteases related to caspase-3 (CPP32), and a protease-resistant Rb was generated when residues in this site were mutated [135]. In fact, the nondegradable Rb actually protected Rb-/- 3t3 cells from TNF cytotoxicity but could not inhibit anti-CD95-induced death of Jurkat T cells. Thus, failure to degrade pRb inhibited apoptosis in a cell type-specific and/or inducing agent-specific manner. This suggests that pRb is a key substrate for caspases and that its cleavage may constitute a commitment point as to whether the cell death pathway gets executed. Janicke et al. [136] have shown that in many tumour cell lines, the C-terminal 42 amino acid peptide of the Rb protein is specifically removed by an ICE-like protease as an early event during TNF- and staurosporine-induced apoptosis. pRb cleavage was blocked in vitro and in vivo by two specific ICE-like protease inhibitors, and in vitro by a point mutation at the cleavage site (Asp886 to Ala). Moreover, the extent of Rb cleavage correlated with TNF-induced apoptosis in all the tumour types examined. While the cleaved form of pRb was still able to bind cyclin D3 and inhibit E2F-1 transcriptional activity, it failed to bind to mdm-2, which itself is implicated in apoptosis. Chen et al. [137] show that breast epithelial cells treated with anti-Fas leads to the cleavage of a 5-kDa fragment from the C-terminus of pRb that coincided with both cysteine protease activation and cleavage at a CPP32-like recognition sequence. The truncated product, termed p100cl, has enhanced E2F-binding affinity, leading the authors to suggest that distinct post-translational pathways may also exist for the function of pRb during growth arrest (as p105Rb) or apoptosis (as p100cl).

Thus, it appears that pRb cleavage is an early and possibly pivotal event in the execution of a death programme in some cells. In fact, failure to activate ICE-like proteases and cleave Rb protein has been associated with drug resistance in tumour cells failing to undergo PCD upon treatment with many chemotherapeutic agents [139].

Regulation of caspases

Caspase regulation is best illustrated by the Fas receptor and the TNF receptor-1 (TNFR1) signal transduction pathways. Mutational analyses indicate that both receptors confer apoptosis via the association with specific intracellular death domain-containing proteins, forming a DISC (death-inducing signalling complex) [140–144] (see fig. 3). Previously, it was shown that an ICE-like protease is involved in Fas-mediated cell death [145, 146] and that caspase-8 is the focal point for sequential activation of the caspase cascade during Fas signalling [147]. Both FLIP (an inhibitor of caspase-8) [148] and mutations in the catalytic domain of caspase-8 [149] prevent Fas-induced apoptosis, implicating this caspase as an early upstream protease active in apoptosis. Recently, Harvey et al. [150] demonstrated that caspase-2 is also active early in apoptosis. Caspase-2 and caspase-8 share large prodomains, and these enzymes function to regulate the smaller effector caspases [151]. Although caspase-2 and caspase-8 are the upstream modulators of PCD, caspase-3 and caspase-6 are the most active in apoptotic cells regardless of stimuli [152, 153].

Activation of caspases via fas and TNFR1. Much work has been done to elucidate how signals through Fas and TNFR1 activate caspases during apoptosis. Boldin et al. in 1995 [154] discovered a unique protein which interacts with the death domain of the Fas receptor, designated MORT1[154]/FADD[155] for mediator of receptor-induced toxicity and Fas-associating protein with a death domain, respectively. Overexpression of MORT1/FADD induced ligand-independent apoptosis [154, 156], and N-terminal truncation of the protein inhibits Fas/TNFR1-induced apoptosis [156], suggesting downstream regulation by MORT1/FADD. Interestingly, caspase-3 activation by Fas is inhibited by the truncation as well. Figure 3 shows the signalling mechanism of Fas and TNFR1. Briefly, FADD associates with Fas through death domains, as does tumour necrosis factor receptor associated death domain (TRADD) with TNFR1 [154, 157]. Although there is a weak association between FADD and TNFR1 directly, TRADD is likely the adaptor molecule necessary for

the functional interaction of FADD with TNFR1 [158, 159]. Caspase-8 is activated by a death domain interaction with FADD [160].

A homologue of caspase-8 exists, FLICE2 (caspase-10), which interacts with FADD (fig. 3). Mutations in the active site of caspase-10 prevent Fas/TNFR1-induced apoptosis. Conversely, I-FLICE is an inert caspase similar to caspases-8 and -10 but which lacks a catalytic domain and substrate-binding pocket. This protein inhibits apoptosis induced by Fas/TNFR1 by binding to caspase-8 and caspase-10 and thereby preventing FADD-mediated activation [161]. FLIP (FLICE inhibitory protein) [148] is expressed early in T-cell activation and also protects cells from Fas-induced apoptosis either by sequestering FADD or by interacting directly with caspase-8 and caspase-10 to prevent their activation.

