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Cell Death: Critical Control Points

Review

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Programmed cell death is a distinct genetic and biochemical pathway essential to metazoans. An intact death pathway is required for successful embryonic development and the maintenance of normal tissue homeostasis. Apoptosis has proven to be tightly interwoven with other essential cell pathways. The identification of critical control points in the cell death pathway has yielded fundamental insights for basic biology, as well as provided rational targets for new therapeutics.

Programmed cell death (Lockshin and Williams, 1965) and its morphologic manifestation of apoptosis (Kerr et al., 1972) is a conserved pathway that in its basic tenets appears operative in all metazoans. Cell deaths during embryonic development are essential for successful organogenesis and the crafting of complex multicellular tissues. The evolutionary advent of differentiated cell types may have necessitated controlling death as well as division in order to keep neighboring cells interdependent and insure the proper balance of each cell lineage. Apoptosis also operates in adult organisms to maintain normal cellular homeostasis. This is especially critical in long-lived mammals that must integrate multiple physiological as well as pathological death signals, which for example includes regulating the response to infectious agents. Gain- and loss-of-function models of genes in the core apoptotic pathway indicate that the violation of cellular homeostasis can be a primary pathogenic event that results in disease. Evidence indicates that insufficient apoptosis can manifest as cancer or autoimmunity, while accelerated cell death is evident in acute and chronic degenerative diseases, immunodeficiency, and infertility. Here, we will explore some highlights of this very active field of endeavor that witnessed an explosion of information over the past 15 years. Notably, insights from *C. elegans, Drosophila*, and mammals have focused on different portions of the death pathway, suggesting that each species or perhaps the cell types and signals studied in them have emphasized selected control points.

Programmed Cell Death in *C. elegans*

The *C. elegans* hermaphrodite undergoes a distinct and invariant pattern of programmed cell death where the same 131 cells out of 1090 cells die in the development of this 959 cell nematode. Sydney Brenner envisioned that this nematode would be an ideal model organism to define specific genes responsible for developmental cell fates (Brenner, 1974). John Sulston mapped cell lineages in *C. elegans,* noting they were invariant and that specific cells always die (Sulston, 1976). H. Robert Horvitz had the insight to mutagenize *C. elegans*, in order to identify genes regulating all 131 somatic cell deaths (Ellis and Horvitz, 1986). For their pioneering contributions to developmental genetics and programmed cell death, the triumvirate of Brenner, Horvitz, and Sulston received the 2002 Nobel Prize. Initially, two genes, *ced-3 and ced-4* were noted to be absolutely required for all deaths. Whereas, another gene, *ced-9* is required to prevent cell death and was first identified by a gain-of-function mutation *n1950*, which dominantly blocked all somatic cell death (Hengartner and Horvitz, 1994a). *ced-9* proved to be the worm homolog of the mammalian *BCL-2* oncogene, which had been shown to prevent apoptotic cell death. Moreover, mammalian *BCL-2* was capable of functioning in *C. elegans* (Vaux et al., 1992; Hengartner and Horvitz, 1994b) suggesting the evolutionary conservation of this cell death pathway.

The cloning and characterization of *ced-3* provided a critical insight into how the core apoptotic machinery executes cell death. *ced-3* encoded a protein related to the mammalian interleukin 1β converting enzyme (ICE) involved in inflammation (Yuan et al., 1993). Expression of either *ced-3* or *ICE* in mammalian cells induced cell death. ICE became the first member (caspase-1) of a family of proteases dependent on a cysteine nucleophile to cleave motifs possessing aspartic acid (aspase), thus the name caspase (Thornberry and Lazebnik, 1998). Caspases are produced as inactive zymogens possessing a large and a small subunit preceded by an N-terminal prodomain. Two Asp cleavage sites are processed sequentially. The large and the small subunits associate to provide the active site of the enzyme. Crystallographic studies revealed that the active caspase is a tetramer of two heterodimers, thus containing two active sites. Upstream caspases known as initiators are capable of autocatalytic activation and generally have a long prodomain. Downstream effector caspases need initiator caspases for their activation by transprocessing. An elegant amino acid library scan identified an optimum four amino acid motif N-terminal to the aspartic acid cleavage site for each caspase which helped define substrate specificity as well as specific peptide inhibitors for caspases (Thornberry et al., 1997).

Select members (caspase-1, -11) of this protein family are involved in specific processing of proinflammatory cytokines, including IL-1 and IL-18. Other effectors, such as caspase-3 and –7, are executioners of apoptosis as processing of their substrates leads to morphological changes associated with apoptosis, including DNA degradation, chromatin condensation, and membrane blebbing. Importantly, activation of CPP32/caspase-3 was shown to cause an apoptotic nuclear morphology, which could be blocked by a peptide inhibitor of CPP32 (Nicholson et al., 1995). Examination of *ced-3* substrate specificity revealed that this enzyme is more similar to mammalian CPP32/caspase-3 than to ICE/caspase-1 (Tewari et al., 1995; Xue et al., 1996). Another line of evidence for the importance of caspases in cell death came from

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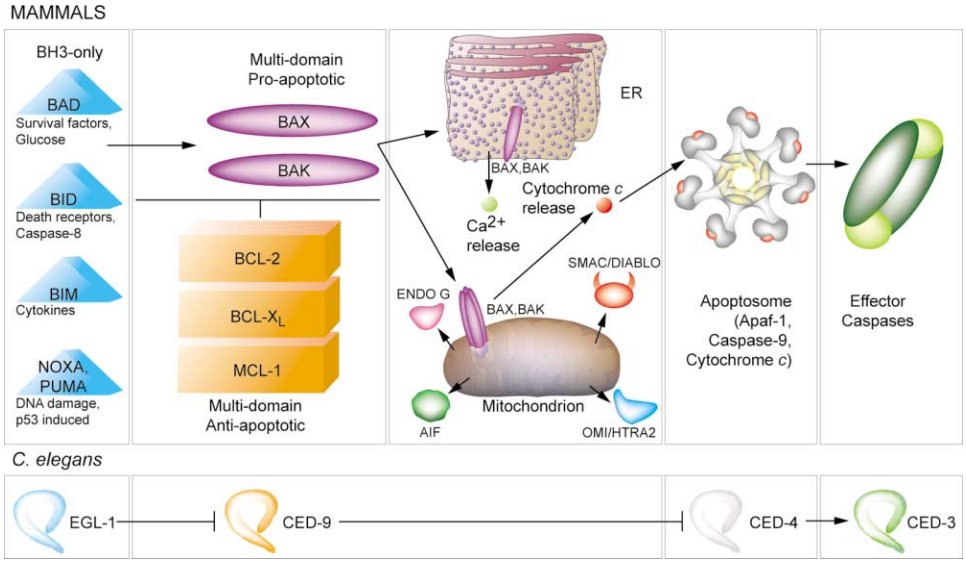

Figure 1. Intrinsic Apoptotic Pathway
See text for details.

studies indicating this protease family mediates apoptosis downstream of death receptors.

