Death Receptors in Cancer Therapy

CANCER DRUG DISCOVERY AND DEVELOPMENT

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Humana Press
Totowa, New Jersey

999 Riverview Drive, Suite 208
Totowa, New Jersey 07512

www.humanapress.com

Production Editor: Tracy Catanese
Cover design by Patricia F. Cleary

This publication is printed on acid-free paper. ∞
ANSI Z39.48-1984 (American National Standards Institute)
Permanence of Paper for Printed Library Materials

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Printed in the United States of America. 10 9 8 7 6 5 4 3 2 1

e-ISBN: 1-59259-851-X

Library of Congress Cataloging-in-Publication Data

Death receptors in cancer therapy / edited by Wafik S. El-Deiry.
p. ; cm. -- (Cancer drug discovery and development)
Includes bibliographical references and index.
ISBN 1-58829-172-3 (alk. paper)
1. Apoptosis. 2. Cell receptors. 3. Cancer--Molecular aspects. 4. Cellular signal transduction.
[DNLM: 1. Neoplasms--immunology. 2. Neoplasms--therapy. 3. Cell Death--immunology. 4. Gene Therapy--methods. 5. Receptors, Tumor Necrosis Factor. QZ 266 D285 2005] I. El-Deiry, Wafik S. II. Series.
QH671.D43 2005
571.9'36--dc22

2004006682

PREFACE

The study of cell death, or apoptosis, has turned into a very large field. Both the extrinsic and intrinsic cell-death pathways appear to have fundamental importance to tumor progression and cancer therapy. It has become clear that the extrinsic pathway provides a number of mechanisms for host immune surveillance of tumors and their suppression. Because this is a fast-moving area that is generating a huge literature, there is an ongoing need in the scientific community to distill the knowledge and to organize it so that students as well as experienced investigators can both learn it and build upon it.

The chapters comprising *Death Receptors in Cancer Therapy* have been written by experts in the field of cell-death research, particularly those interested in death receptors and their relevance to cancer and cancer therapy. The basic information about signaling, as well as conservation of the pathways in *Drosophila* or *Caenorhabditis elegans*, can be found herein. There is information on the role of death domains and receptors in development, and there is secondary and tertiary structural information about receptors and ligands. One of the important aspects of the text that will be of use for experts is the cross-talk in signal transduction pathways. It is clear that pathways are networked and cross-regulated through other signaling pathways that may be on or off depending on physiological or cellular state. Finally, with a firm foundation in the understanding of the molecular events in cell death, the major emphasis of *Death Receptors in Cancer Therapy* is on both alterations in cancer as well as therapeutic strategies and combination therapies.

It is important to note that, although there is a great deal of preclinical translational research on death receptors and ligands, the history of drug development is complex and subject to many forces and hurdles. As such, it is important to mention that the opinions or conclusions of the contributors to this text are theirs, and not necessarily endorsed by the editor or the publisher. However, it is very important in a fast-moving field with exciting possibilities for new cancer therapies to provide readers with the views of leaders in the field from their own perspectives.

One of the chapters in this book was a contribution from Dr. Vincent Kidd and colleagues at St. Jude Children's Research Hospital. In reviewing the proofs, I became aware that Dr. Kidd passed away suddenly on May 7, 2004. His colleagues have dedicated the chapter on caspase methylation, to which he made a major contribution, to his memory. We will all miss him.

I wish to take this opportunity to personally thank each and every contributor to this volume. I believe a useful resource has been created that will serve as a reference in the field and will also provide an excellent introduction of the cell death field to the beginner. There are many acronyms in this field, and this text describes the many molecules involved in death signaling and allows the reader to get a handle on their many names. The extrinsic death pathway and death receptors are of great interest to cancer biologists, immunologists, developmental biologists, medical oncologists, hematologists, radiation therapists, and rheumatologists as well as to those in the biotech and pharmaceutical industries.

W. El-Deiry

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1 Mammalian Cell Death Pathways
Intrinsic and Extrinsic

E. Robert McDonald III, PhD
and Wafik S. El-Deiry, MD, PhD

SUMMARY

Programmed cell death results from a conserved cascade of events essential in the development and maintenance of tissue homeostasis. "Extrinsic" cell-death pathways initiate at the cell surface, leading to execution through substrate cleavage, and may involve mitochondrial amplification. Multiple "intrinsic" death pathways converge and require signaling through the mitochondria. Extrinsic cell death is integral to cell-mediated immunity and host immune surveillance/suppression of cancer. Caspase activation is highly regulated and defects at virtually all levels of death regulation are observed in cancer. This chapter focuses on the cell biology, biochemistry, and genetics of programmed cell death.

ORIGINS OF APOPTOSIS IN *CAENORHABDITIS ELEGANS*

Even after numerous reports in the early to mid-1900s of "programmed cell death" with characteristic morphological changes such as cell shrinkage and nuclear condensation and fragmentation, the importance of this process in normal cellular physiology went largely unexplored *(1)*. However, with the description of the genetically controlled deletion of a subset of cells within the nematode *C. elegans* and the subsequent cloning of the genes responsible for this process, the field of programmed cell death or apoptosis gained popularity *(2)*. The realization that apoptosis is an evolutionarily conserved, genetic event has sparked interest in understanding the regulation of the process in various model systems. Furthermore the deregulation of apoptosis in human disorders such as neurodegenerative disease and cancer has lead to the manipulation of these pathways in order to combat these diseases *(3)*. It was, however, seminal work in *C. elegans* that laid the foundation for the central themes of apoptosis found throughout the animal kingdom.

While Richard Lockshin coined the term programmed cell death in 1964 *(4)*, John Kerr is credited with early microscopic observations of cell death distinct from necrosis called "apoptosis" which he, Wyllie, and Currie perceived to be controlled by a series of con-

From: *Cancer Drug Discovery and Development: Death Receptors in Cancer Therapy*
Edited by: W. S. El-Deiry

served events *(1)*. However, Robert Horvitz was responsible for providing the first molecular clues of how apoptosis is controlled *(5)*. The identification of *C. elegans* cell death abnormal or *ced* mutants with defective development *(5,6)* established three families of highly conserved proteins, which oversee apoptosis in most organisms (Fig. 1): caspases (ced-3), caspase-activating adaptor proteins (ced-4) and the bcl-2 family of proteins (ced-9). Caspases are the enzymes responsible for dismantling the cell and for eliciting the cellular phenotypes first described by Kerr, therefore their regulation is paramount when considering apoptotic pathways. In the worm, these three gene products act in a linear fashion to either suppress or activate ced-3, with ced-9 acting as the only antiapoptotic protein in the pathway. ced-9 inhibits ced-4 function, which is required for ced-3 caspase activation *(7)*. Whereas the loss of ced-3 or ced-4 did not compromise the longevity of the organism, suppression of apoptosis by ced-9 was crucial for its long-term survival *(6)*. Subsequently, the lone BH3-only protein, egl-1, was placed genetically upstream of ced-9 due to the ability of egl-1 to bind and negatively regulate ced-9 *(8)*. These four genes constitute the core apoptotic machinery in *C. elegans* required for the execution phase of cell death.

INCREASED APOPTOTIC COMPLEXITY OF HIGHER EUKARYOTES

Cloning of the core apoptotic genes in *C. elegans* led to the discovery that higher eukaryotes adhered to this basic blueprint but had predictably evolved to include novel gene families to regulate further complexity (Fig. 1). Mammalian systems, being the most complex, contain 14 caspases (ced-3), 2 proapoptotic adaptor proteins (ced-4), at least 10 bcl-2 family proteins (ced-9), and a similar number of BH3-only proteins (egl-1) to date *(9,10)*. BH3-only proteins antagonize the antiapoptotic members of the bcl-2 family in order to facilitate downstream adaptor-mediated caspase activation *(11)*. However, unlike *C. elegans*, antiapoptotic bcl-2 proteins do not directly interact with adaptors but rather regulate adaptor assembly by influencing mitochondrial homeostasis *(12)*. This pathway involving mitochondria and subsequent caspase activation is referred to as the intrinsic pathway and is the functional equivalent of the *C. elegans* cell death pathway (Fig. 1).

Following mitochondrial dysfunction, formation of a caspase activation complex known as the apoptosome initiates the death program. The apoptosome is comprised of the ced-4 homolog, Apaf-1, along with procaspase-9, ATP, and cytochrome c, which has been extruded from the mitochondria. Activation of caspase-9 within the apoptosome in turn leads to the activation of caspase-3, the true mammalian ced-3 homolog, committing the cell to death *(13)*. The basic principle from *C. elegans* of bcl-2 mediated inhibition of adaptor-driven caspase activation is therefore represented at the mammalian level by the intrinsic pathway. In addition to the intrinsic pathway, however, mammals have evolved an alternative pathway—the extrinsic pathway—which is initiated at the cell surface by death receptor/death ligand interactions *(14)*. Activation of this pathway also results in adaptor-driven caspase activation. The adaptor, FADD, and caspase-8 and -10, through a series of protein interactions with the death ligand-associated receptors, form a death-inducing signaling complex (DISC) which is sufficient for caspase activation *(15)*. The last major difference between *C. elegans* and higher eukaryotes is the creation of another protein family, the inhibitor of apoptosis proteins, or IAPs *(16)*. These proteins have evolved to bind to and negatively regulate caspases and will be discussed in more detail in a subsequent section (Fig. 1).