RIP is a death domain protein designated receptor interacting protein for its ability to interact with Fas and weakly with TNFR1 [162, 163]. RIP contains a kinase domain at its N-terminus with structural similarity to tyrosine and serine-threonine kinases. Although no enzymatic activity has been ascribed to this domain, it is worth considering that Fas-induced apoptosis requires tyrosine kinase activity [164]. RAIDD (RIP-associated ICE-homologous protein with death domain) [166]/CRADD (caspase and RIP adaptor with death domain) [165] is an adaptor molecule involved in apoptosis signalling in response to TNFR1 stimulation. The N-terminal domain of CRADD interacts with the prodomain of caspase-2, while the carboxy terminus binds RIP. Since the kinase domain of RIP is now in close proximity to caspase-2, a phosphorylation event may be involved in caspase regulation. It is clear that a caspase cascade is active in apoptosis and that TNFR1 and Fas signalling through death domain proteins regulate this cascade. Caspase-8 and caspase-2 are regulated proximally at the level of the death receptors, while caspase-3 and caspase-6 function as the distal effectors. Ultimately, the apoptotic signal initiated through Fas and caspase-8 activation is transduced by caspase-4 as it cleaves procaspase-3 [167]. The net result is specific substrate cleavage by caspase-3 and final execution of apoptosis.

Transcription factors and caspases. Nuclear events mediating apoptosis have been demonstrated in some cell types. NF-kB is a transcription factor whose activity prevents apoptosis despite signalling through a death receptor (TNFR1) [168–170]. TNFR1 promotes cell death with caspase activation, but a second pathway through NF-kB is capable of suppressing apoptosis. TRAF2 (TNF receptor-associated factor 2) binds RIP and associates indirectly with TNFR1 through TRADD [171], activating NF-kB to promote cell survival (fig. 3), while mutant TRAF2 prevents activation of NF-kB [172]. Thus, the balance between life and death may depend on the activation or attenuation of transcription factors. Indeed, other factors modulate apoptosis through nuclear triggers. STAT (signal transducer and activator of transcription) proteins are activated by protein tyrosine kinases [173]. Growth factors such as IGF-1 and EGF promote cell survival and proliferation through tyrosine kinase activity [174, 175], while inhibitors of the EGF receptor tyrosine kinase can activate apoptotic pathways [176]. Interferons (IFN) also function through tyrosine kinase signals, yet they induce cell cycle arrest through STAT activation [177, 178]. Tyrosine kinases regulate both cell survival and cell death via initially similar signalling pathways. The flexibility of these signal cascades results from specific gene activation and/or repression. In support of this theory, Chin et al. [179] demonstrated that IFN-γ and EGF receptor activation induce apoptosis through JAK (Janus kinase)-STAT signalling and that STAT1 was necessary to increase caspase-1 messenger RNA (mRNA). The increase in caspase-1 mRNA was required for IFN-γ-mediated PCD, while the specific caspase inhibitor (ZVAD) [180] prevented apoptosis. This evidence suggests that nuclear regulation of caspases is important for apoptosis and that ubiquitous signalling cascades contribute to the regulation of PCD. The transcription factor c-jun is an early immediate gene switched on by various kinases in response to growth stimuli [181]. However, c-jun can also signal apoptosis with caspase-3 activation [182]. DPC4 is a transcription factor capable of inducing cell death when expressed through a SAPK cascade [183]. The pleiotropy of signal transduction is dependent on specific gene regulation, and caspase gene expression may be an important factor in regulating PCD.

Bcl-2 and caspases: from nematode to mammal

There now appears to be convincing evidence that mammalian cytosolic proteins exist which have the ability to functionally and possibly physically connect Bcl-2-like proteins to caspases [117, 184, 185]. These studies stem from the fact that Bcl-2 family members can prevent the activation of those caspases which implement the cell death programme. Indeed, Bcl-2 prevented E1A-induced caspase-3 (CPP32) activation and PARP cleavage normally seen during E1A-induced apoptosis [184]. An explanation for this occurrence began by using the Bcl-2 and caspase homologues of *C. elegans*, *ced-9* and *ced-3* [15, 93], respectively, to search for a direct and conserved link between death inhibitors and promoters [15, 151, 186, 187] that is similar to the Ced-4 protein. Mechanistically, Ced-9 requires Ced-4 to block