APAF-1 and the Apoptosome

The genetic studies in *C. elegans* were seminal in ordering *ced-4* upstream of *ced-3* caspase, however *ced-4* proved to be a "pioneer" sequence (Yuan and Horvitz, 1992). Biochemical fractionation of mammalian cells dedicated to reconstituting apoptosis in vitro shed light on this intermediate step in the core pathway. Three activities designated as Apafs (apoptotic protease activating factors) were required to reconstitute caspase activity in vitro. Apaf-1 turned out to be an adaptor/amplifier molecule with homology to *ced-4*, while Apaf-2, and Apaf-3 were identified as cytochrome *c* and caspase-9, respectively (Li et al., 1996, 1997; Zou et al., 1997). Caspase-9, an initiator caspase, is capable of self-processing when bound to Apaf-1, which provides a complex to ensure high local concentration and proper protein conformation suitable for activation. Caspase-3, an effector caspase, is cleaved and activated by caspase-9. Structure/function studies have offered a sophisticated model for caspase-9 activation. Apaf-1 binds cytochrome *c* via its WD40 domains. Elegant studies revealed that upon binding to cytochrome *c*, Apaf-1 becomes competent to recruit caspase-9 in the presence of ATP/dATP. This interaction is mediated by caspase recruitment domains (CARD) present in both Apaf-1 and caspase-9 (Li et al., 1997). The CARD domain of Apaf-1 is usually bound by 2 of its WD40 domains and is dislodged when cytochrome *c* binds WD40 domains within Apaf-1. Subsequent binding of ATP/dATP to Apaf-1 is proposed to cause a conformational change facilitating heptamer assembly in the shape of a wheel, known as the apoptosome (Acehan et al., 2002). The CARD and CED-4 homology domains form the hub, while the spokes consist of WD40 domains, and procaspase-9 binds the hub (Figure 1).

Studies of the pathway in *C. elegans* indicate *ced-9* negatively regulates *ced-4* preventing activation of *ced-3* (Shaham and Horvitz, 1996). Like other caspases, CED-3 is an inactive zymogen until it undergoes proteolytic activation. The death machinery is activated when EGL-1, a proapoptotic BH3-only BCL-2 family homolog, binds CED-9 at the mitochondria and displaces CED-4 (Conradt and Horvitz, 1998), which then translocates to the perinuclear region (Chen et al., 2000). Released CED-4 undergoes oligomerization and bound CED-3 is proposed to autocatalytically activate by an induced proximity mechanism (Yang et al., 1998). Unlike Apaf-1, CED-4 does not have WD40 domains and hence does not bind cytochrome *c*. Unlike CED-4, Apaf-1 is not localized to mitochondria and does not bind BCL-2, supporting alternative modes of activating effector caspases in mammalian cells.

The BCL-2 Family

The BCL-2 family of proteins constitutes a critical intracellular checkpoint in the intrinsic pathway of apoptosis. The founding member, the *BCL-2* protooncogene, was first identified at the chromosomal breakpoint of t(14;18) bearing human follicular B cell lymphoma (Bakhshi et al., 1985; Cleary and Sklar, 1985; Tsujimoto et al., 1985). Expression of BCL-2 proved not to promote cell proliferation, like other oncogenes of that day, but instead blocked cell death following multiple physiological and pathological stimuli (McDonnell et al., 1989; Vaux et al., 1988). Specifically, the plasma membrane blebbing, volume contraction, nuclear condensation, and endonucleolytic cleavage of DNA termed apoptosis (Kerr et al., 1972) was blocked by BCL-2, which unexpectedly localized to the mitochondrion, nominating this intracellular organelle for a prominent role in apoptosis (Hockenbery et al., 1990). As a stringent test of BCL-2's oncogenic activity, transgenic mice bearing a *BCL-2*-immunoglobulin minigene, that recapitulates the t(14;18)

developed a polyclonal follicular hyperplasia comprised of resting B cells, which accumulate because of extended cell survival not increased proliferation (McDonnell et al., 1989). Over time, such *BCL-2*-Ig mice progress to life-threatening high grade, monoclonal lymphoma in which their resistance to apoptosis is often spontaneously complemented by the activation of *c-myc* (McDonnell and Korsmeyer, 1991). When *bcl-2/myc* doubly transgenic mice were created, they developed undifferentiated hematopoietic leukemia (Strasser et al., 1990). This potent synergy of a proliferative aberration plus an apoptotic defect has subsequently proven common, perhaps even universal to cancer. Loss-of-function analysis uncovered a critical role for BCL-2 in maintaining normal cellular homeostasis in that *Bcl-2*-deficient mice display apoptosis of lymphocytes, developmental renal cell death and loss of melanocytes (Veis et al., 1993). Thus, BCL-2 constituted the cardinal member of a new category of oncogenes: regulators of cell death.

Mammals possess an entire family of BCL-2 proteins that includes proapoptotic as well as antiapoptotic members. The first proapoptotic homolog, BAX, was identified by its interaction with BCL-2 (Oltvai et al., 1993). *Bax*-deficient mice displayed selective expansion of cell populations. The ratio of anti- to proapoptotic molecules such as BCL-2/BAX constitutes a rheostat that sets the threshold of susceptibility to apoptosis for the intrinsic pathway, which utilizes organelles such as the mitochondrion to amplify death signals (Figure 1). The BCL-2 family can be divided into three main subclasses, defined in part by the homology shared within four conserved regions termed BCL-2 homology (BH) 1-4 domains, roughly corresponding to α helices which dictate structure and function. The antiapoptotic members include BCL-2, BCL-X_L (Boise et al., 1993), MCL-1 (Kozopas et al., 1993), A1 (Choi et al., 1995), and BCL-W (Gibson et al., 1996) and display conservation in all four BH1-4 domains. The structure of a BCL-X_L monomer revealed that its BH1, BH2, and BH3 domains are in close proximity and create a hydrophobic pocket which can accommodate a BH3 domain of a proapoptotic member (Muchmore et al., 1996; Sattler et al., 1997). The "multidomain" proapoptotic members (BAX, BAK) possess BH1-3 domains, although they appear to require an activation event, perhaps to expose the hydrophobic face of their BH3 domain before they can interact with BCL-X_L or BCL-2. In contrast, the proapoptotic molecule BID, isolated based on its ability to bind both BAX and BCL-2, has homology only within the minimal death domain, the BH3 amphipathic α helix, prompting the title "BH3-only" (Wang et al., 1996). Cells doubly deficient for the pair of "multidomain" proapoptotic molecules BAX and BAK proved resistant to all tested intrinsic death pathway stimuli (Lindsten et al., 2000; Wei et al., 2001). BAX and BAK together constitute a requisite gateway to the intrinsic pathway operative at both the mitochondrion (Wei et al., 2001) and the endoplasmic reticulum (ER) (Scorrano et al., 2003). In viable cells, multidomain BAX and BAK exist as monomers. Inactive BAX resides in the cytosol or is loosely attached to membranes and its pocket is occupied by its C-terminal helix (Suzuki et al., 2000). Upon receipt of a death signal BAX inserts into the mitochondrial outer membrane (MOM) as homooligomerized multimers. Inactive BAK that resides at the mitochondria also undergoes an allosteric conformational activation in response to death signals, which includes its oligomerization and the permeabilization of the MOM with release of intermembrane space (IMS) proteins including cytochrome *c*. The precise mechanism whereby IMS proteins are released is still under active investigation. One model holds that oligomerized BAX or BAK may form pores capable of releasing cytochrome *c*. This thesis has origins in the structural similarity between BCL-2 family molecules and the pore-forming helices of bacterial toxins (Muchmore et al., 1996) and evidence that BAX can form channels in artificial membranes and release cytochrome *c* from liposomes. Alternatively, BCL-2 molecules have been proposed to interact with intrinsic mitochondrial proteins and trigger permeability transition (PT); however, substantial cytochrome *c* release clearly occurs prior to swelling or rupture of the mitochondrion. Finally, more global mechanisms of MOM permeabilization including altered membrane curvature and lipid pores are also being investigated.