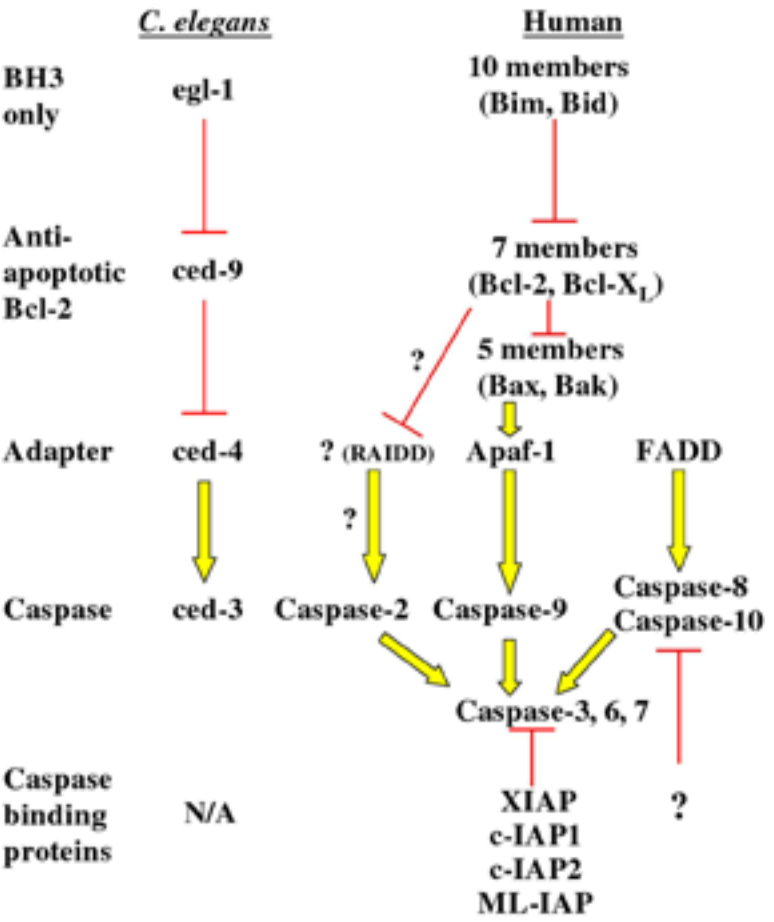

Fig. 1. Increased apoptotic complexity of Higher Eukaryotes as compared to the *Caenorhabitis elegans* system. Although the linear system of adaptor-mediated caspase activation is conserved in higher eukaryotes, a number of important differences exist. The expansion of each gene family is dramatic in addition to a new family of proapoptotic Bcl-2 proteins that regulate mitochondrial homestasis in mammalian systems. The death receptor pathway and caspase binding proteins are completely absent in the worm. Unanswered points within a putative mitochondrial-independent, caspase-2-dependent pathway are denoted by a question mark.

THE GENETICS OF THE *DROSOPHILA* APOPTOTIC RESPONSE

Another genetically tractable organism used for the study of cell death regulation is *Drosophila melanogaster*. As would be expected, the complexity of the apoptotic program of this organism is intermediate between that of *C. elegans* and humans. The intrinsic pathway mediated by mitochondrial homeostasis and apoptosome formation in humans has conserved elements in *Drosophila* (Fig. 2). Dark, the *Drosophila* Apaf-1 homolog, and Dronc, the *Drosophila* caspase-9 homolog, are able to interact consistent with a model using adaptor-driven caspase activation *(17)*, and furthermore, cytochrome c interacts with Dark in *Drosophila* tissue culture cells *(18)*. Despite these similarities with higher organisms, initial studies suggest that cytochrome c is not released from

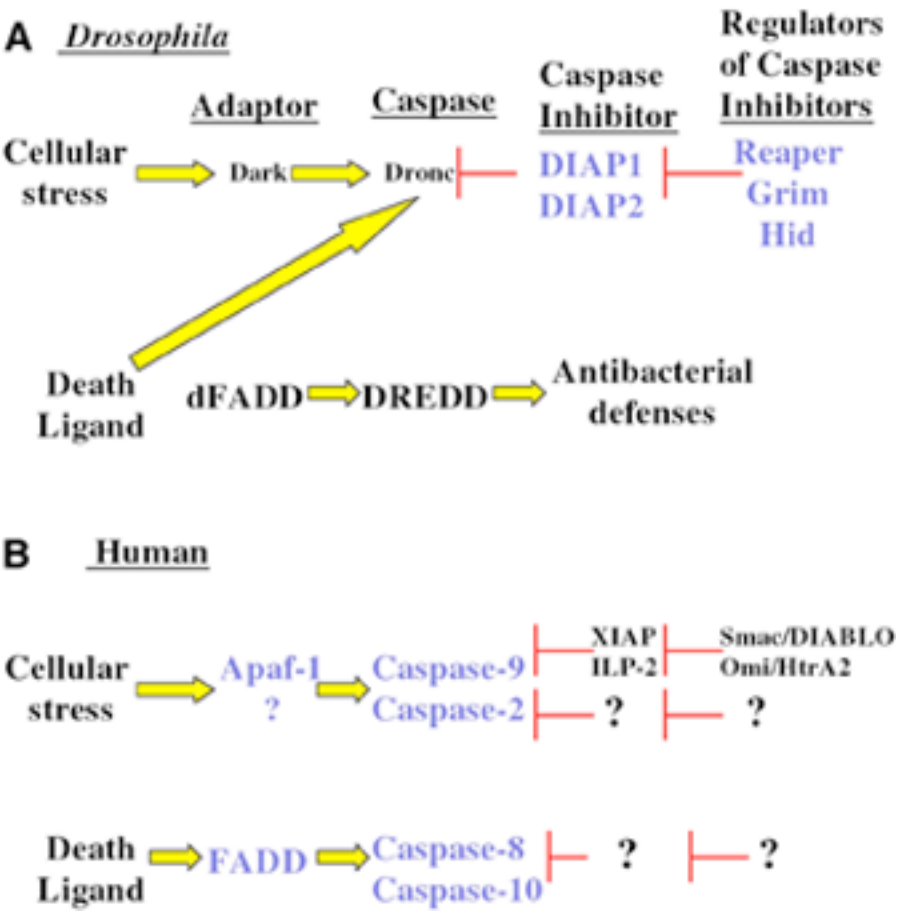

Fig. 2. Differences between the *Drosophila* and mammalian cell death pathways. **(A)** The main regulators of apoptosis in *Drosophila* are Reaper, Grim, and Hid that negatively regulate IAP function. The IAPs in turn control caspase activity that is a triggered in cytochrome c-independent manner. The only "putative" death ligand described in *Drosophila* activates a Dark, Dronc pathway while FADD and caspase-8 homologs regulate antibacterial defenses. **(B)** The human pathways are dominated by caspase regulation and activation. Caspase inhibitory proteins only delay the death process which is highly regulated by adaptor-mediated caspase activation. Cytochrome c-dependent caspase-9 activation and cytochrome c-independent caspase-2 regulation both contribute to death in response to cellular stress.

mitochondria in *Drosophila* and is not required for Dronc or downstream Drice activation *(19,20)* inconsistent with a role for cytochrome c in cell death. Further experiments must be carried out, but this preliminary evidence suggests that mechanistically the core machinery of the intrinsic pathway may not require cytochrome c and therefore may more closely resemble *C. elegans*.

Unlike *C. elegans* however, *Drosophila* contains FADD (dFADD) and caspase-8 (DREDD) homologs consistent with the existence of an extrinsic pathway in *Drosophila*. Despite the absence of recognizable death receptors in the genome, an apoptosis-inducing death ligand, Eiger, was recently cloned *(21)*. Surprisingly however, this ligand did

not require DREDD (caspase-8) for cell death but rather used a DRONC/DARK-mediated pathway induced by JNK activation *(21)*. Furthermore, recent experiments with both dFADD and DREDD demonstrate that they play a role in the antibacterial response to Gram-negative bacteria *(22,23)*. These studies imply that the extrinsic death pathway exists in *Drosophila* but that caspase-8 (DREDD) may not be necessary; instead, it may be required for antibacterial defenses (Fig. 2). The identification of putative death receptors for the *Drosophila* death ligand should assist in determining the biological relevance of the extrinsic pathway in *Drosophila*.

IAPs are also found in *Drosophila* and appear to play a more significant role in apoptotic regulation than any other system studied (Fig. 2). Loss of DIAP1 leads to embryonic lethality due to constitutive caspase activation *(24)*, reinforcing the role of IAPs in the negative regulation of caspases. Furthermore, the lethality due to DIAP1 loss is suppressed by subsequent loss of Dark or DRONC *(20,25)*, suggesting that DIAP1 plays a crucial role in regulating the apoptosome in *Drosophila*, an observation not made in mammalian systems. The importance of IAPs in *Drosophila* is further supported by the presence of three genes— rpr, hid, *and* grim (collectively known as the RHG proteins)— that inhibit IAPs to induce apoptosis *(26)*. Interestingly, rpr, hid, *and* grim control almost all apoptosis in the fly, as was initially described by a deletion mutant, deficiency H99 *(27)*. However, structural homologs of RHG proteins appear to be absent in other species, although functional homologs (Smac/DIABLO and HtrA2/Omi) in mammals have recently been described *(28–34)*. More recent experiments suggest that select RHG proteins can promote the degradation of DIAP1 as a means to promote cell death *(35)*. Therefore, *Drosophila*, despite their acquistion of components of the extrinsic pathway and IAPs, differ from mammals significantly in the control of apoptosis by relying on a novel group of proteins, the RHG family, to regulate IAP function which is critical in controlling an intrinsic pathway devoid of cytochrome c involvement (Fig. 2). All further discussions of the apoptotic pathways will focus on the mammalian systems garnered over the past twenty years.