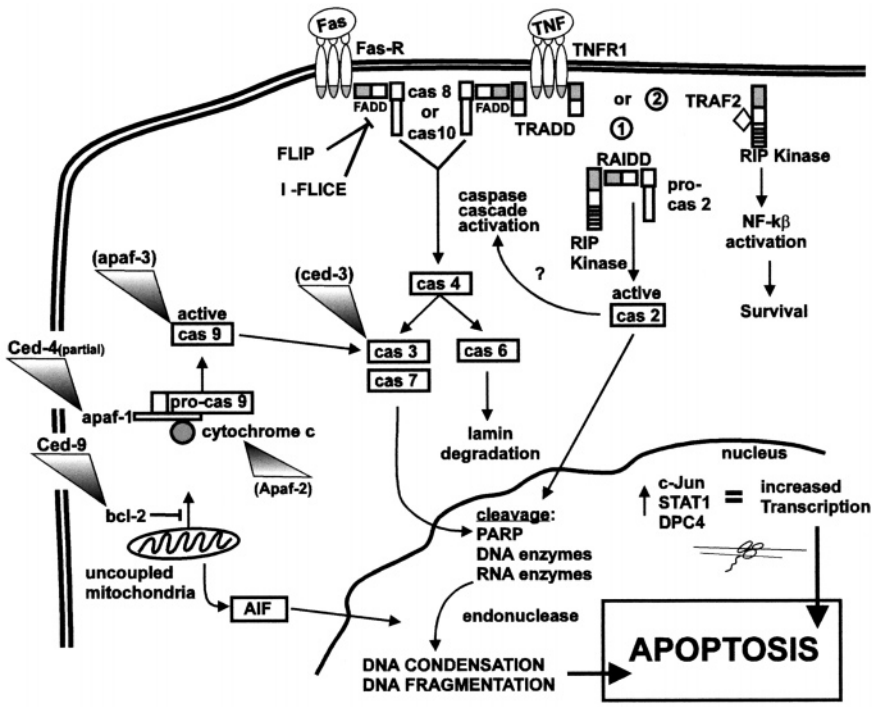

Figure 3. Fas-R and TNFR1 signal transduction and caspase regulation. Activation of either Fas-R or TNFR1 results in recruitment of death domain proteins (FADD, TRADD etc.) and the activation of a caspase cascade. Bcl-2 modulates the activity of caspase-9, and potentially the entire caspase cascade, by preventing cytochrome c release from the mitochondria. Specific cleavage of PARP and lamins by caspases-3 and -6 activates an endonuclease which cleaves DNA, leading to programmed cell death. TNFR1 dual interactions include (1) TRADD activation of caspase-2 through RAIDD, leading to PCD, or (2) NF-κB activation through TRAF2, promoting cell survival. Specific prodeath genes are regulated by separate transcription factors (c-jun, STATs etc.).

apoptosis, while Ced-4 also appears to regulate Ced-3 [15, 188], since Ced-3-enforced death requires Ced-4. Ced-9 acts upstream of Ced-3 with Ced-4 in between. It now appears that Ced-4 physically interacts with Ced-3 and Ced-9 simultaneously, and cell death induced by Ced-3/Ced-4 is inhibited by either Ced-9 or Bcl-x_L (substituted for Ced-9) during constant association with Ced-4 [151, 186, 187]. The same Ced-4 - caspase interaction occurred when caspase-8 was substitiuted for Ced-3 [151]. In fact, only in the presence of Ced-4 could Ced-9 and Ced-3 be coimmunoprecipitated from the cytosol of mammalian cells, suggesting that a trimeric complex is regulating the death programme. Mutations introduced into regions of Bcl-x_L or Ced-9 which inhibit their ability to interact with Ced-4 rendered both survival proteins unable to block apoptosis [151]. This again supports the idea that conserved pathways may exist between nematodes and humans and that a mammalian Ced-4 homologue should definitely contribute to this molecular complex. In addition, transfection and expression of *ced-4* alone in mammalian cells induces apoptosis which could be blocked by Ced-9 or caspase inhibitors. The fact that caspase inhibitors block Ced-4-induced death in mammalian cells suggested that a physical interaction, and possibly activation, occurred between Ced-4 and certain mammalian caspases. This was indeed true, but although Ced-4 could directly bind Ced-3, it could only bind to mammalian caspase-1 and caspase-8, and not to caspases-3 or -6 [151]. This is apparently due to the presence of a large amino-terminal domain, or 'pro-domain', on caspases-1 and -8 which is cleaved off during caspase activation. That the large prodomain of Ced-3, separate from the remainder of the protein, is sufficient to associate with Ced-4 in vivo further supports the idea that this large prodomain is a key region for these interactions. Unlike that of Ced-3, caspase-1 and caspase-8, small prodomains are found in caspases-3 and -6 which presumably do not allow such an interaction. On the other hand, Bcl-x_L could associate with caspase-1 and caspase-8 only when coexpressed in mammalian cells and without much affinity in vitro [151]. Moreover, this was an indirect association which again suggests that an uncharacterized mammalian linker protein exists which is in some

