I. Introduction

Apoptosis was first discovered by Carl Vogt in 1842 (243). After more than a century of dormancy, Kerr, Wyllie, and Currie reinitiated apoptosis research. They coined the term apoptosis in 1972 to describe the unique morphology and ladder-like DNA fragmentation found in the dying adrenal cells after the withdrawal of ACTH support (119, 255, 257). The morphological hallmarks of apoptosis include cell shrinkage, plasma membrane blebbing, and apoptotic body formation.

In multicellular organisms, apoptosis or programmed cell death is an important physiological process to remove superfluous cells including those that are generated in excess, have already completed their specific functions, or are harmful to the whole organism (119, 220, 230). Apoptosis is particularly important during embryonic development, metamorphosis, tissue renewal, hormone-in-
duced tissue atrophy, and many pathological conditions. During embryonic development, apoptotic cell death is widespread and is essential for the sculpturing of body shape. It is estimated that as much as 85% of embryonic neurons undergo apoptosis during development of the central nervous system (8, 9, 174, 187, 230). During metamorphosis in different animals, extensive apoptosis is necessary for the remodeling of body structure in essentially every part of the organism (104, 117, 220).

In adult tissues, the occurrence of apoptosis is also common and can be separated into three broad categories. Minimal cell proliferation and a low rate of cell turnover characterize the first group of tissues, such as neuron, heart, liver, and kidney. Generally, little apoptosis can be observed in these tissues (10, 11). The second group, including hematopoietic tissues, the epithelium lining intestinal crypts, and male germ cells (spermatogonia) in the testis, exhibits constant cell turnover and has high rates of stem cell proliferation accompanied by massive apoptosis (18, 255–257). In the third type of tissues, exemplified by the ovary, a high rate of follicular cell apoptosis continues during the reproductive life but with no replenishment of the original stockpile of follicles (91).

The maintenance of a balance between apoptosis and cell proliferation is crucial for the well being of the multicellular organism. Abnormal apoptosis regulation often leads to tissue degeneration and/or malformation, thus leading to pathogenesis found in diseased states (230). Certain forms of autoimmune disease are due to the suppression of apoptosis in the immune system and the ensuing accumulation of autoreactive lymphoid cells. In more extreme conditions, the failure of cells to die could lead to an accumulation of excess cells and, eventually, tumorigenesis. Similar to the abnormal suppression of apoptosis, excessive cell death could also lead to pathological states. Increased apoptosis has been associated with ischemic injury of vascular tissues, graft versus host diseases, and the development of neurodegenerative disorders such as Alzheimer’s disease, Parkinson’s disease, Huntington’s disease (230), Charcot-Marie-Tooth type 1A demyelinating neuropathy (62), spinal muscular atrophies (200), and toxic epidermal necrolysis (242). Likewise, excessive apoptosis may be the cause of premature ovarian failure due to an early depletion of ovarian follicle reserve. Moreover, adverse environmental factors could also alter apoptosis such as injuries that could be incurred by environmental toxins, X-irradiation and chemotherapeutic agents, or after a viral infection (Table 1).

With the exception of blastomeres, possibly all differentiated tissues in multicellular organisms are “programmed” to undergo apoptosis through an evolutionarily conserved “suicide” program (186–188). To stay alive, the healthy cells require signals provided by specific survival factors acting on receptors found intracellularly or on the cell surface. Once the survival signals are absent, the “survival surveillance system” switches on the decision step of the apoptotic program, thus leading to a subsequent activation of a set of death genes in the execution phase of apoptosis (186). Because extracellular signals and cellular receptors that are important for the survival and apoptosis of different cell types are extremely diverse, redundant but overlapping genes in the apoptotic pathway have evolved, and different subsets of these apoptosis regulatory genes are responsible for the decision of cell fate in a tissue-specific manner.