CASPASES—STRUCTURE, CLASSIFICATION, AND ACTIVATION

The first mammalian homolog of *C. elegans* ced-3 identified was the interleukin (IL)-1-converting enzyme, or ICE *(36)*. This protease, responsible for the processing and subsequent maturation of IL-1β, was then demonstrated to possess apoptotic potential when expressed in rat fibroblasts *(37)*. This was the first demonstration that the overexpression of a ced-3 -like protease could cause programmed cell death in mammalian cells. The identification of numerous mammalian ced-3-like proteins led to the adoption of a common nomenclature for the family. Due to the presence of a *c*ysteine residue within the active site of the protease coupled with the substrate specificity following *asp*artic acid residues, the proteins were designated as the *caspase* family *(38)*. All caspases are synthesized as inactive precursors (procaspases) containing a regulatory prodomain of varying length and a large subunit (p20) as well as a small subunit (p10). Activation of procaspases (with the exception of initiator caspases) requires cleavage to form the active enzyme, which is a tetramer of two large subunits and two small subunits creating two active sites *(39)* (Fig. 3). Examination of cleavage sites within procaspases reveals that they adhere to caspase substrate sites, suggesting that caspases are activated in a sequential fashion forming a caspase cascade *(40)*.

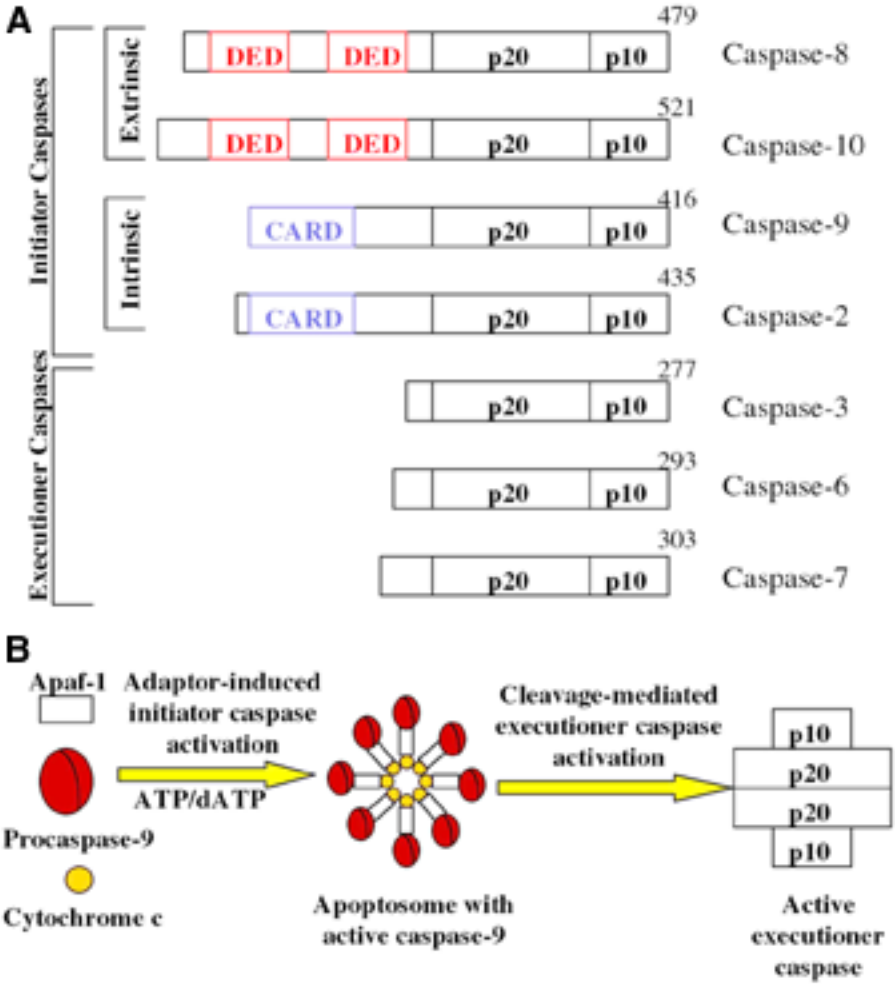

Fig. 3. Mammalian apoptotic caspase classification and activation mechanisms. **(A)** Caspases are divided into initiator and executioner caspases based on mechanism of activation and the presence or absence of a prodomain. Initiator caspases of the extrinsic pathway contain DEDs to allow FADD interaction while initiator caspases of the intrinsic pathway utilize a CARD for adaptor interaction. **(B)** Initiator caspase activation is driven by adaptor-mediated oligomerization that in turn leads to cleavage-induced executioner caspase activation. In this example, procaspase-9 is activated within the apoptosome after CARD-mediated recruitment by Apaf-1 and cytochrome c in a process driven by ATP. Apoptosome formation leads to cleavage and activation of executioner caspase-3.

To date, 14 mammalian caspases have been cloned and belong to one of two functional subgroups: inflammation or apoptosis. Incidentally, the first mammalian caspase cloned, ICE or caspase-1, is involved in inflammation *(41)*, not apoptosis as was originally suggested *(37)*. The expansion of the caspase family can be explained by the increase in the number and complexity of the pathways as well as the different substrate specificity of each caspase defined by the tetrapeptide recognition motif with an aspartic acid requirement in the first position *(42)*. The apoptotic caspases have more recently been subdivided into two categories: initiator and executioner (Fig. 3A). This distinction reflects the

caspase's overall structure, which determines at what point in the pathway it participates *(39)*. Initiator caspases are activated by adaptor-mediated processes and, as the name implies, begin the caspase cascade. These caspases include caspase-9 and -2 as well as caspase-8 and -10. The adaptor-driven activation of these caspases depends on conserved motifs within their long prodomains. The caspase activation and recruitment domain (CARD) of caspase-9 and -2 and the death effector domain (DED) of caspase-8 and -10 facilitate their interactions with CARD- or DED-containing adaptor proteins, leading to the local aggregation and activation of these initiator caspases *(41)* (Fig. 3B). Knockout studies in mice have demonstrated a requirement in signaling for caspase-8 downstream of all known death receptors *(43)*, whereas caspase-9 is required for most death stimuli using the intrinsic pathway, with a few notable exceptions *(44,45)*.

Once activated, the main function of initiator caspases is in targeting the specific cleavage and activation of the second set of apoptotic caspases, the executioner caspases including caspase-3, -6, and -7 (Fig. 3A,B). Executioner caspases are mainly responsible for cleaving various intracellular target proteins containing consensus caspase cleavage sites in order to dismantle the cell as quickly as possible, avoiding an inflammatory response *(46)*. Similar to observations with caspase-9, caspase-3 knockout mice display gross brain malformations and die prematurely, suggesting a role for caspase-3 in normal development of the brain *(47,48)*. Furthermore, apoptotic defects are stimulus- and tissue-dependent, as seen with the caspase-9 knockout, but death in response to most stimuli of the intrinsic and extrinsic pathways display a defect in some cell type with the loss of caspase-3. Therefore caspase-3 has been proposed to be the crucial executioner caspase responsible for most of the nuclear phenotypes associated with apoptosis *(48)*. Lesser roles are postulated for the remaining executioner caspases; however, caspase-7 likely plays a more predominant role in the execution phase of cell death than caspase-6 based on knockout studies *(49)*.

CASPASE ACTIVATING ADAPTOR PROTEINS—APAF-1 AND FADD

Initiator caspases become activated in response to a number of apoptotic stimuli including death ligands, serum withdrawal, and DNA damage due to replication dysfunction, irradiation, or exposure to chemotherapeutic agents. Initiator caspase activation is contingent on their local aggregation via adaptor proteins. DED-containing initiator caspase-8 and -10 function in the extrinsic pathway and undergo autoproteolytic cleavage due to induced aggregation *(50–52)*. Aggregation is a result of the ligation of death ligands to their cognate receptors, leading to recruitment of the adaptor protein FADD followed by DED-caspase recruitment (Fig. 4A). A conserved motif termed the death domain (DD) *(53)*, present in both the receptor and the adaptor, is responsible for their interaction *(54,55)*. FADD, in turn via its DED, is able to recruit and concentrate DED-caspases at the cell surface; thus, the compilation of these factors (ligand, receptor, adaptor, and caspases) are the minimal requirements within the DISC *(56,57)*. Formation of the DISC is sufficient to increase the local concentration of DED-caspases, leading to their cleavage *(15)* (Fig. 4A). This cleavage event was considered the DED-caspase activating event; however, more recent studies suggest that dimerization, not cleavage, leads to DED-caspase activation, with the cleavage event serving a stabilizing role *(58–60)*. Knockout studies in human and mouse cells demonstrate an absolute requirement for FADD and caspase-8 in death receptor-mediated apoptosis *(61–64)*.