way the functional equivalent to Ced-4. Interestingly, Ced-4 binding to Ced-3/caspases was found to be independent of any Ced-9 association. Consequently, studies in mammalian cells revealed that the proapoptotic activity of Ced-4 requires expression of a functional Ced-3 protease [189], and expression of Ced-4 enhances the proteolytic activation of Ced-3. In addition, Ced-9 inhibits the proteolytic activation of Ced-3 as well as inhibiting the enzymatic activity of Ced-3 promoted by Ced-4. Thus, a multimeric protein complex is formed between Ced-3 and Ced-4 with Ced-9 in vivo [189].

One important clue as to what the mammalian homologue of Ced-4 might be came from the knowledge of its intracellular location. Studies provided evidence that Ced-9 regulates the subcellular location of Ced-4 and thus its ability to associate and cooperate with Ced-3 in implementing cell death [187]. In mammalian cells, Ced-9 localized to intracellular and perinuclear membranes, whereas expression of Ced-4 alone resulted in a cytosolic distribution. However, using the yeast two-hybrid system, expression of Ced-9 targeted Ced-4 to the same intracellular membrane sites as Ced-9. This suggests that in mammalian cells, Ced-9 (or Bcl-x_L) is able to sequester Ced-4 (and possibly the mammalian equivalent) from the cytosol and thereby prevent it from activating downstream caspases. Discoveries over the past 6 months further clarify Ced-4 function on the molecular level, providing a framework for a functionally homologous mammalian Ced-4.

The caspase-activating Apafs

It had been shown that Ced-4 contained a phosphate-binding loop (P loop) which when mutated disrupts its ability to induce chromatin condensation in yeast [190]. Subsequent in vivo studies showed that a P loop mutant was also defective in its ability to cause Ced-3 processing [191]. Even so, pro-Ced-3 was unable to be processed and activated by Ced-4 alone [191, 192]. Studies from these two groups demonstrated that Ced-4 catalyses Ced-3 self-processing and that this requires the prodomain of Ced-3, which itself was previously shown to interact with Ced-4 [193]. They show that using its nucleotide-binding site, Ced-4 binding to adenosine triphosphate (ATP) was required for its activity as a catalyst in Ced-3 processing, presumably due to the activity of the putative Ced-4 ATPase domain.

Using a previously established method whereby deoxyribose-ATP is added to human cell extracts to cause proteolytic activation of caspase-3 [194], Zou et al. [195] purified and cloned a novel protein from HeLa cell cytosol called Apaf-1 for apoptosis protease-activating factor. Three Apafs have been isolated using this system, Apaf-2 being cyctochrome c [194] and Apaf-1 being resolved into two factors designated Apaf-1 and Apaf-3. Apaf-1 is essentially the partial functional equivalent of Ced-4, since addition of deoxy adenosine triphosphate (dATP) in this system led to caspase-3 processing and activation only in the presence of Apaf-1. It should be noted that Apaf-1 homology relates only to the death-inducing pathway, since Ced-4 interacts with Ced-9 to promote survival, but Apaf-1 is not known to interact with and support the function of Bcl-2. The ability of Apaf-1 to function in this capacity relates to its protein sequence. A portion of Apaf-1, including the nucleotide-binding site region, shows remarkable sequence similarity to Ced-4 via alignment studies. The NH_2-terminus, however, shows high similarity to the prodomain of Ced-3. These domains probably act as caspase recruitment domains (CARD) which are thought to bind to caspases directly [196]. Ced-3, Ced-4 and other cell death proteins with long prodomains (including caspase-1, caspase-2 and caspase-9) contain CARD domains as well [197]. Unlike Ced-4, however, the COOH-terminus of Apaf-1 contains multiple WD-40 repeats suspected to mediate protein-protein interactions, such as that observed by Zou et al. [195] between Apaf-2/cytochrome c and Apaf-1. Not only does the activation of caspase-3 by Apaf-1 and Apaf-3 require cytochrome c and dATP, but cytochrome c directly binds to Apaf-1. Interestingly, binding of cytochrome c to Apaf-1 was not influenced by the presence or absence of dATP. Since Bcl-2 prevents caspase-3 activation during many proapoptotic conditions [184, 194, 198, 199], Zou et al. proposed a model whereby Bcl-2 prevents this activation by preventing cytochrome c release from the mitochondria, previously shown to occur in Bcl-2-overexpressing cells [69, 70]. If released, cytochrome c would bind Apaf-1 possibly in its WD repeat region, which would join cytochrome c and Apaf-3 and thereby trigger caspase-3 activation only in the presence of dATP. Activated caspase-3 then cleaves and activates DNA fragmentation factor [194], leading to internucleosomal DNA fragmentation. The recent characterization of Apaf-3 in this system helps to complete the protease signalling cascade.