In this review, present understandings on the role of Bcl-2 proteins in the apoptosis decision step are summarized using ovarian follicle apoptosis regulation as an experimental paradigm. Several novel Bcl-2 protein partners were isolated from the ovary [Bcl-2-related ovarian killer (Bok) and Bcl-2-related ovarian death gene (BOD)] using a yeast two-hybrid protein-protein interaction assay (85–87, 90). The function of these genes is discussed to provide a framework for the understanding of the hormonal regulation of apoptosis in ovarian and other systems. In addition, unique structure-functional attributes of three subgroups of Bcl-2-related proteins (antiapoptotic channel forming, proapoptotic channel forming, and proapoptotic ligand) and their role in apoptosis is elucidated.

II. CED-9/BCL-2 PROTEINS ARE PRESENT IN THE DECISION STEP OF APOPTOSIS: LESSONS LEARNED FROM THE NEMATODE

The genetic analysis of programmed cell death during Caenorhabditis elegans embryogenesis has contributed significantly to the understanding of molecular mechanisms underlying apoptosis (73, 83). In combination with biochemical studies using mammalian cells, it is now clear that programmed cell death in diverse animals is controlled by evolutionarily conserved cellular machinery. Programmed cell death in C. elegans shares morphological features of apoptosis commonly observed in both vertebrates and invertebrates. Of the 1,000 somatic cells formed during development of the nematode, 131 of them undergo programmed cell death that is under stringent genetic control (73, 83).

<table>
<thead>
<tr>
<th>TABLE 1. Conditions associated with abnormal apoptosis</th>
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<tr>
<td>Autoimmune diseases</td>
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<td>Neurodegenerative disorders</td>
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<td>Environmental toxins</td>
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<td>Radiation</td>
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Genetic analyses have demonstrated that about a dozen genes are essential for the regulation of programmed cell death in *C. elegans* (79). In addition to genes responsible for morphological changes and phagocytosis (55, 253, 254), four genes have been shown to be important in the “decision” and “execution” steps of apoptosis (Fig. 1). Among these four genes, *ced-9* encodes a protein that inhibits the cell death program (78, 79) (Fig. 1). Mutations that abnormally activate *ced-9* can prevent cell death that occurs during normal *C. elegans* development. Conversely, when *ced-9* is inactivated, most of the cells that would normally live now undergo programmed cell death, and the animal dies early in development. Of interest, *ced-9* can function properly only if both *ced-3* and *ced-4* are functionally intact, suggesting that *ced-9* functions upstream of the *ced-3* and *ced-4* genes and might prevent cell death by keeping the *ced-3/ced-4*-dependent death program in check in normal cells (78). The mammalian Bcl-2 family proteins are homologous to the *ced-9* gene, and overexpression of Bcl-2 blocks apoptosis in nematode, insect, and mammalian cells (6, 79, 240) (Table 2).

Unlike the antiapoptotic role of *ced-9*, the two downstream genes, *ced-3* and *ced-4*, are necessary for apoptosis. Mutation of these genes prevents cell death that normally occurs during embryogenesis, leading to the development of worms with excessive cells (60, 77, 262, 265). The *ced-3* gene encodes a cysteine protease homologous to proteins of the caspase family in mammals (262). These cysteine proteases are important for the execution of apoptosis through proteolytic catalysis of cellular components; unrestricted expression of these proteases leads to apoptosis in a variety of cells.

The nematode *ced-4* gene encodes a hydrophilic polypeptide containing a P-loop nucleotide (ATP) binding motif. It presumably functions by forming complexes with the single polypeptide zymogen form of *ced-3*, thus promoting its activation by autoprocessing. In living cells, the *ced-4/ced-3* complexes also include the antiapoptotic *ced-9* protein which blocks death by suppressing the *ced-4* from activating *ced-3* caspase. In mammalian cells, the *ced-4* homolog apoptosis activating factor (Apaf)-1 (265), is essential for caspase activation and forms complexes with specific initiator caspases upstream of the apoptosis caspase cascade (93, 178, 217). Because *ced-4/-Apaf-1* physically forms complexes with both *ced-9/Bcl-2* proteins and *ced-3/caspases* in mammalian cells, it has been hypothesized that *ced-4/-Apaf-1* can bridge between the *ced-9/Bcl-2* family members in the apoptosis decision step and caspases in the execution phase. Although mammalian Apaf-1 shares significant sequence homology with *ced-4*, it contains additional functional motifs including the NH2-terminal CARD caspase recruitment domain (CARD) domain and the COOH-terminal WD-40 repeats.