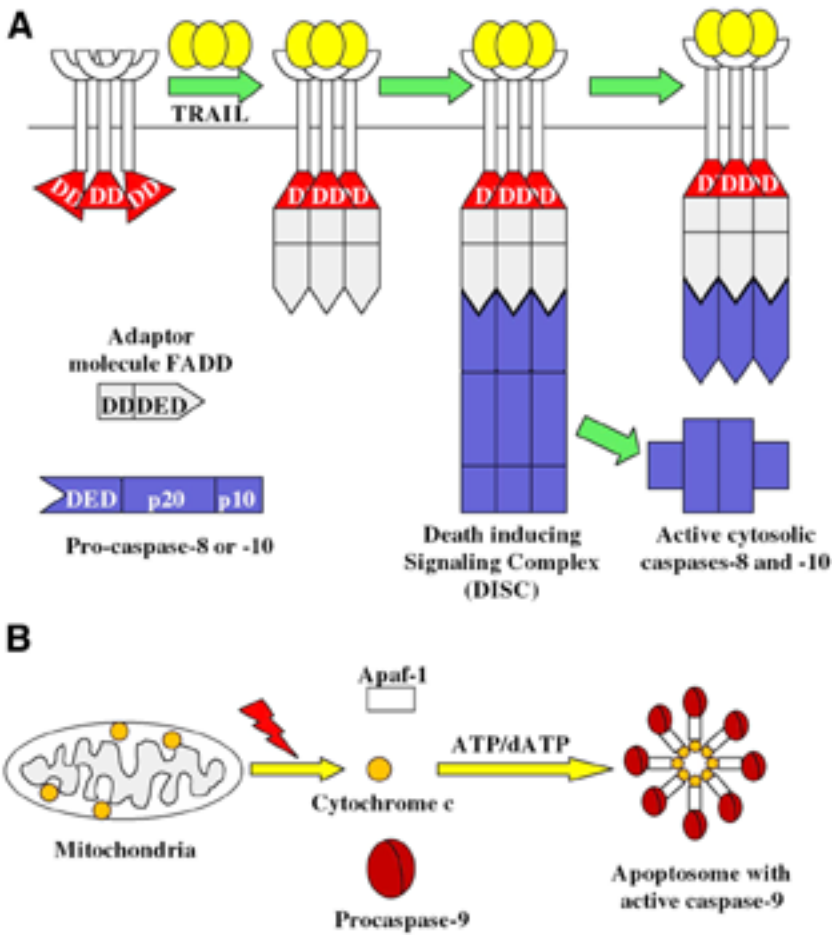

Fig. 4. Adaptor-mediated initiator caspase activation. **(A)** FADD-mediated procaspase-8 and -10 recruitment following death ligand exposure leads DISC formation and subsequent DED-caspase activation. **(B)** Following cellular stress, cytochrome c release from mitochondria in conjunction with ATP, Apaf-1, and procaspase-9 leads to apoptosome formation and subsequent caspase-9 activity. Apaf-1 undergoes a conformational change to allow binding to procaspase-9.

The other adaptor protein responsible for initiator caspase activation is Apaf-1 *(65)*, the ced-4 homolog, which functions in the intrinsic pathway leading to procaspase-9 activation *(66)* (Fig. 4B). Besides Apaf-1, other cofactors are necessary for the activation of caspase-9. Following mitochondrial dysfunction in response to growth factor withdrawal or DNA damage, cytochrome c is released from the mitochondria *(67)*. Cytosolic cytochrome c interacts with the WD-40 repeats of Apaf-1, enhancing the recruitment of ATP *(68)*. This causes a conformational change within Apaf-1, exposing the CARD and allowing for adaptor-mediated procaspase-9 recruitment via CARD/CARD interactions. Cytochrome c in conjunction with ATP, Apaf-1, and procaspase-9 form the holoenzyme known as the apoptosome *(66)* (Fig. 4B). Unlike other known caspases, active caspase-9 remains associated with the apoptosome without undergoing cleavage events to achieve its

maximal activity *(69)*. The apoptosome is then competent to activate executioner caspases such as caspase-3 *(66)*. The phenotype and death characteristics of Apaf-1 knockout mice *(70,71)* support a pathway from Apaf-1 to caspase-9 to caspase-3, regulating the intrinsic pathway.

CASPASE INHIBITORY PROTEINS—MAMMALIAN AND VIRAL

Due to the central role that caspases play in apoptosis, mammals as well as *Drosophila* regulate caspase activity in part by a family of caspase binding proteins called IAPs. The first IAP was identified by Lois Miller in baculovirus as a protein that increases host cell survival following infection *(72)*. Initial studies suggested that two features of these genes were necessary for antiapoptotic function: the baculovirus IAP repeat (BIR) domain and the RING domain *(73)*. Subsequently, a number of mammalian IAP genes were recognized and cloned based on homology *(16)*. A number of these genes, including the first human IAP discovered (NAIP) *(74)*, survivin, and Bruce, contain only BIR domains. Although when overexpressed they inhibit apoptosis to varying degrees, they are not considered classical IAPs but rather BIR-containing proteins (BIRPs) *(75)*and will not be discussed further. The five remaining IAPs—namely, XIAP (ILP-1), ILP-2 (not found in mouse), c-IAP1 (hIAP2), c-IAP2 (hIAP1), and ML-IAP (Livin)—contain a variable number of BIR domains (anywhere from one to three) with intervening linker regions as well as a C-terminal RING domain (Fig. 5).

The most studied family member, XIAP *(76)*, provided the first clues as to how IAPs inhibit cell death. XIAP directly interacts with executioner caspase-3 and -7 to inhibit caspase function *(77)*. Caspase-9 was subsequently identified as an XIAP target for inhibition as well *(78)*. These effects are mediated in part by the BIR domains that have different caspase specificity, BIR2 for caspase-3 and -7 and BIR3 for caspase-9 *(79)*. Surprisingly, subsequent crystal structure analysis revealed a role for linker binding directly to caspase-3 and -7, preventing substrate binding and allowing only a limited number of contacts between caspase and the actual BIR domain *(80–83)*. Therefore, the linker region between the BIR1 and 2 domains directly binds caspase-3 and -7 and helps in determining the caspase specificity along with the BIR2 domain. Recently the crystal structure of the BIR3 domain of XIAP complexed with caspase-9 revealed the mechanism behind the inhibition. Due to the necessity of initiator caspase dimerization for activation *(59,84)*, the BIR3 domain acts to prevent caspase dimerization, trapping the caspase in an inactive monomeric state *(85)*.

XIAP is generally considered the most potent of the IAP family due to its ability to bind and inhibit caspase-9, -3, and -7. On the other hand, a recently cloned, highly homologous human protein, ILP-2, has specificity for only caspase-9 due to the presence of only one BIR domain, most similar to BIR 3 of XIAP *(86)*. Interestingly this gene is absent in mice, does not contain any introns, and is only expressed in the testis. Other family members, c-IAP1 and c-IAP2, distinguish themselves based on their cloning as tumor necrosis factor (TNF) receptor 2-associated proteins which interact with TRAF1 and TRAF2 *(87)*. These two proteins were subsequently shown to inhibit caspase-3 and -7 *(88)* in studies similar to those initially performed with XIAP. c-IAP1 and c-IAP2, two highly similar proteins, also contain a CARD, the significance of which is still not understood. Despite their recruitment to the TNF receptor complex that contains caspase-8, c-IAP1 and c-IAP2 do not demonstrate any specificity for this caspase *(78,88)*. The same can be said for all