The BH3-only members serve as upstream sentinels that selectively respond to specific, proximal death, and survival signals (Figure 1). For example, the extrinsic pathway is triggered by the engagement of cell surface death receptors, which then activate caspase-8 that cleaves p22 BID to connect with the intrinsic death pathway. A newly exposed glycine following cleavage in an unstructured loop is N-myristoylated enhancing the translocation and targeting of a p7/myr-p15 BID complex to mitochondria. A reconstituted mitochondrial assay reveals that tBID serves as a membrane-targeted ligand, which requires its intact BH3 domain to trigger oligomerization of BAK or BAX to release cytochrome *c* (Desagher et al., 1999; Wei et al., 2001). The proapoptotic activity of BH3-only molecules is apparently kept in check by either transcriptional control or posttranslational modification. For example, NOXA and PUMA are under p53 mediated transcriptional control in response to DNA damage (Nakano and Vousden, 2001; Oda et al., 2000; Yu et al., 2001). BAD is switched on and off by its phosphorylation in response to growth/survival factors (Zha et al., 1996), providing a connection to the established importance of extracellular factors in promoting cell survival (Raff, 1992). BIM, which is complexed with dynein light chain LC8, responds to multiple stimuli (Puthalakath et al., 1999). Activation of BH3-only molecules either directly or indirectly results in the activation of BAX, BAK and actually requires BAX, BAK for executing apoptosis. In contrast, antiapoptotics, such as BCL-2 or BCL-X_L, serve a principal, although perhaps not an exclusive role of binding and sequestering BH3-only molecules preventing BAX, BAK activation (Cheng et al., 2001). This ordering is consistent with the pathway in *C. elegans*, which places the BH3-only EGL-1 upstream of the multidomain CED-9 molecule (Conradt and Horvitz, 1998).

Unresolved issues include whether all BH3-only molecules function identically or whether subsets exist that might reflect their marked variation in binding preferences. Recently, short peptides of the α helical BH3 domains provided evidence for a two-class model in which BAD-like BH3 regions occupy antiapoptotic pockets serving as "sensitizing" domains capable of displac-

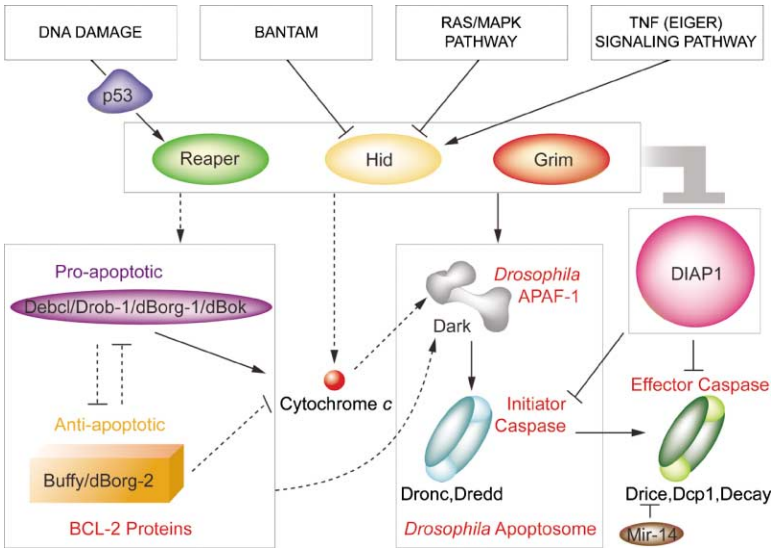

Figure 2. Apoptosis in *Drosophila*

Multiple upstream pathways regulate expression and activation of Reaper, Hid, and Grim (RHG), three proapoptotic proteins central to regulation of cell death in *Drosophila*. RHG proteins appear to control caspase activation by multiple mechanisms, including formation of an apoptosome-like complex containing Dark and Dark-independent activation of downstream caspases through antagonizing caspase inhibitors such as DIAP1. RHG proteins may further impact caspase activation by regulating conformational change or release of cytochrome *c*. Anti- and proapoptotic BCL-2 family homologs in *Drosophila* reside downstream or in parallel to RHG proteins and further influence caspase activation. The microRNAs Bantam and Mir-14 impact apoptosis in flies by suppressing Hid and Drice, respectively. Possible pathways are shown as dashed lines.

ing BID-like "activating" domains which induce the oligomerization, activation of BAX, BAK. Small molecules or peptidomimetics that mimic BH3 domains represent prototype therapeutics targeting the apoptotic pathway.

Drosophila BCL-2 Proteins

Fly homologs of BCL-2 family proteins identified to date include a proapoptotic protein Debcl/Drob-1/dBorg-1/dBok and an antiapoptotic protein Buffy/dBorg-2 (Figure 2) (Brachmann et al., 2000; Quinn et al., 2003). Both Debcl and Buffy possess BH1-3 domains and a hydrophobic membrane segment for localization to the mitochondrion. They have been shown to associate and counteract each other—typical of BCL-2 members—functioning upstream of caspase activation. Whether they act downstream of RHG proteins (see following section) or in a parallel pathway is still being resolved.

Tiered Antiapoptotics: MCL-1 as an Apical Checkpoint

Biochemical fractionation indicted MCL-1 as a cytosolic inhibitory factor whose degradation was required to initiate cytochrome *c* release following genotoxic damage to HeLa cells (Nijhawan et al., 2003). Degradation of MCL-1 was needed prior to mitochondrial translocation of BCL-X_L and BAX. Mice conditional for the expression of MCL-1 reveal it is essential early in development and again later in the maintenance of resting B and T lymphocytes, two stages heavily dependent upon cytokines. Consistent with this IL-7 both induced and required MCL-1 to mediate lymphocyte survival. At the molecular level, MCL-1 selectively counters BIM, not BAD, to protect BAX, BAK and promote survival (Opferman et al., 2003). Collectively, this in vivo and in vitro evidence supports a notion that antiapoptotics, like their proapoptotic counterparts, are also ordered in which MCL-1, a short half-life protein, serves as a critical upstream checkpoint.

Regulatory Mechanisms Converging on Caspases: Lessons Learned from Flies

The regulation of caspase activation is a major strategy by which *Drosophila* regulates apoptosis. This traces to inhibitors of apoptosis (IAPs) initially characterized as baculovirus-encoded proteins, such as p35, that suppressed apoptosis in infected host cells (Clem et al., 1991). A family of IAPs, including cellular homologs, all bear one or several signature BIR (baculovirus IAP repeat) domains thought to directly or indirectly inhibit caspases (Salvesen and Duckett, 2002).

Structural and functional studies have provided important insights into the molecular mechanism by which cellular IAPs inhibit caspase-3, -7, and -9 (Deveraux et al., 1997; Chai et al., 2001; Huang et al., 2001; Riedl et al., 2001). A flexible linker N-terminal to the BIR2 domain binds the substrate groove of caspase-3, -7 adopting a reverse orientation as compared to that of classic caspase substrates, thus blocking the substrate's access to the enzyme. Inhibition of the initiator caspase-9 by XIAP has a distinct molecular basis relying on direct interaction of XIAP's BIR3 domain with the small subunit of caspase-9 (Srinivasula et al., 2001).

In *Drosophila*, IAPs constitute a critical apoptotic control point. *rpr*, *hid*, and *grim* figure prominently in controlling death in this organism (Figure 2). These genes are encoded by a genomic region (H99) which when deleted, eliminates cell death during embryogenesis and following γ-irradiation (White et al., 1994; Chen et al., 1996a). Genetic analysis indicates that the fly apical caspase Dronc is essential for the proapoptotic activity of Reaper, Hid, and Grim (RHG) proteins. Binding of Dronc or RHG proteins to *Drosophila* IAP, DIAP1, is mutually exclusive. At their N terminus, RHG proteins contain an IAP binding motif (IBM) also known as the RHG motif including the tetrapeptide consensus A-(V/T/I)-(P/A)-(F/Y/I/V/S) implicated in binding to the BIR2 domain of DIAP1. Dronc competes with RHG proteins for binding to the DIAP1 BIR2 domain. A 12 amino acid region between the CARD and the protease domains of Dronc mediates this binding. Subsequent to these interactions, complex regulatory events can lead to ubiquitin-mediated proteolysis of Dronc or DIAP1 with differential effect on cell fate.