![Fig. 1. Conserved regulation of apoptosis from nematodes to human. On the basis of genetic analyses, four genes have been shown to function at different points of the apoptotic pathway in *Caenorhabditis elegans* during embryogenesis. These genes are indispensable for the normal progression of apoptosis during early development, and they can be categorized into 3 different groups encoding apoptosis decision factors (antiapoptotic *ced-9* and proapoptotic *egl-1*), a bridging factor (*ced-4*), and apoptosis execution proteolytic enzyme (*ced-3*). The *ced-9* gene has evolved into a large group of anti- and proapoptotic channel-forming proteins (represented by Bcl-2 here) in the mammals, whereas the proapoptotic *egl-1* is related to the mammalian proapoptotic ligands with BHI domain only (represented by BAD). The mammalian *ced-4* gene counterpart is Apaf-1, whereas the *ced-3* gene has evolved into a large group of cysteine proteases known as caspases that are essential for the degradation of cellular structures. The antagonistic relationship between Ced9/Bcl-2 and EGL-1/BAD proteins are reflected by broken arrows.](http://physrev.physiology.org/)

<table>
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<th>TABLE 2. The expanding Bcl-2 family of apoptosis-regulating proteins</th>
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<td><strong>Antiapoptotic channel members</strong></td>
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<tr>
<td><em>Ced-9, Bcl-2, Bcl-x, Bcl-w, McI-1, BH-U1, NR-13</em></td>
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<tr>
<td>BHRF1 (Epstein-Barr virus), LMW5-HL (African swine fever virus),</td>
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<td>ORF16 (herpes virus saimiri), KSbcl-2 (Kaposi sarcoma-associated</td>
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<tr>
<td><strong>Proapoptotic channel members</strong></td>
</tr>
<tr>
<td>BAX, Bak, Bok/Mtd, Diva/Boo</td>
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<td><strong>Proapoptotic ligands with BHI domain only</strong></td>
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<td>EGL-1, BAD, Bik/Nbk/Bik, BOD/Bim, BD, Harakiri/DP5, NIP3, NIP3L/</td>
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suggesting that the mammalian Apaf-1 could regulate apoptosis using newly evolved mechanisms. Recent studies suggested that the CARD domain and WD-40 repeats are important for interactions with specific caspases and the Apaf-1 cofactor cytochrome c, respectively (98, 178, 217).

Another nematode gene egl-1 encodes a proapoptotic protein sharing a conserved BH3 (Bcl-2 homology) domain originally characterized in mammalian Bcl-2 family proteins but without other common functional motifs (41, 48). EGL-1 activates apoptosis by binding to and directly suppressing the activity of ced-9, and loss-of-function mutations of egl-1 in worms prevent cell death. It is likely that the binding of EGL-1 to ced-9 releases the cell death activator ced-4 from the constraint imposed by ced-9, thus allowing ced-4 to activate ced-3 and apoptosis. A subgroup of mammalian Bcl-2-related proapoptotic proteins, including BAD, Bim/BOD, Bik/Nbk/Nlk, BID, Nip3, Nip3L/Nix, and Harakiri/DP5, is homologous to the nematode egl-1 gene in having only the BH3 domain (25, 26, 76, 90, 97, 98, 115, 171, 245, 263) (Table 2). These proteins are likely to have the same evolutionary origin and functional characteristics as the nematode egl-1 gene. Structural and functional analyses have revealed that these proapoptotic proteins are ligands to antiapoptotic Bcl-2 proteins and require the conserved BH3 domain for apoptosis induction.