Wang et al. have now identified Apaf-3 as caspase-9 [197]. They recently demonstrated that Apaf-3/caspase-9 is activated upstream of caspase-3 and that Apaf-3/caspase-9 mediates the subsequent cleavage and activation of caspase-3. Caspase-9 activation preferentially occurred in the presence of dATP rather than ATP, and nonhydrolysable ATP analogues prevented their cleavage, suggesting that hydrolysis is required for the reaction. In addition, in the presence of both dATP and cytochrome c, a complex was formed between Apaf-1 and caspase-9 – an interaction occurring via the amino terminal CARD domains of both caspase-9 and Apaf-1. Most important, mutation of the caspase-9 active site blocked both caspase-3 activation and apop-

tosis in vivo. Thus, the proposed model begins with an apoptotic stimulus causing release of cytochrome c and the subsequent binding of caspase-9 to Apaf-1 triggered by the presence of cytosolic cytochrome c and dATP. The binding of caspase-9 to Apaf-1 via their CARD domains leads to caspase-9 cleavage and activation, resulting in caspase-3 processing/activation and ultimately apoptosis. The authors suggest that Apaf-1 functions as a docking protein, or bridge, for caspase-9 and cytochrome c. The inability of caspase-9 to bind Apaf-1 without cytochrome c and dATP further supports the notion that the CARD of Apaf-1 may not be normally exposed or accessible unless cytochrome c is present in the cytosol. Furthermore, the cytochrome c-Apaf-1 complex (able to form in the absence of dATP [195]) cannot bind caspase-9 unless dATP (or a high concentration of ATP) is present. Ultimately, nucleotide binding with hydrolysis may provide the necessary energy to cause a conformational change in Apaf-1, exposing its CARD region and thus triggering a committed cascade towards cell suicide.

Conclusion

Studies investigating the genetic regulation of apoptosis have provided insight into the numerous genes and proteins that act either to protect or to induce apoptosis in a variety of cell types. One of these is the proto-oncogene *bcl-2* which plays a central role in cell survival programmes. The function and regulation of Bcl-2 proteins during apoptosis of mammalian cells is increasingly complex. Some members of this family, such as Bcl-2 and Bcl-x_L, act as survival promoters, whereas others, including Bad, Bax, Bak and Bcl-x_s, act as death promoters. Membrane-targeted Bcl-2 family members that inhibit apoptosis may do so by forming channels to prevent cytochrome c holoenzyme release during a death stimulus. They may also recruit and activate cytosolic kinases, such as Raf-1, in an attempt to possibly regulate these channels and/or to negatively regulate death-promoting family members such as BAD. Alternatively, stress-activated protein kinases, such as SEK1 and JNK/SAPK, under final duress may target survival proteins to initiate a committed death programme, while growth-associated ERK-1/ERK-2 MAP kinases phosphorylate substrates to promote survival. Despite rapid advances in this area, the information about other upstream or downstream apoptotic effectors or inhibitors of these kinases and how they integrate into the death programme is still lacking. In addition, enzymes such as those of the PKC family are known to be activated during both survival and death responses, indicating the difficulty of generalizing which molecules function in an absolute fashion. Caspase substrates and the effects of their cleavage cause multiple proapoptotic influences in the execution of PCD. Cleavage of Rb is one instance where growth and death stimuli converge, which suggests that caspases cleave substrates for biological as well as morphological reasons. Thus, regulatory signals are diverse in nature and require cell processing and integration before a final response is evident. The Fas-induced signalling pathway and the activation of stress-related kinases are only two important examples. Cellular fate will undoubtedly be an environmentally altered tissue-specific response.

The importance of apoptosis to clinical biology will ultimately require the identification of molecules acting as close to the cell death transition point as possible. This should allow the design of agents that selectively manipulate each molecular player to obtain a desired outcome on cell fate. Treatment of cancer and other apoptotic-related disease conditions will ultimately depend on the effectiveness of therapies and specificity with which they alter the regulation of the cell death programme. Further characterization of the genes and cellular signals mediating mammalian cell apoptosis is therefore essential.

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