Degradation of DIAP1 is under complex regulation by two distinct ubiquitin pathways. One pathway operates

upstream of caspases and requires the RING domain of DIAP1 (Ryoo et al., 2002; Wing et al., 2002). This is thought to be ultimately proapoptotic by lowering the threshold of caspase activation. In this case, RHG proteins regulate DIAP1 ubiquitination by recruiting a ubiquitin-conjugating E2 enzyme, UBCD1 (Ryoo et al., 2002), or E2-like protein Morgue (Wing et al., 2002). A second pathway of ubiquitin-mediated DIAP1 degradation has been shown to reside downstream of caspases and operate independently of an intact DIAP1 RING domain. This second mechanism appears to be antiapoptotic and is felt to protect cells from basal caspase activity in the absence of any death stimuli. Caspase-mediated cleavage of DIAP1 at residue 20 uncovers an N-terminal Asn residue, which ultimately subjects the protein to degradation by the N-end rule pathway (Ditzel et al., 2003). Cleaved DIAP1 remains bound to caspases or any other associated protein so that bound proteins are codegraded. The compilation of these observations suggests the manner by which DIAP1 is degraded actually matters. It is conceivable that one ubiquitin pathway evolved to protect cells from basal caspase activation while the other influences the switch from life to death when cells receive an apoptotic signal.

Mammalian IAPs are controlled by several mechanisms, including binding of SMAC, DIABLO and OMI, HTRA2; two mitochondrial IMS proteins released during apoptosis (Du et al., 2000; Suzuki et al., 2001; Verhagen et al., 2000). Both molecules possess the tetrapeptide IAP binding motif (IBM) and antagonize IAP inhibition of caspases. Structural studies indicate that a SMAC dimer binds the BIR2 domain of XIAP and allows caspase-3 activation (Chai et al., 2000; Liu et al., 2000). In its monomeric form, SMAC displaces caspase-9 from XIAP by utilizing an IBM similar to that found in caspase-9. Interestingly, a feed forward amplification ensures that caspase-9 remains uninhibited. The IAP binding domain of caspase-9 is released upon cleavage by activated caspase-3 and subsequently binds to XIAP to keep XIAP inert (Srinivasula et al., 2001).

The extent to which IAPs and their regulatory proteins are essential regulators of apoptosis appears to vary among different organisms. While RHG proteins and IAPs in *Drosophila* are prominently featured (Goyal et al., 2000), ablation of SMAC, a functional homolog of RHG proteins in mammals, or deletion of XIAP in mice have indicated that apoptosis can proceed in their absence. Mammals may use IAPs in a more cooperative context. For example, in sympathetic neurons deprived of NGF, release of cytochrome *c* alone is not sufficient to activate the caspase cascade, but can be augmented by the degradation of IAPs (Deshmukh et al., 2002).

Cell Engulfment

The apoptotic pathway and the engulfment process are part of a continuum that helps ensure the noninflammatory nature of this death paradigm. In *C. elegans*, phagocytosis can help promote cell killing and an intact engulfment process requires *ced-3* (Hoeppner et al., 2001; Reddien et al., 2001). The cast orchestrating clearance of apoptotic cell bodies in nematodes consists of at least seven genes, which have homologs in higher organisms (Figure 3). These genes were further divided into two partially redundant classes such that the most dramatic engulfment defects were seen when one gene from each category was altered in double-mutant animals (Ellis et al., 1991). *ced-1* which encodes an engulfment receptor (Zhou et al., 2001), *ced-6* which is homologous to the mammalian PTB domain-bearing adaptor GULP (Liu and Hengartner, 1998) and *ced-7* which encodes a protein with homology to ABC-1 transporter (Wu and Horvitz, 1998a) belong in one category and help recognize apoptotic cells. *ced-2* (CrkII) (Reddien and Horvitz, 2000), *ced-5* (DOCK-180) (Wu and Horvitz, 1998b), *ced-10* (small GTPase Rac-1) (Reddien and Horvitz, 2000), and *ced-12* (ELMO) (Gumienny et al., 2001) constitute the second class of genes and influence cytoskeletal remodeling.

Phagocytes recognize the surface of the dying cell most likely through an "eat me" signal. In mammalian systems, the best characterized "eat me" signal is phosphatidylserine (PS) displayed on the plasma membrane of dying cells (Fadok et al., 2000). Evidence has been marshaled for the participation of multiple engulfment receptors including CD91, CD14, CD36, and $\alpha_v\beta_3$ integrin, as well as the phosphatidylserine receptor (PSR) (Figure 3) (Savill and Fadok, 2000).

The disposal of the apoptotic corpse is plotted once "eat me" signals on its surface are engaged by engulfment receptors. In *C. elegans*, the receptor encoded by *ced-1* clusters around the dying cell in a manner that utilizes *Ced-7* (Zhou et al., 2001). Interestingly, ABC-1, the ortholog of CED-7, is believed to regulate the distribution of PS in the membrane (Hamon et al., 2000). *ced-7* is unique among cell engulfment genes in that it functions both in phagocytes and apoptotic cells (Wu and Horvitz, 1998a). Binding of engulfment receptors to apoptotic cells ultimately signals cytoskeletal events. An interaction between the CED-1 cytoplasmic tail and CED-6 (Su et al., 2002) may serve this role consistent with genetic studies ordering *ced-1* upstream of *ced-6* (Liu and Hengartner, 1998).

Studies in mammals have highlighted the importance of proper disposal of corpses by phagocytic cells (Savill and Fadok, 2000). In addition to engulfment of apoptotic cells, macrophages are important regulators of proinflammatory responses by releasing cytokines such as TNFα. While proinflammatory factors are necessary in immune reaction against infection, their suppression during apoptotic corpse clearance is essential. This is accomplished at least in part by release of anti-inflammatory factors including TGFβ and IL-10 by macrophages engaged in corpse engulfment. Furthermore, regulatory mechanisms help ensure that when phagocytosing dendritic cells present peptides from apoptotic corpses to T cells, no immune reaction against self peptides is initiated. Defects in clearance of corpses are predicted to create a proinflammatory milieu that may predispose to autoimmune disorders.

DNA Degradation

Condensation and fragmentation of nuclei is a morphological hallmark of apoptosis. Downstream of caspase activation, degradation of DNA first occurs at A/T rich regions within the nuclear scaffold sites to produce 50–

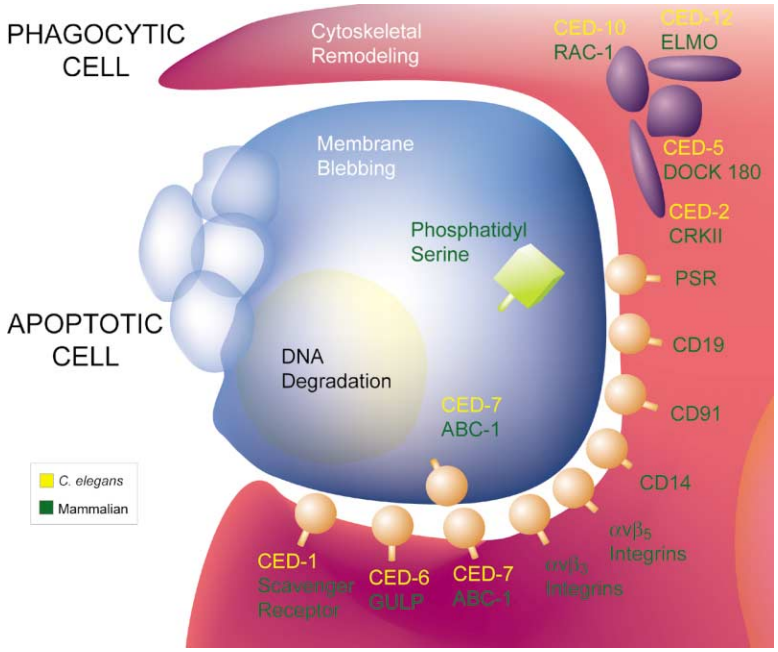

Figure 3. Engulfment of Apoptotic Cells

The engulfment machinery in mammals and *C. elegans* share evolutionarily conserved elements. Proteins encoded by two partially redundant categories of genes in *C. elegans* involved in this process are labeled in yellow and their mammalian counterparts are labeled in green.