In addition to the decision and execution steps of apoptosis, removal of dying cells is also an important process to maintain tissue homeostasis. The corpses of dead cells in developing embryos of the nematode are quickly engulfed by neighboring cells and degraded. A group of genes, which includes ced-1, -2, -5, -6, -7, -8, and -10, has been found to be important for the efficient removal of corpses of dying cells (54, 55, 60). Although the identity of these genes has not been fully characterized, ced-5 and its mammalian homolog DOCK180 are essential for cell surface extension of engulfing cells (254), whereas ced-7 and its homolog ABC transporters probably translocate molecules that mediate homotypic cell surface adhesion between the dying and engulfing cells (253).

It is clear that a number of evolutionarily conserved genes could regulate a common cell death pathway in different organisms. Although genetic studies in nematode provided valuable insight into the basic mechanism of apoptosis conserved in diverse species, recent biochemical studies indicated that the increasing complexity in cellular differentiation of higher organisms has led to the development of additional regulatory mechanisms in the apoptosis program. It appears that Bcl-2 proteins, in addition to forming dimers with each other and ced-4/ Apaf-1, may also form channels in the mitochondria outer membrane to regulate the release of cytochrome c, an important Apaf-1 cofactor for the activation of specific caspases in the “apoptosome” which comprises of homologs of ced-3, ced-4 and ced-9 as well as other characterized factors (69, 109, 146, 152, 190, 191, 204, 222).

III. APOPTOSIS REGULATION BY THREE SUBGROUPS OF MAMMALIAN BCL-2 PROTEINS

The nematode ced-9 gene and its mammalian homolog bcl-2 protect cells from apoptosis. The bcl-2 (B-cell leukemia/lymphoma 2) gene was first isolated as a protooncogene at the breakpoint of a (t(14,18) chromosomal translocation associated with follicular B-cell lymphoma (40, 234). This translocation leads to overexpression of Bcl-2 in lymphoid cells due to an aberrant regulation of the Bcl-2 gene under the control of the immunoglobulin heavy chain gene promoter/enhancer. Because of the gained survival advantage provided by Bcl-2, lymphoid cells become susceptible to the accumulation of additional genetic alterations (e.g., c-Myc mutation), thus leading to tumorigenesis (42, 221).

The Bcl-2 protein is localized to the mitochondria, perinuclear membrane, and smooth endoplasmic reticulum (29, 80). When overexpressed, the Bcl-2 protein suppresses apoptosis induced by a variety of agents both in vitro and in vivo (5, 7, 169, 170). In transgenic mice, overexpression of Bcl-2 in the lymphoid system results in increased immunoglobulin-secreting cells, elevated serum immunoglobulins, exaggerated antibody response to immunization, development of spontaneous systemic autoimmune disease, and an increased incidence of malignant B-cell lymphoma in aging transgenic animals (42, 155, 213, 221).

However, the apoptosis-suppressing action of Bcl-2 is not universal, and this protein cannot prevent apoptosis in many occasions (45, 237). For example, cell death incurred by withdrawal of trophic factors such as interleukin-2, interleukin-6 (170), or ciliary neurotrophic factor from dependent cells (5) is not altered by Bcl-2 overexpression. In transgenic mice overexpressing Bcl-2 in the lymphoid system, apoptosis induced by superantigens in immature thymocytes is also not suppressed (114, 209, 239). Likewise, mice deficient in Bcl-2 do not show an overt abnormality during development, and only cells in hair follicles, kidney, and lymphoid systems show defects in apoptosis (216, 241). Thus the suppression of apoptosis by Bcl-2 is restricted to selected apoptotic stimuli in restricted tissues.