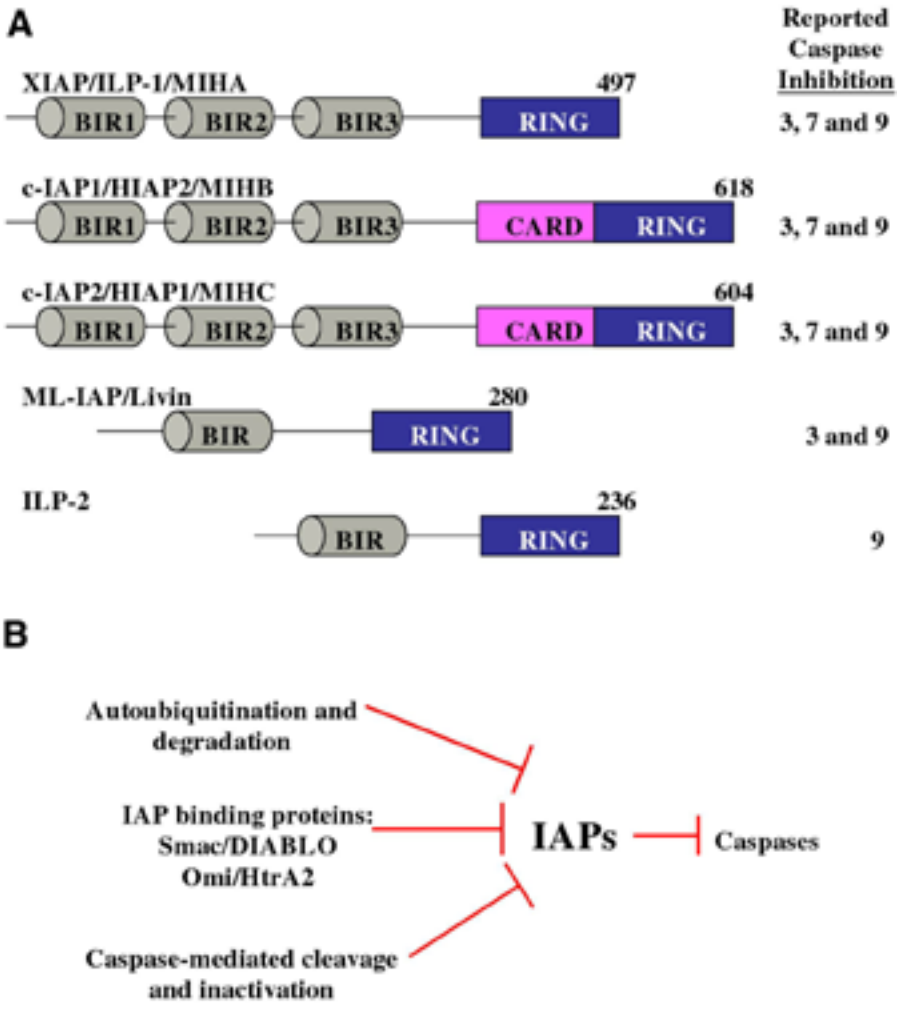

Fig. 5. The human antiapoptotic IAP family members and mechanisms of IAP inhibition. **(A)** The five human IAP proteins thought to directly bind caspases are listed above along with their cellular caspase targets. Conserved motifs include the BIR domain, the RING domain and the CARD. The BIR domain of ILP-2 most closely resembles the BIR3 domain of XIAP while the BIR domain of ML-IAP looks like a hybrid between XIAP's BIR2 and BIR3 domain. **(B)** The IAPs are inhibited by three distinct mechanisms: auto-ubiquitination and degradation, caspase-mediated cleavage and sequestration by IAP binding proteins, Smac/DIABLO and Omi/HtrA2.

other IAP family members. No IAP has been found to bind or inhibit the DED-caspases of the extrinsic pathway. Lastly, ML-IAP was recently identified as a single BIR domain containing IAP with a RING domain and is overexpressed in a majority of melanoma cell lines *(89)*.

Despite the controversy surrounding its requirement for the suppression of apoptosis *(90)*, the IAP RING domain has recently been recognized as a regulator of IAP and associated protein stability *(91)*. The realization that RING domains, in a variety of unrelated proteins, serve as E3 ubiquitin ligases in the ubiquitin-mediated proteolytic

pathway *(92)* initiated subsequent work on the IAPs. Both XIAP and c-IAP1 were demonstrated to contain ubiquitin ligase activity that was dependent on their RING domains. This activity was shown to mediate the autoubiquitination of both proteins in response to glucocorticoids or etoposide in thymocytes, suggesting that the degradation of both proteins was necessary for death progression *(93)*. Recent studies have attempted to identify targets of IAP-mediated ubiquitination and degradation. In vitro data with c-IAP2 suggests that it promotes the monoubiquitination of caspase-3 and -7 *(94)*. Similarly, XIAP has been shown to ubiquitinate caspase-3 in vitro and a constitutively active caspase-3 mutant could be degraded by XIAP in a RING-domain dependent manner *(95)*. Both of these studies suggest that caspases could be a target of IAP-mediated ubiquitination and degradation, supplying another means by which IAPs inhibit cell death. These observations suggest that the E3 ligase function is crucial for the ability of IAPs to suppress cell death. The identification of more physiologic E3 IAP targets will further our understanding of IAP biology.

With the creation of the IAP family of proteins in higher organisms to inhibit caspase activity also comes the necessity to counteract this inhibition when a death stimulus is received (Fig. 5B). As was discussed above, the ubiquitin ligase activity of the IAPs is one mechanism by which the cell targets IAPs for degradation to allow death progression *(93)*. Furthermore, caspase-dependent IAP cleavage provides another means by which the cell eliminates IAP function *(96,97)*. Lastly, IAP binding proteins, functionally homologous to the *Drosophila* RHG proteins, have been discovered in mammals *(28–34)*. Unlike their *Drosophila* counterparts, Smac/DIABLO and HtrA2/Omi are resident mitochondrial proteins that are released upon mitochondrial dysfunction in order to bind and sequester IAPs. Despite the lack of overall amino acid conservation between the *Drosophila* and mammalian genes, all of the IAP binding proteins including caspase-9 contain a common tetrapeptide sequence or an IAP-binding motif (IBM) that is either constitutively exposed (RHG proteins) or exposed following posttranslational processing (mammalian proteins), which mediates IAP binding *(98)*. Although their relative contribution in different physiological settings remains unclear, these three mechanisms of IAP inhibition cooperate to eliminate IAP function following an apoptotic signal (Fig. 5B).

In addition to the IAPs, baculoviruses encode a second protein, p35, that appears to be required for host cell survival *(99)*. p35 encodes a broad spectrum caspase inhibitor whose cleavage generates a tight inhibitor/caspase complex *(100,101)*. With the evolution of an extrinsic pathway in mammalian cells, viruses began to develop inhibitors of caspase-8 in order to subvert the host's immune response. The cowpox virus utilizes the gene product CrmA to target caspase-8 as means to shut down the extrinsic pathway *(102–104)*. Two other recently described viral caspase-8 binding proteins include vICA from cytomegalovirus *(105)* and the 14.7kDa adenovirus protein *(106)*. A more indirect means of caspase-8 inhibition can be seen with some herpesviruses as well as with molluscipoxvirus. These viruses encode DED-containing proteins termed viral FLICE-inhibitory proteins (v-FLIPs) that compete with cellular caspase-8 and -10 for recruitment to the DISC *(107–109)*. This competition leads to decreased DED-caspase activation and cell survival. All of the aforementioned viral strategies have evolved to target apoptosis at its core, the caspase, in order to allow viral propagation in the face of an attacking immune system.

CELLULAR CASPASE SUBSTRATES

The morphological changes associated with programmed cell death are a direct result of the systematic cleavage of intracellular proteins by caspases. In the initiation phase, the apical caspases begin the caspase cascade by directly cleaving and activating executioner caspases *(41)*. The executioner caspases are therefore responsible for dismantling the cell by cleaving specific substrates leading to DNA fragmentation, membrane blebbing, and cell shrinkage *(110)* (Fig. 6). However, besides executioner caspases, there are two notable noncaspase substrates of caspase-8: the BH3-only protein, Bid, as well as plectin involved in cytoskeletal integrity. Bid was initially described as a proapoptotic BH3-only protein that interacted with both pro- and antiapoptotic Bcl-2 family members *(111)*. The identification of Bid as a caspase-8 target established a link between the extrinsic pathway and the intrinsic pathway, resulting in cytochrome c release following death ligand treatment *(112,113)*. Cleavage of Bid by caspase-8 results in the subsequent myristoylation of an exposed glycine that assists in the translocation to mitochondrial membranes *(114)*. This mitochondrial amplification step through Bid serves to further activate caspases, which in some cell types is absolutely necessary for cell death.

Plectin is the only known cytoskeletal protein that is targeted by initiator caspases, namely caspase-8. Cleavage of plectin is thought to be important for cytoskeletal reorganization because plectin-deficient mouse embryonic fibroblasts (MEFs) do not undergo the characteristic actin rearrangements seen in response to death stimuli *(115)*. Additional cytoskeletal and cytosolic caspase cleavage targets involved in the cell reshaping, blebbing, and shrinkage process include gelsolin *(116)*, keratins *(117,118)*, PAK2 *(119)*, α-fodrin *(120)*, and ROCK I *(121,122)*; however, these proteins are among a growing list of over 100 proteins which are targeted by the executioner caspase group, caspase-3, -6, and -7 *(9)* (Fig. 6). The executioner caspases can further amplify the death signal through further caspase activation. Caspase-3 has been shown to be necessary for the activation of caspase-6 in response to cytochrome c release *(123)* while caspase-6 has the ability to activate caspase-3 *(124)*.

One of the most characteristic changes associated with apoptosis is chromatin condensation and DNA fragmentation creating a laddering effect due to cleavage between nucleosomes *(125)*. The identification of the caspase-activated DNase (CAD) revealed that CAD was latent in undamaged cells due to the association of an inhibitor (ICAD) *(126–128)*. An apoptotic stimulus then led to a caspase-3 dependent cleavage of ICAD, releasing CAD to cleave cellular DNA. More recent experiments have also attributed roles for the cleavage/degradation of cellular DNA to endoG *(129)* released from mitochondria and DNase II contributed by phagocytic cells *(130)*. Apoptotic chromatin condensation is both a caspase-dependent and -independent process. The caspase-3 target, acinus *(131)*, as well as the apoptosis inducing factor (AIF) *(132)*, normally sequestered in the mitochondria, have the capacity to trigger chromatin condensation following cellular insult. Elimination of DNA repair enzymes such as PARP *(133,134)* and DNA-PK *(135)* through executioner caspase-mediated cleavage prevents the cell from attempting to fix apoptotic DNA fragmentation.

Caspase-3 was originally identified as the enzyme responsible for the majority of the cellular phenotypic changes due to mouse knockout studies that demonstrate impaired PARP cleavage and DNA fragmentation *(47,48)*. However, compensatory mechanisms mediated by caspase-6 and -7 may exist in cells that lose caspase-3 expression *(136,137)*.