200 kb fragments. A caspase activated DNase (CAD) was purified (Enari et al., 1998; Liu et al., 1997) and is normally kept in check by its inhibitor ICAD, DFF-45 which is eliminated when cleaved by caspase-3 and -7.

DNA degradation in apoptotic cells is under the regulation of CAD within the dying cell and DNase II within the lysosomes of phagocytes. Loss-of-function mouse models for CAD and DNase II revealed a prominent role for DNase II in degrading DNA during mammalian apoptosis. CAD null cells can undergo apoptosis and their DNA is digested efficiently after engulfment by macrophages. DNase II-deficient cells, however, accumulate undigested DNA. Mice doubly deficient for these proteins show increased undigested DNA, thought to activate innate immunity and arrest T cell development.

In mammalian cells, caspase-independent apoptotic DNA degradation has been attributed to two mitochondrial proteins endonuclease G and apoptosis-inducing factor (AIF) that translocate to the nucleus upon release (Li et al., 2001; Susin et al., 1999). AIF induces nuclear condensation and large-scale DNA fragmentation and is required for apoptosis during embryoid body cavitation (Joza et al., 2001). Genetic studies indicate that nuclear translocation of AIF is dependent upon poly (ADP-ribose) polymerase-1 (PARP-1) (Yu et al., 2002). PARP-1 attaches poly ADP-ribose to nuclear proteins such as histones and its activation leads to apoptosis under several conditions, including DNA damage. AIF function is in turn required for PARP-1 proapoptotic activity.

Precisely how AIF and endonuclease G affect DNA degradation is not fully understood. Genetic studies in *C. elegans* suggest that they may work in concert. However, unlike their mammalian counterpart, worm AIF and endonuclease G function in a caspase-dependent manner. In *C. elegans*, genes implicated in apoptotic DNA degradation include the DNase II *nuc-1* (Wu et al., 2000), *wah-1* (AIF) (Wang et al., 2002), and *cps*-6 (endonuclease G) (Parrish et al., 2001). WAH-1 and CPS-6 are both believed to be mitochondrial proteins and their release during apoptosis suggests a role for mitochondria in *C. elegans* cell death.

Signal Transduction in Apoptosis

Elucidation of the pathways activated by death receptors, including Fas (APO-1/CD95) and other TNF receptor family proteins have provided a major advance in understanding the role of apoptosis in maintaining tissue homeostasis, especially in the immune system. Monoclonal antibodies, which recognized cell surface APO-1/Fas, induced apoptotic death of the target cell (Trauth et al., 1989). The molecular cloning of this new cell surface receptor, Fas, revealed a gene that mapped to the chromosomal location of a lymphoproliferative disorder known as *lpr* (Itoh et al., 1991). *Lpr* represented a mutation in the Fas death receptor indicating its loss-of-function violated cellular homeostasis (Watanabe-Fukunaga et al., 1992). Expression cloning of the Fas ligand revealed a novel TNF family member (Suda et al., 1993). The generalized lymphoproliferative, autoimmune disorder *gld* proved to be a mutant Fas ligand (FasL) (Takahashi et al., 1994). The cloning of TNF and TNF receptors (Tartaglia et al., 1993) together with Fas and FasL propelled an explosion of studies detailing the signal transduction pathways downstream of death receptors.

Death receptors rely on signaling proteins possessing a distinct set of modular protein motifs capable of homotypic interaction, including death domains (DD) and death effector domains (DED) (Itoh and Nagata, 1993; Tartaglia et al., 1993; Hsu et al., 1995). Remarkably, structural studies of DD and DED domains have revealed that their overall fold is very similar to that of the CARD domain (Fesik, 2000) indicative of an evolutionarily conserved structure in the assemblage of proapoptotic cascades. Nature exploited the necessity for such domains by evolving antagonistic decoy receptors which seques-

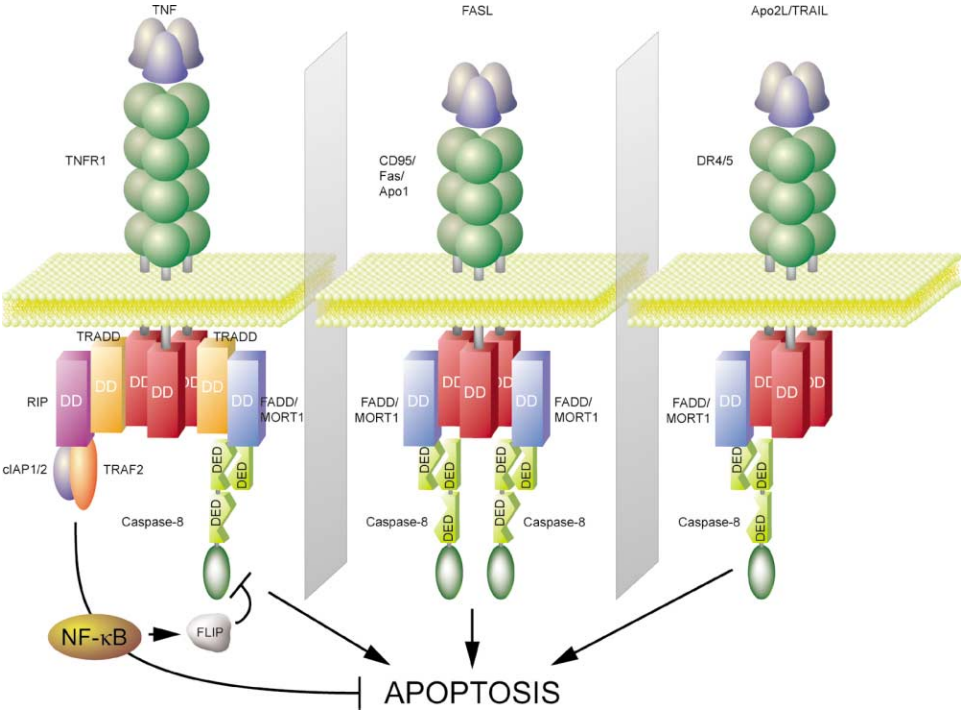

Figure 4. Extrinsic Death Receptor Pathways

The distinct composition of the *D*eath-*I*nducing-Signaling *C*omplex (DISC) downstream of the various death receptors TNFR1, CD95, and DR4/5 is illustrated.

ter death ligands but cannot propagate downstream signals due to nonfunctional or absent death domains.