With the use of homologous gene isolation, protein-protein interaction screen, subtraction cloning, and viral gene analysis, an expanding family of Bcl-2-related proteins has been identified in mammals. On the basis of structural and functional attributes, Bcl-2 proteins can be divided into three subgroups (Fig. 2): 1) the antiapoptotic channel-forming Bcl-2 proteins with four BH domains (BH1 to -4) and a transmembrane anchor sequence, 2)
proapoptotic channel-forming proteins with all but the BH4 domain, and 3) the proapoptotic ligands containing only the BH3 domain (2, 69, 191). The first two subgroups of proteins are believed to be anchored on the mitochondria membrane, whereas the third subgroup of proteins acts as ligands that dimerize with the membrane anchored, channel-forming Bcl-2 “receptors” (116, 158). The BH3 domains in the third subgroup are essential for the binding activity of these ligands.

The mitochondria-anchored antiapoptotic Bcl-2 proteins, represented by ced-9 and Bcl-2, likely form ion channels capable of maintaining membrane homeostasis and preventing cytochrome c release, thus enhancing cell survival. These proteins also interact with ced-4/Apaf-1 to prevent their activation of associated caspases, thus leading to the suppression of the caspase cascade and apoptosis. In addition to ced-9 and Bcl-2, several other Bcl-2 proteins, including Bcl-xL, Bcl-w, Mcl-1, and Bfl-1, have similar antiapoptotic activity and functional motif arrangement but with a unique and overlapping tissue distribution pattern. Bcl-xL and Bcl-w were identified by homologous screens (19, 68), whereas Mcl-1 (myeloid cell lymphoma-1) and Bfl-1/A1 were identified by subtraction cloning (35, 36, 56, 138, 194). Viral infection can trigger host cell apoptosis to limit viral propagation, and some viruses have developed mechanisms to prevent host cell demise. Of interest, several viral genes, the adenovirus E1B 19K (251), the Epstein-Barr virus BHRF-1 (106, 120, 225), the African swine fever virus LMW5-HL (166), ORF16 (165), and KsbcI-2 (28), encode proteins that are structurally and functionally homologous to the mammalian antiapoptotic Bcl-2 proteins. Suppression of apoptosis by these viral proteins extends the life span of host cells and increases the efficiency of viral replication.

Unlike the channel-forming antiapoptotic Bcl-2 members, Bcl-2 family proteins in the second subgroup (Bax, Bak, and Bok) not only antagonize the survival action of antiapoptotic Bcl-2 proteins but also actively trigger apoptosis in transfected cells (33, 34, 63, 86, 121, 173). This subgroup of Bcl-2 proteins has BH1, -2, and -3 domains and the membrane-anchoring region but is missing the NH2-terminal BH4 domain important for apoptosis inhibition (94). They can dimerize with antiapoptotic Bcl-2 proteins, thus relieving the ced-4/Apaf-1 from the suppression exerted by antiapoptotic Bcl-2 proteins and promoting caspase activation (178, 217). These mitochondria-anchored proapoptotic Bcl-2 proteins could also promote apoptosis by altering mitochondria membrane homeostasis and increasing cytochrome c release (70, 109, 137, 217, 236).

The third subgroup of Bcl-2 proteins, homologous to the recently identified nematode EGL-1 protein, consists of proapoptotic ligands with only the BH3 domain. These proteins are capable of interacting with selective channel-forming Bcl-2 proteins to promote cell death and may serve as adaptor proteins linking upstream signaling pathways to the decision step of the apoptosis program. Several Bcl-2 proteins in this subgroup have been identified based on protein-protein interaction screens using the yeast two-hybrid system or bacterial expression cloning. Like the nematode EGL-1, several of the proapoptotic ligands (BAD, BOD/Bim, and BID) can be structurally and functionally homologous to the mammalian antiapoptotic Bcl-2 proteins. Suppression of apoptosis by these viral proteins extends the life span of host cells and increases the efficiency of viral replication.