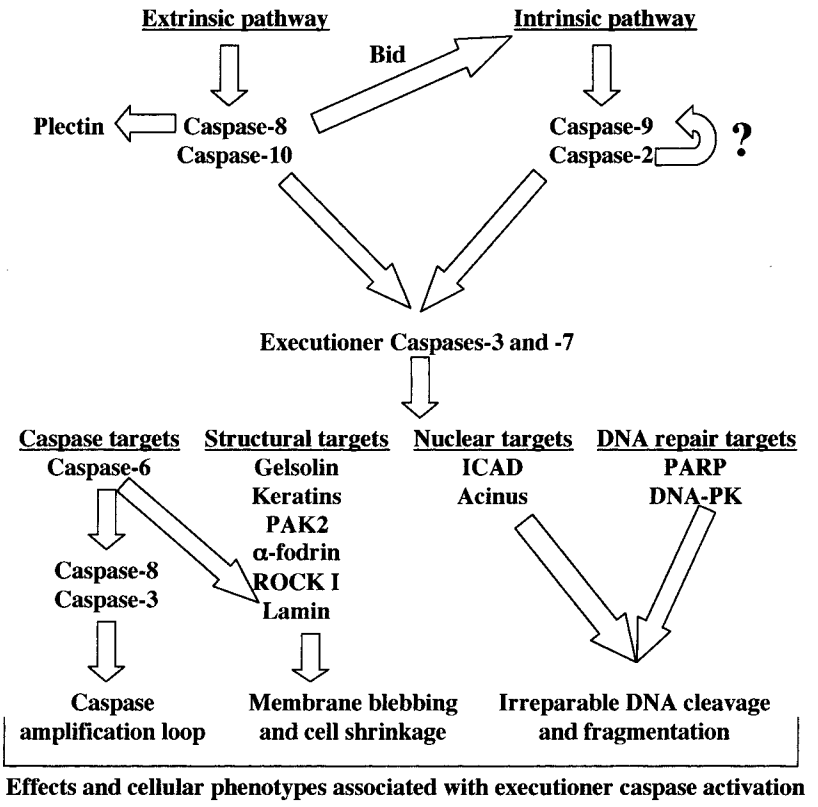

Fig. 6. The caspase cascade of the intrinsic and extrinsic pathways and the relevant caspase targets mediating cellular breakdown. Following death stimuli, initiator caspases are activated and primarily function to activate executioner caspases. Two relevant noncaspase-8 targets are shown which participate in the cell reshaping process (Plectin) as well as initiating the mitochondrial amplification loop (Bid). Activation of the executioner caspases however are primarily responsible for the large scale phenotypic changes associated with programmed cell death.

Due to the substrate specificity similarities between caspase-3 and -7 *(42)*, they are believed to act on a similar substrates with caspase-3 being the predominant effector. Caspase-6, however, has a different substrate specificity than the other executioner caspases *(42)*. Besides caspase-3 *(124)*, Lamin A/C is also recognized as a caspase-6 target for cleavage involved in nuclear breakdown *(138)*. Recently, caspase-6 was biochemically purified as the caspase-8 activating enzyme in response to cytochrome c release *(139)*, identifying the mechanism by which the intrinsic pathway activates the caspases of the extrinsic pathway.

CLEARANCE OF APOPTOTIC CELLS BY PHAGOCYTOSIS

Elimination of cells via apoptosis avoids an immune response that is contingent on the apoptotic cell being cleared via phagocytosis *(140)*. In mammalian cells, macrophages (professional phagocytes) or neighboring cells (nonprofessional phagocytes) carry out this function. One active area of research focuses on identifying "eat me" signals on dying cells. Although a number of signals probably cooperate to initiate phagocytosis *(141)*, the

phospholipid, phosphatidylserine (PS) is the best characterized signal to date. PS is normally only exposed on the inner leaflet of the plasma membrane but becomes permanently externalized when aminophopholipid translocase is switched off in apoptotic cells *(142)*. With the discovery of the phosphatidylserine receptor (PSR) on macrophages, fibroblasts, and epithelial cells *(143)*, one component of the mechanism by which phagocytes recognize apoptotic cells was elucidated. More recently, a protein produced and secreted by macrophages, MFG-E8, binds apoptotic cells via aminophospholipids such as PS and serves to assist in engulfment by macrophages *(144)*. Roles for additional extracellular proteins such as lectins, integrins, and scavenger receptors on the surface of phagocytes in the recognition of dying cells are still ongoing *(141)*.

Following the recognition of "eat me" signals on dying cells, understanding the cytoskeletal rearrangements involved in carrying out the engulfment process is a major task. The use of phagocytosis mutants from *C. elegans* has identified a Rac-dependent cell reshaping process involving ELMO-1, DOCK180, and Crk11 that is conserved in mammalian cells *(145)*. Experiments on this pathway in *C. elegans* have led to the theory of "assisted cell suicide." The idea stems from data in ced-3 partial loss of function mutant worms (caspase compromised) in which engulfment genes were also mutated leading to an increase in cell survival. This supports the idea that cells surrounding apoptotic cells can actively participate in the life or death decision of that cell (i.e., assisted suicide) via phagocytosis *(146,147)*. Evidence of a similar phenomenon is being studied in mammalian cells as well *(148)*.

REGULATION OF THE INTRINSIC PATHWAY BY THE BCL-2 FAMILY OF PROTEINS

Recent studies have revealed that mitochondria function at the core of the intrinsic pathway by not only sensing cellular stress but also by responding to this stress by releasing necessary components of the pathway into the cytosol. Therefore, understanding the maintenance and subsequent disruption of mitochondrial integrity has become paramount in delineating these pathways. Even though the mechanism(s) involved in altering mitochondrial integrity are not completely understood, the importance of pro- and antiapoptotic Bcl-2 family members in apoptosis is irrefutable *(11)* (Fig. 7). The genesis of the field dates back to the realization that Bcl-2, a gene overexpressed in follicular lymphoma due to a translocation, promoted cell survival without affecting cell proliferation directly *(149)*. This was the first demonstration that alteration of an apoptotic pathway could lead to tumor development. Similar to the revelation that *C. elegans* ced-3 represented the primordial caspase gene, subsequent studies demonstrated that Bcl-2 could functionally substitute for the loss of ced-9 in *C. elegans (150)*. In the case of *C. elegans* where one pro- (egl-1) and one antiapoptotic (ced-9) Bcl-2-like genes exist, the pathway seems straightforward; however, when considering that multiple pro- and antiapoptotic Bcl-2 members, including at least 10 BH3-only proteins, exist in humans, the situation becomes more complex.

Classification as a Bcl-2 family protein requires the presence of at least one Bcl-2 homology (BH) domain. The Bcl-2 family is divided into three groups based on BH structure/function: one antiapoptotic family with three to four BH domains (Bcl-2, Bcl-X_L, BCL-w, A1, Mcl-1, Bcl-B and Boo), and two proapoptotic families, the Bcl-2-like (Bax) group with two to three BH domains (Bax, Bak, Bok, Bcl-G_L, and Bcl-X_S) and the BH3-

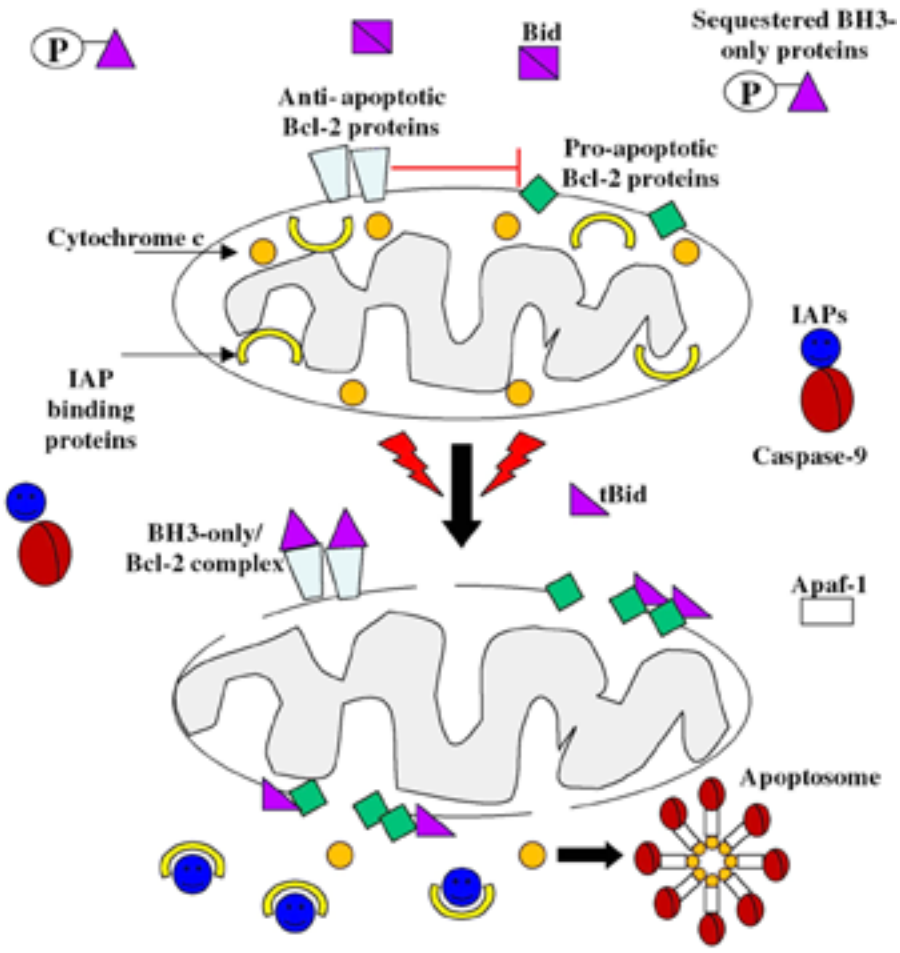

Fig. 7. Intrinsic pathway mediated by mitochondrial homeostasis. Although the exact mechanism by which mitochondria become permeabilized is of much debate, Bcl-2 proteins impact this decision. In the absence of stress, BH3-only proteins are sequestered and thereby inactivated by a number of posttranslational mechanisms. Antiapoptotic Bcl-2 proteins inhibit the pro-apoptotic members on the surface of the mitochondria. Following stress, BH3-only proteins target the antiapoptotic proteins on the surface of the mitochondria. This relieves the inhibition on proapoptotic members such as Bax and Bak allowing multimerization in conjunction with membrane targeted tBid. These events can lead to membrane disruption by a number of mechanims resulting in the release of cytochrome c, IAP binding proteins and subsequent apoptosome formation.