Fas, which is preassembled as a trimer, undergoes a conformational change following ligand binding and assembles on its cytoplasmic tail a signaling complex known as the DISC (*D*eath-*I*nducing *S*ignaling *C*omplex) (Muzio et al., 1996) (Figure 4). The adaptor FADD/MORT, bearing both DD and DED motifs, binds the DD of Fas and recruits procaspase-8 via its DED domain (Kischkel et al., 1995). Activation of caspase-8 in the DISC complex is believed to follow an "induced proximity" model where high local concentration of procaspase-8 leads to its autoproteolytic activation and subsequent activation of caspase-3 and -7. Fas-induced apoptosis can follow two pathways (Scaffidi et al., 1998). In type I cells, such as thymocytes in-vitro, Fas-induced apoptosis is refractory to BCL-2 since sufficient caspase-8 cleavage and activation of caspase-3, -7 occurs. In type II cells, such as hepatocytes, BCL-2 blocks Fas-mediated death as a mitochondrial amplification loop is required in which caspase-8-mediated cleavage of BID results in its translocation to mitochondria and cytochrome *c* release in order to generate sufficient effector caspase activity to kill such cells (Li et al., 1998; Luo et al., 1998). This is consistent with the susceptibility of thymocytes but resistance of hepatocytes to Fas-mediated death in *Bid*-deficient and *Bax, Bak* doubly deficient mice (Lindsten et al., 2000; Wei et al., 2001; Yin et al., 1999).

Another set of death receptors (DR 4/5) have been characterized that share a different death ligand, known as Apo2L,TRAIL (*T*NF-*R*elated *A*poptosis *I*nducing *L*igand) (Ashkenazi and Dixit, 1998). Cancer cells may display relative sensitivity to TRAIL-mediated apoptosis and recent reports have highlighted the importance of a mitochondrial/postmitochondrial program. DISC complex formation and BID cleavage downstream of DR4/5 is similar to the Fas pathway (Figure 4).

In addition to cell death, signaling by death receptors has been reported to activate proliferation in select settings. The decision between apoptosis versus survival appears to be governed in part by differential complex formation between various DD or DED proteins. In the case of TNFR1 (Figure 4), a recent report indicates various DD-containing proteins can form distinct complexes in a temporal manner once the receptor is activated (Micheau and Tschopp, 2003). A TNFR1 complex possessing the DD-containing protein TRADD, TRAF2, cIAP1, and the kinase RIP1 (known as complex I) assembles at the plasma membrane within minutes after activation in order to recruit IKK leading to NF-kB activation and survival. In a second step, complex II forms after the TRADD-based complex dissociates from the receptor and recruits FADD and the initiator caspase-8. In this model, the balance of effects by complex I versus II rests with cFLIP, an inhibitor of caspase-8. When complex I NF-κB activation is sufficient, adequate cFLIP is expressed to inhibit caspase-8 of complex II. By this model, complex II can mediate apoptosis only when complex I-mediated NF-κB activation is insufficient.

Depending on their biochemical milieu, DED-containing proteins coordinately regulate lymphocyte homeostasis. Humans with caspase-10 deficiency as well as those with heterozygous Fas mutations develop *a*utoimmune *l*ympho*p*roliferative *s*yndrome (ALPS) marked

by defective lymphocyte apoptosis and autoimmunity, whereas caspase-8 mutations lead to a distinct disorder involving defects in T, B, NK cell activation, and immunodeficiency (Chun et al., 2002; Siegel et al., 2000; Wang et al., 1999). Although, caspase-8 and -10 are both involved in the DISC complex, the human diseases imply an additional function for caspase-8 in regulation of lymphocyte proliferation. One model for further testing proposes that the location and extent of protein-protein interactions mediated by DED-containing proteins determines a "cell renewal set point" to integrate the pathways of apoptosis and proliferation.

Protein Quality as a Checkpoint for Cell Death: Roles in Degenerative Disorders

Proper folding of proteins is aided by multiple chaperones functioning in the endoplasmic reticulum (ER). Misfolded or unfolded proteins and stimuli that disrupt ER function initiate the *u*nfolded *p*rotein *r*esponse (UPR), a stress response pathway marked by a transcriptional program ensuring upregulation of key proteins necessary to restore proper "protein homeostasis" (Kaufman, 1999). In the extreme, if proper protein folding is not achieved, unfolded proteins can trigger apoptosis. ER stress has been implicated in the etiology of multiple neurodegenerative disorders, including Huntington's Disease and Alzheimer's Disease (Mattson, 2000).

Huntington's Disease appears to result from an aberrant expansion of CAG repeats encoding a polyglutamine repeat in the Huntingtin protein. The mutant protein pathogenesis involves caspase-8 activation (Sanchez et al., 1999), which is thought to occur because of an altered complex formed by mutant Huntingtin protein (Gervais et al., 2002). Under normal circumstances, Huntingtin forms a complex with *H*untingtin *i*nteracting *p*rotein 1 (Hip1), clathrin and AP2, possibly to regulate vesicular transport during neurotransmitter release. Mutant Huntingtin is in a different complex containing Hip1, caspase-8, and the DED-containing protein Hippi leading to caspase-8 and subsequent caspase-3 activation. One model holds that active caspase-3 cleaves Huntingtin into self-aggregating fragments that prove toxic to the affected neurons. Many questions remain, including whether this complex can account for all parameters of damage including the mitochondrial dysfunction in Huntington's Disease.

Induction of apoptosis by malfolded proteins in Alzheimer's disease and disorders associated with prion proteins involves abnormal ER Ca^{2+} signaling. In Alzheimer's Disease, neurotoxicity has been attributed to amyloid-β peptides (Yankner et al., 1990). Under normal conditions, *a*myloid *p*recursor *p*rotein (APP) is cleaved by α secretase, released in the extracellular space leading ultimately to activation of cyclic GMP-activated protein kinase, NF-κB activation, and the survival of cells. Neurotoxicity ensues when instead of the α secretase-cleaved APP, amyloid-β peptides accumulate. This has been traced to alterations of one of 3 genes: APP, persenilins 1, or 2. Amyloid-β peptides cause lipid peroxidation, increased Ca^{2+} release by ryanodine or IP3 receptors, and are associated with activation of caspase-12 and mitochondrial dysfunction (Mattson, 2000; Nakagawa et al., 2000).

In addition to the UPR, the cell's response to misfolded proteins can utilize *ER-A*ssociated *D*egradation (ERAD) through the ubiquitin pathway. Recent insight implicated defects in the ubiquitin pathway in some forms of Parkinson's Disease. Mutations in the E3 ubiquitin ligase Parkin causes the death of dopaminergic neurons in autosomal recessive juvenile parkinsonism (AR-JP) (Kitada et al., 1998). Parkin binds to E2 enzymes at the ER. The G protein-coupled receptor Pael was identified as a Parkin substrate. Consistent with this finding, aggregates of Pael are detected in brains from AR-JP patients.

Future Directions

Other Molecules that Traffic to Mitochondria During Apoptosis

Multiple studies have reported translocation of selected proteins including the nuclear orphan receptor TR3, p53, JNK/SPAK, PKCδ, and histone H1.2 to mitochondria during apoptosis (Brenner and Kroemer, 2000). How these molecules influence mitochondrial dysfunction during apoptosis is not known, and whether they interact with the BCL-2 pathway or reflect unique effector pathways is under evaluation.

Activation of the mitochondrial pathway of apoptosis is one attractive explanation for the transcription-independent portion of p53-influenced apoptosis (Chen et al., 1996b; Haupt et al., 1995). Mitochondrial translocation of p53 following DNA damage (Mihara et al., 2003) and its ability to engage BCL-2 family proteins to regulate cytochrome *c* release have been noted (Chipuk et al., 2004).

An unexpected molecule, histone H1.2, has recently been implicated in a mitochondrial pathway of apoptosis (Konishi et al., 2003). Histone H1.2 is released into the cytosol upon DNA double-strand breaks in a p53-dependent manner. Histone H1.2 traffics to the mitochondria and while it possesses no obvious BH3 domain, BAK oligomerization, and cytochrome *c* release follow. These observations suggest an enticing model in which the linker histone H1.2 could serve as a signaling intermediate, sensing changes in chromatin status, and communicating this to the apoptotic machinery at the mitochondrion.

Intrinsic Apoptotic Pathway Operates at Organelles

It is striking how many of the critical control steps in the intrinsic apoptotic pathway are localized to the surface of intracellular organelles. The mitochondria and the endoplasmic reticulum (ER) are best documented, but localization of BCL-2 members to the nuclear membrane and translocation of CED-4 to the outer nuclear membrane raises questions about that locale as well. These observations have prompted inquiries into whether there may be a more overarching rationale for the localization of apoptosis at organelles.

An ER Gateway to Apoptosis

Accumulating evidence suggests that in addition to mitochondria, the ER serves as an important apoptotic control point. Antiapoptotic BCL-2 and proapoptotic BAX, BAK also localize to the ER. Overexpression of BCL-2 was noted to prevent cell death by the passive release of ER Ca^{2+} when thapsigargin was used to block

the *s*arcoplasmic/*e*ndoplasmic *r*eticulum Ca^{2+} *A*TPase (SERCA) reuptake pump (Lam et al., 1994). Either overexpression of BCL-2 or loss of BAX, BAK leads to reduced resting ER Ca^{2+} concentrations and a secondary decrease in Ca^{2+} uptake by mitochondria (Pinton et al., 2000; Scorrano et al., 2003). Selective reconstitution of individual organelles enabled the classification of death signals based on their dependence on an ER Ca^{2+} gateway and/or a mitochondrial gateway to apoptosis (Scorrano et al., 2003). Signals reliant upon the ER gateway include the Ca^{2+}-dependent lipid second messengers such as C_2-ceramide and arachidonic acid as well as pathologic oxidative stress. In contrast, activated BH3-only proteins kill cells as long as BAX or BAK is present at the mitochondria irrespective of Ca^{2+} stores. Finally, a number of classic death signals utilize both gateways.

Why the Mitochondrion: Integration of Cellular Metabolism and Apoptosis

Cellular energy metabolism and the core apoptotic pathway are the two major determinants of cellular survival. Growth/survival factors such as IGF-1 or IL-3 stimulate glucose transport and the translocation of hexokinase to mitochondria, stimulating glycolysis as well as inhibiting apoptosis (Gottlob et al., 2001; Vander Heiden et al., 2001). Withdrawal of growth/survival factors leads to metabolic decline including a decreased glycolytic rate, lowered O_2 consumption, decreased ATP levels, and reduced protein production as well as triggering the apoptotic pathway. One line of investigation suggested that these processes were distinct in that activated myr-AKT prevented the metabolic decline and promoted cell survival, but required glucose to do so. In contrast, anti-apoptotic BCL-2 or BCL-X_L blocked apoptotic death of factor-deprived cells, but in a glucose-independent fashion that did not prevent the metabolic decline.

Recent studies suggest a more intimate integration of glucose metabolism and apoptosis. At the surface of liver mitochondria, proapoptotic BAD nucleates a macromolecular complex containing glucokinase (hexokinase IV), a resident kinase and phosphatase pair (PKA and PP1α), an A-kinase anchoring protein (WAVE-1) and BAD itself (Danial et al., 2003). *Bad*-null mice revealed BAD is important in regulation of mitochondrial-based glucokinase (GK) activity and glucose-driven mitochondrial respiration. A deficit in GK activity at this organelle in both the *Bad*-null and nonphosphorylatable Bad^{3SA} knockin mouse models manifests in a systemic diabetic phenotype. Whether regulation of glucose metabolism by BAD extends beyond the high *K*m glucose-sensing GK found in liver and pancreatic β islets to the low *K*m hexokinase isoforms elsewhere awaits study. The unexpected role for BAD in regulation of glucose homeostasis suggests a model in which BH3-only proteins serve as specific "sentinels" for death signals by being embedded as integral participants in the pathways they monitor.

Another interrelationship of apoptosis and mitochondrial function has been suggested by an interface between VDAC (voltage dependent anion channel) and BCL-2 family proteins. One school of thought proposes BCL-X_L stimulates VDAC closure while proapoptotic BAX and BAK would promote its opening and perhaps form a BAX/VDAC hybrid channel to release cytochrome *c*, implicating VDAC as a positive regulator of apoptosis (Shimizu et al., 1999). Other evidence supports BCL-X_L opposing VDAC closure, perhaps by preventing mitochondrial alterations in response to death stimuli (Vander Heiden et al., 1997). A recent finding implicated VDAC-2 as a negative regulator of a BAK-driven apoptotic program (Cheng et al., 2003). Genetic loss of function studies, together with reconstitution assays, indicate that VDAC-2, amongst the three mammalian VDAC isoforms, is a specific inhibitor of the potentially lethal BAK molecule in viable cells. This physical link between the core apoptotic pathway and mitochondrial physiology raises a question for future pursuit as to whether BAK reciprocally influences VDAC-2-mediated metabolic function.

An Intraorganelle Program: Membrane Remodeling and Morphology

Studies of the kinetics of cytochrome *c* release in response to apoptotic stimuli indicated that the release can be rapid and the extent remarkably complete (Goldstein et al., 2000). However, high-voltage electron microscopic tomography of mitochondria has revealed a very narrow *inter*membrane *s*pace (IMS) that possesses only 15%–20% of total cytochrome *c*. The majority of cytochrome *c* ($\sim$85%) resides in pleomorphic involutions of the *i*nner *m*itochondrial *m*embrane (IMM) termed tubular cristae, a highly sequestered compartment separated from the IMS by narrow cristae junctions. *P*ermeability *t*ransition (PT) that ultimately leads to swelling of the mitochondria, altered cristae, and secondary rupture of the MOM has been noted in certain apoptotic and necrotic cell deaths (Lemasters et al., 1998). In this fully open conformation the *PT p*ore (PTP), a high conductance IMM channel whose precise components are still being explored, is permeable to solutes up to 1500 Da (Bernardi et al., 1999). However, in most apoptotic deaths substantial cytochrome *c* is released prior to any swelling of the mitochondria. Several studies have addressed changes in the mitochondrial ultrastructure during apoptosis (Scorrano et al., 2002; von Ahsen et al., 2000). In one model the BH3-only molecule tBID induced striking remodeling of the IMM, where individual cristae were fused and the junctions between cristae and the IMS opened, mobilizing the stores of cytochrome *c*. Notably, this reorganization occurred without swelling of the organelle, but correlated with transient opening of the PTP (Scorrano et al., 2002). Apoptotic stimuli coordinately result in the oxidation of cardiolipin and release of cytochrome *c* from tethered sites (Ott et al., 2002). It will be of interest to see if this remodeling program relates to BID's capacity to bind selected lipids (Esposti et al., 2001).

Accumulating evidence suggests that the dynamin family of GTPases impact mitochondrial morphology and membrane remodeling, including events during apoptosis. Dynamins are mechanoenzymes known to regulate membrane pinching during vesicular/membrane transport. Remarkably, RNAi knockdown of OPA-1, a mitochondria-resident dynamin, results in a morphology resembling tBid-driven IMM remodeling and cytochrome *c* release (Olichon et al., 2003). Another dynamin family protein Drp-1, known to regulate mitochondrial fission, appears to participate in the fragmentation of this organelle noted in certain cell deaths (Frank et al., 2001). These observations suggest that mechanisms dominating normal mitochondrial morphology may be coopted in apo-

ptosis. Interestingly, an axis between the ER and mitochondrial morphology during apoptosis is suggested by the Ca^{2+}-dependent recruitment of Drp-1 to mitochondria, proposed to be regulated by caspase cleavage of the ER protein BAP31 (Breckenridge et al., 2003).