only group (Bid, Bad, Bik, Bim, Hrk, Blk, Bmf, Noxa, Puma, and Bcl-G_S) *(10)*. The antiapoptotic group relies on its ability to either inducibly or constitutively localize to intracellular membranes (mitochondria, ER, and nucleus) via a C-terminal transmembrane tail to prevent cell death *(13)*. Evidence for their importance comes from mouse knockout studies as well as transgenic animals. Bcl-2, the prototype member, causes enhanced hematopoetic cell survival when overexpressed in different lineages *(151–153)* or leads to kidney and mature lymphocyte cell loss when deleted *(154)*.

Similar to *C. elegans*, the BH3-only proteins are upstream of and negatively regulate the antiapoptotic Bcl-2 members through direct protein/protein interactions (Fig. 7). The conserved BH3 domain, consisting of only nine amino acids, is sufficient to interact with the antiapoptotic family, nullifying their protective effects *(155)*. The large expansion of this group in mammalian cells has led to the hypothesis that the different members respond in a stimulus-dependent, tissue-specific manner, which is being tested in BH3-only knockout mice *(10)*. For instance, the Bim knockout mice respond normally to DNA damage but are partially resistant to cytokine withdrawal while displaying increased numbers of only lymphoid and myeloid cells *(156)*. The combined knockout of Bim and Bcl-2 also confirms that BH3-only proteins regulate the activity of the antiapoptotic members because loss of only one Bim allele in a Bcl-2 –/– background rescues the degenerative kidney disease in these mice *(157)*. Because the BH3-only proteins are the most upstream regulators within this family, their regulation has become an active area of research. Transcriptional mechanisms of increasing cellular protein levels as well as posttranslational modifications affecting subcellular localization and/or conformation has begun to unravel the signaling pathways upstream of the mitochondria *(10)*.

The remaining family of multi-BH domain proapoptotic Bcl-2 proteins appears to act most distally in the mitochondrial pathway, based on studies of mice lacking two of these proteins, Bax and Bak. Single gene knockout of either protein yielded no dramatic (or lethal) organismal phenotype; however, deletion of both genes rendered most animals nonviable due to a variety of developmental defects stemming from loss of apoptosis *(158)*. Subsequent studies demonstrated that overexpression of BH3-only proteins was unable to induce apoptosis in the absence of Bax and Bak, placing these two genes most downstream genetically in the Bcl-2 pathway *(159–161)*. Cytosolic Bax and mitochondrial Bak both undergo conformational changes and oligomerize in the mitochondrial membrane upon activation of the pathway, relieving inhibition by the antiapoptotic family members *(11)* (Fig. 7). Nevertheless, how oligomerization of Bax family members contributes to mitochondrial dysfunction and effects the permeability transition (PT), and whether it occurs directly through pore formation or other means, is still under investigation *(162)*. Regardless of the mechanism(s) involved, mitochondrial dysfunction leads to the release of proapoptotic factors from the mitochondrial intermembrane space that serves to activate procaspase-9 through apoptosome formation (Fig. 7).

MITOCHONDRIA—INITIATOR OR AMPLIFIER OF THE INTRINSIC PATHWAY?

Recently, the classical view of the mammalian intrinsic pathway, as described above, has been challenged as a few inconsistencies have become apparent *(155)*. Despite the fact that *C. elegans* and mammalian systems contain the same basic core machinery (namely a BH3-only protein, Bcl-2 protein, an adaptor, and a caspase), mechanistically the two systems operate very differently. In *C. elegans*, after a death signal is received, the BH3-only protein binds to and negatively regulates the Bcl-2 protein (similar to what is observed in the mammalian systems). However, Bcl-2 sequestration in *C. elegans* releases the adaptor protein, leading directly to caspase activation, unlike what is observed in mammalian systems. In addition, the mitochondria play no active role in this process in *C. elegans* or *Drosophila*. This has led to the hypothesis that Bcl-2 proteins may play a similar role in sequestering unknown adaptors in mammalian cells *(155)*. Such adaptor

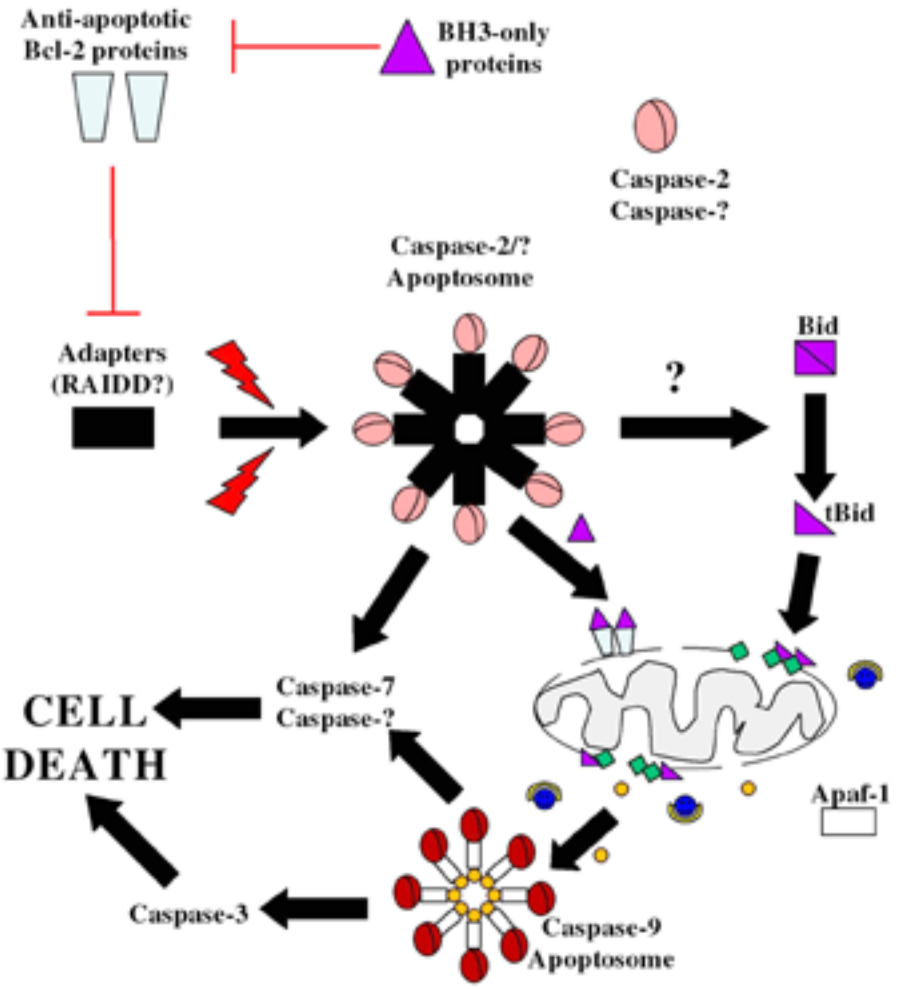

Fig. 8. Alternative model of the intrinsic pathway. This model more closely reflects the basic cell death machinery of C. elegans in which antiapoptotic Bcl-2 family members antagonize an adaptor molecule necessary for caspase activation. A role for caspase-2 upstream of the mitochondria has recently been suggested in which an unknown adaptor molecule triggers its activation. Caspase-2 activation (and potentially other unidentified caspases) simultaneously activates the mitochondrial pathway as well as an alternative pathway not requiring caspase-9. In this model, mitochondrial involvement only serves as an amplification loop, as is seen in the death receptor pathway. The other pathway activated by caspase-2 is sufficient to carry out the death program via the intrinsic pathway in the absence of Apaf-1 or caspase-9.

proteins in turn may activate caspase(s) upstream of mitochondrial engagement. Mitochondrial dysfunction therefore may be a consequence of this upstream caspase activation rather than the event responsible for caspase activation, similar to what is seen after activation of the extrinsic pathway (Fig. 8). Two recent reports give support to this model *(163,164)*; however, many questions still remain.