Regulation of Apoptosis by MicroRNAs

Another class of cell death regulators consists of microRNAs, which are capable of dampening translation of apoptotic components in a rapid and reversible fashion. Two such microRNAs have been identified in the *Drosophila* cell death pathway (Figure 2). Bantam binds the 3′UTR region of *hid* and suppresses its translation independently of the Ras/MAPK pathway (Brennecke et al., 2003). Mir-14 is a microRNA regulating the *Drosophila* caspase Drice (Xu et al., 2003). It will be of interest to determine whether microRNAs regulate critical control points in mammalian apoptosis.

Alternative Death Pathways

Granzymes and Calpain

T cells and natural killer (NK) cells utilize a granule-exocytosis pathway for the elimination of virus-infected cells. Cytotoxic granules deliver a pore-forming protein, perforin, and a family of serine proteases known as granzymes into a tightly sealed intercellular synapse, presumably to ensure their selective uptake into target cells. Gene knockouts coupled with biochemistry are revealing distinct apoptotic pathways downstream of each granzyme. Granzyme B can cleave caspase-3, but also cleaves other substrates including BID and ICAD which results in activation of the CAD DNase as well as alternative pathways of apoptosis (Heusel et al., 1994; Alimonti et al., 2001). Granzyme A targets the SET complex resulting in the degradation of selected components, freeing the NM23-H1 DNase and resulting in single-stranded DNA nicks (Lieberman, 2003). Granzyme C induces yet another caspase-independent death distinct from either granzyme A or B. Notably, the Ca^{2+}-dependent cysteine protease, calpain also shares some common substrates with the caspases including cleavage of caspases themselves (Gil-Parrado et al., 2002). Perhaps further elucidation of these protease-induced deaths will uncover caspase-independent programs, that while triggered by granzymes, are also intended to operate within cells following intrinsic death signals.

Apoptosome-Independent Death

In the extrinsic pathway, type I cells can run a direct, initiator (caspase-8) to effector (caspase-3) cascade. The inability of caspase inhibitors to completely protect cells and their organelles from damage following intrinsic death signals suggested that caspase-independent death might also occur. It is also plausible for the intrinsic pathway that mechanisms to activate caspases beyond the apoptosome might exist. Deletion of the downstream effectors Apaf-1 or caspase-9 initially protects from apoptotic stimuli, yet cells can go on to die without measurable caspase activity, but with substantial organelle dysfunction. In contrast, cells doubly deficient for BAX, BAK demonstrated long-term resistance indicating the most profound commitment point to apoptosis is upstream at the BAX, BAK gateway proximal to organelle damage (Cheng et al., 2001). Consistent with this, neither Apaf-1 nor caspase-9 deficiency afforded protection to B and T cells equal to that provided by a BCL-2 transgene (Marsden et al., 2002).

Activation of alternative caspases including caspase-2 has been noted in certain apoptotic paradigms, which don't appear to require Apaf-1 (Lassus et al., 2002). Caspase-2 activity may represent an upstream initiating event or may be part of a downstream amplification loop. The final mechanism of demise in deaths that do not utilize the apoptosome and perhaps not even caspases needs further resolution. Possibilities include metabolic deaths resulting from irreversible damage to organelles including mitochondria and ER, other apoptogenic factors released from mitochondria (OMI, ENDO G, or AIF) that run caspase-independent deaths, or a novel path to activate alternative caspases. One candidate OMI relies both on its IAP binding and serine/threonine protease activity. Neither the release nor the proapoptotic activity of OMI is caspase dependent. The release of OMI may cause caspase-independent mitochondrial dysfunction as well as inhibition of IAPs. Notably, a recent report indicated that the serine protease activity of OMI plays a role in maintenance of mitochondrial homeostasis under nonapoptotic conditions, as its loss is associated with mitochondrial dysfunction and neurodegeneration (Jones et al., 2003).

Additional Death Pathways in C. elegans

Several observations suggest that alternative death pathways may exist in *C. elegans*. One candidate is a newly discovered participant, *icd-1* (*i*nhibitor of *c*ell *d*eath) that modulates apoptosis in a *ced-3*-independent but *ced-4*-dependent manner (Bloss et al., 2003).Perhaps another caspase is involved or perhaps *icd-1* function is caspase-independent. *icd-1* encodes the β subunit of the βNAC (*n*ascent polypeptide *a*ssociated *c*omplex) suggesting that functions attributed to βNAC, including protein translation, folding, and translocation to mitochondria may relate to the death inhibitory capacity of *icd-1*.

Necrotic Death

Necrotic cell death occurs following a wide variety of cellular injuries. In distinction from apoptosis, necrosis is characterized by distortion and degradation of organelles and cellular swelling (Kerr et al., 1972). While much remains to be learned about the genetic and biochemical pathways of necrosis downstream of the individual insults, studies examining excitotoxic neuronal deaths, including those following ischemia, have proven instructive. Excess glutamate hyperactivates NMDA channels with consequent increase in intracellular Ca^{2+} and Ca^{2+}-dependent pathways (Nicotera et al., 1997). A necrotic death model in *C. elegans* which results from a mutant degenerin Na^{2+} channel MEC-4 is reminiscent of mammalian excitotoxic death. Notably, a genetic screen for suppressors of these deaths identified four genes known to regulate ER Ca^{2+}: calreticulin, an intralumenal ER Ca^{2+} binding protein; calnexin, an ER Ca^{2+} binding chaperone; the IP3R Ca^{2+} release channel; and the ryanodine receptor, another ER Ca^{2+} release channel (Xu et al., 2001). This argues that the serial steps in necrotic cell death are also definable and will ultimately yield targets for therapeutic intervention.

Autophagy

Autophagy is a documented pathway of disposal for intracellular organelles that has also been implicated in cell death. In particular, macrophagy is a dynamic

process of membrane engulfment in which portions of the cytoplasm are sequestered within double membrane vesicles known as autophagosomes. Morphologically the process is similar from yeast to mammals. The biogenesis and consumption of such vesicles has been divided into four distinct steps: induction and cargo packaging; formation and completion; docking and fusion; and breakdown. Genetic screens in yeast identified overlapping set of genes designated *apg* (autophagy) and *aut* (autophagocytosis) (Klionsky and Emr, 2000). Of these, *apg-6* functions in multiple cellular processes, is required to induce autophagy upon starvation and is homologous to Beclin, a tumor suppressor in mammals, and to a gene required in *C. elegans* to complete dauer morphogenesis (Liang et al., 1999). Certain cell deaths, for example the elimination of intersegmental muscles during morphogenesis, is more reminiscent of autophagy than apoptosis. The induction of autophagy is prominently controlled by the nutrient sensing mTOR kinase. Obvious overlaps in signal transduction pathways whereby the PI3-kinase and mTOR paths can influence shared intermediates, such as p70S6K, suggest autophagy and apoptosis may prove to be coordinated.

Conclusions

Our talented colleagues in this field have marshaled an extraordinary effort that uncovered the many truths governing cell death. In return, the identification of critical control points in the apoptotic pathway has provided rational targets for the development of a new generation of therapeutics. Inhibitors that block caspase activity, molecules that intervene at the BCL-2 control point, and mimetics of the small RHG motif that binds IAPs are but three examples of compounds under active development or clinical evaluation. Yet, much remains to be done to further extend and integrate death pathways. We anticipate that apoptosis will become more fully interwoven with the fabric of other physiological pathways it is charged with monitoring. However, history argues that the unexpected will make the greatest imprint on our understanding of cell death, and that we can fully expect it from this exceptional field of investigators.

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