One study to support this idea comes from observations comparing wild-type mice reconstituted with fetal liver stem cells from Bcl-2 transgenic mice, Bim –/– mice, Apaf-1 –/– mice, or caspase-9 –/– mice *(163)*. The survival of thymocytes from a Bcl-2 transgenic mouse or a Bim –/– mouse is enhanced compared with Apaf-1 –/– or caspase-9 –/– mice when challenged with cytokine withdrawal. In addition, thymocytes isolated directly from Bcl-2 transgenic mice or Apaf-1 or caspase-9 knockout mice demonstrate that Bcl-2 plays a major role in their clonogenic survival after cytokine withdrawal, whereas the loss of Apaf-1 or caspase-9 does not rescue their survival. These observations suggest that Bcl-2 and Bim play a major role in the apoptotic process of hematopoetic cells whereas caspase-9 and Apaf-1 are largely dispensible *(163)*. In contrast to previous reports *(44,45,70,71)*, the Apaf-1 –/– and caspase-9 –/– thymocytes undergo caspase-mediated cell death with traditional hallmarks (PARP cleavage, DNA fragmentation) in response to cytokine withdrawal and irradiation *(163)*. The kinetics of death are delayed, presumably as a result of the lack of a mitochondrial amplification loop, but cytochrome c is released in a caspase-dependent manner ultimately leading to caspase-7 activation followed by PARP and ICAD cleavage *(163)*. These observations are consistent with a model in which an initiator caspase acts upstream of the mitochondria, possibly inhibited by antiapoptotic bcl-2 family members (Fig. 8).

One potential initiator caspase to serve this role upstream of the mitochondria has recently been identified through the use of RNAi technology. The knockdown of caspase-2 protein levels by RNAi resulted in resistance to etoposide, cisplatin, and ultraviolet (UV) irradiation as robustly as knockdown of Apaf-1 protein *(164)*. However, depletion of caspase-2 results in a lack of cytochrome c and Smac release from the mitochondria and loss of Bax translocation, whereas Apaf-1 ablation does not effect these events. Caspase-2 activation in the absence of Apaf-1 in response to etoposide suggests that it may occur upstream of the mitochondria in an initiator caspase role. The lack of a significant phenotype in the caspase-2 knockout mouse does not support a major role in the intrinsic pathway *(165)*; however, another caspase may compensate for the loss of caspase-2 in the mouse or it may represent a difference between humans and mice. Preliminary experiments with caspase-2 suggest that it may also form a multiprotein complex leading to activation upstream of cytochrome c release, but the constituents of the activating complex have not been identified *(166)* (Fig. 8). More experiments are required to discern what initiator caspase(s) and cofactors form upstream of the mitochondria and which substrates contribute to mitochondrial dysfunction.

RELEASE OF PROAPOPTOTIC FACTORS FROM THE MITOCHONDRIA

Regardless of whether the intrinsic pathway initiates at the mitochondria or upstream of it, mitochondrial dysfunction occurs in response to both the intrinsic and extrinsic pathway and this leads to the release of proapoptotic proteins from the intermembrane space *(167)*. The first to be identified was cytochrome c *(67)*, previously known to function only within the electron transport chain. Subsequently it was shown to play a crucial role in the activation of caspase-9. Caspase-9 is monomeric in unstimulated cells *(84)*, however all caspase-9 activity following cytochrome c release is associated with a large molecular weight complex *(69)*. Activation of caspase-9 minimally requires dimerization *(59,84)*, therefore oligomerization is mediated via complex formation with released

cytochrome c, Apaf-1, and ATP (or dATP) to create the holoenzyme known as the apoptosome *(66)* (Fig. 7). Apoptosome formation leads to the activation of executioner caspase-3, -6, and -7 *(66)*. Activation of caspase-3 by the apoptosome leads to a positive caspase feedback loop as caspase-3-mediated caspase-9 cleavage results in more active apoptosome *(168)*.

Because caspase activation and amplification is crucial for cell death, the elimination of caspase inhibitory proteins, such as IAPs, will further enhance the process. The mitochondria sequester two known IAP binding proteins, Smac/DIABLO and HtrA2/Omi (Fig. 7). Smac/DIABLO was simultaneously cloned through its ability to biochemically enhance caspase-3 activation and by its ability to bind to XIAP *(28,29)*. HtrA2/Omi also acts by binding and inhibiting IAPs, but additionally contains serine protease activity which also contributes to its proapoptotic function *(30–34)*. When overexpressed, HtrA2/Omi mutants, which are unable to bind IAPs, can still potentiate death by virtue of this serine protease activity. Consequently, this death is not inhibitable by zVAD, XIAP, or a dominant negative caspase-9. The identification of HtrA2/Omi as a mediator in the apoptotic process could potentially reveal a novel role for serine proteases in apoptosis. IAP binding of both Smac/DIABLO and HtrA2/Omi is mediated by an IAP binding motif (AVPI for SMAC/Diablo and AVPS for Omi) that is revealed once the mitochondrial targeting sequence is removed, thereby preventing IAP binding during their translocation to the mitochondria. Elimination of IAP function by these two proteins serves to release caspase-9, -3, and -7, allowing for their activation.

Activation of caspases leads to DNA fragmentation, one of the earliest recognized hallmarks of programmed cell death, and subsequent work provided evidence that the activation of the nuclease (CAD) by caspase-3 was responsible for this phenotype *(126–128)*; however, the absence of significant defects in mice lacking CAD activity *(169)* prompted the search for another apoptotic nuclease activity. Two mitochondrially localized proteins that cause DNA fragmentation were subsequently discovered, endonuclease G (endoG) and AIF. EndoG was identified as a mitochondrial protein which induces nuclear DNA fragmentation upon release from the mitochondria even in the absence of CAD *(170)*. This pathway appears to be evolutionarily conserved in *C. elegans* because cps6 mutants can be rescued with the murine endonuclease G *(132)*. AIF is also released from the mitochondria, and microinjection of AIF in resting cells results in caspase-independent chromatin condensation and degradation, loss of mitochondrial membrane potential, and phosphatidylserine exposure *(132,171)*. While the mechanism(s) behind these effects remain elusive due to an early embryonic lethality in the knockout (an apparent necessity for AIF in the first wave of developmental apoptosis) *(172)*, an interaction with Hsp70 may begin to shed light on this pathway *(173)*. Nevertheless, EndoG and AIF represent two caspase-independent mechanisms by which the cell can undergo DNA degradation upon mitochondrial disruption. All of the aforementioned proteins released from the mitochondria either serve to enhance caspase activation or directly lead to DNA fragmentation in order to propagate the death signal.

THE EXTRINSIC PATHWAY

The intrinsic cell death pathway involving the mitochondria with the interplay of Bcl-2 family members leading to apoptosome formation and subsequent executioner caspase activation is considered to be the ancestral apoptotic pathway with conservation

of its core components in *C. elegans (9)*. A more recently evolved cell death cascade, termed the extrinsic pathway, employs a ligand/receptor interaction to transduce a death signal inside the cell in order to activate an independent set of initiator caspases (-8 and -10) *(174)* (Fig. 9). Specific ligand/receptor interactions evoke the formation of a DISC that is comprised of a trimeric ligand, trimeric receptor, an adaptor molecule (such as FADD) and caspases (-8 and -10). DISC formation has been postulated to occur due to protein/protein interactions mediated by conserved modules such as the death domain (receptor/adaptor) and death effector domain (adaptor/caspase) *(175)*. DISC formation leads to oligomerization of initiator caspase-8 and -10 that results in caspase activation via induced proximity *(176)*. Initiator caspase activation can either directly activate downstream executioner caspases to lead to cell death or employ the mitochondria through the cleavage of Bid to amplify the signal from the receptors *(177)* (Fig. 9). The intrinsic and extrinsic pathways ultimately converge to use the same executioner caspases to dismantle the cell; therefore, the gross phenotypic changes brought about by programmed cell death are the same regardless of which pathway is employed.

DEATH LIGANDS FROM THE TNF SUPERFAMILY (TNF-α, FAS, AND TRAIL)

Observations from the 1800s that acute bacterial infections caused tumor shrinkage in patients led to the description of a TNF that could kill tumor cells in patients *(178)* and in culture *(179)*. Cloning of the molecule *(180)* marked the beginning of an era that has identified members of the TNF superfamily of ligands, now numbering 18 *(177)*. This superfamily mainly evolved to regulate immune homeostasis *(181)*. Discussions will only focus on three of these ligands that cause cell death in which the molecular mechanisms are fairly well understood: TNF, FasL (CD95L, APO1L), and TNF-related apoptosis-inducing ligand (TRAIL or APO2L) (Fig. 10). Despite having "tumor necrosis" activity and the ability to cause cell death, TNF engages multiple pathways that regulate cell proliferation and inflammatory responses as well, depending on the cellular environment *(182)*. Conversely, FasL and TRAIL primarily mediate cell death when bound to their cognate receptors, although situations in which FasL and TRAIL promote cell proliferation have been described *(183,184)*. The evolution of death ligands was thought to occur specifically in order to respond to threats to the host (bacterial/viral infections and injured/cancerous cells) as well as to regulate immune homeostasis *(177)*. These type II transmembrane proteins are active as trimers with homology of TNF family members restricted to the residues necessary for trimerization *(185)*. Nonconserved residues between family members account for ligand/receptor specificity.

The biological activities of these death ligands in vivo have been explored through the use of neutralizing antibodies and knockout animals as well as studies of hereditary syndromes with defects in these pathways. Bacterial pathogens are recognized by Toll receptors, leading to TNF production in order to mount appropriate inflammatory responses *(181)*. TNF is produced from a variety of hematopoetic cell types in response to inflammation, injury, and environmental stresses. The biological responses to TNF treatment include cell proliferation, differentiation, apoptosis, and necrosis. Deregulation of TNF can lead to conditions such as septic shock, arthritis, irritable bowel disorder, and cachexia *(186)*. Unlike TNF, FasL, due to its propensity to primarily initiate cell death, leads to lymphoproliferation when the signaling pathway is compromised. Physiologic roles of