It is now becoming clear that Bcl-2 and related proteins are multifunctional proteins, and protein-protein interactions play an important role in their regulation of apoptosis. One of the mechanisms by which the Bcl-2 proteins regulate apoptosis is through homodimerization and heterodimerization with proteins in the same family. The BH3 domain in the proapoptotic Bcl-2 proteins serves...
as the ligand for binding to the receptor domain (encompassing the BH3, BH1, and BH2 domains) in antiapoptotic members (162, 203). As shown in Figure 3, the prototypic antiapoptotic Bcl-2 protein contains a unique BH4 domain, believed to be important for interaction with Apaf-1, thus preventing caspase activation. In addition to the COOH-terminal transmembrane region essential for anchoring to the mitochondria, endoplasmic reticulum, or nuclear membrane, the α-helices 5 and 6 encompassing the BH1 and BH2 regions are important for channel formation in the regulation of cytochrome c release by mitochondria (109, 152, 203, 204). Although no specific molecules have been identified to interact with these channel domains of Bcl-2 proteins, recent studies have shown that channel-forming Bcl-2 family proteins could interact with multiple mitochondrial proteins to regulate cytochrome c release through permeability transition pores of mitochondria (146, 147, 164, 210).

The multiple Bcl-2-related proteins found in vertebrates interact in a partner-specific manner and play tissue- and pathway-specific roles in apoptosis regulation (86, 99, 100, 208, 214). The expression pattern of Bcl-2 homologs in different tissues appears to be distinct but overlapping; some are widely distributed, whereas others are more restricted. Despite the wide distribution of Bcl-2 proteins in a variety of tissues, studies on mutant mice deficient in different members of the bcl-2 gene family reinforce the notion that Bcl-2 homologs regulate apoptosis in a tissue-specific manner. In Bcl-2-deficient mice, most tissues develop normally, and only cells in hair follicles, kidney, small intestine, neurons, and lymphoid system show defects in apoptosis (112, 216, 227, 241). In contrast, targeted disruption of Bcl-X in mutant mice leads to lethality around embryonic day 13 (161). Histological studies revealed excessive cell deaths in developing neurons and lymphoid cells of mutant embryos, suggesting that Bcl-X supports the viability of cells in the nervous and hematopoietic systems during fetal life. Likewise, mice deficient in Bax appear healthy, and only cells of lymphoid and gonadal lineage show aberrations in cell death. In these mice, hyperplasia in T and B cells results in enlarged thymus and spleen, whereas the accumulation of premeiotic germ cells in seminiferous tubules leads to male infertility (125). Furthermore, mice that lack the widely distributed Bcl-w gene are viable and healthy except that the males are infertile (182, 198). The testis develop normally, and the initial prepubertal wave of spermatogenesis is largely unaffected; however, the seminiferous tubules are disorganized because of enhanced apoptosis during adulthood. Likewise, mice lacking Bfl-1 are normal and indistinguishable from their wild-type littermates. However, hair loss on the head and face are obvious by 8–12 wk of age in these Bfl-1-deficient mice (74). Although the tissue-specific phenotypes detected in these transgenic mice could be due to differences in the apoptosis regulatory mechanisms in diverse cells, the findings clearly show that the ced-9 homologs in mammalian cells not only have diverged in their functional characteristics but also have adopted unique roles in specific cell lineage.

IV. OVARIAN FOLLICLE ATRESIA AS A MODEL OF APOPTOSIS: CONSTRUCTION OF AN OVARY-SPECIFIC PROTEIN-PROTEIN INTERACTION MAP IN APOPTOSIS REGULATION

Although it appears that the intracellular regulators of cell death are conserved during evolution, the death of
a cell is usually under the control of multiple extracellular factors, and a balance of survival and apoptotic signals determines a cell’s fate. The multifactorial mode of apoptosis regulation is exemplified by the hormonal control of follicle demise in the mammalian ovary. In the ovary, apoptosis occurs mainly at the early antral follicle stage, and >99% of ovarian follicles undergo atresia during reproductive life (84, 91, 156). Morphological and biochemical studies have demonstrated that the death of both somatic and germ cells in the ovary is mediated by apoptosis (16, 91, 96, 179, 232). Ovarian follicle cell apoptosis is regulated by multiple hormones including pituitary glycoprotein hormones as well as local growth factors, cytokines, a diffusible gas (nitric oxide), and steroids (16, 91). Of interest, many of these factors are important both for promoting cell proliferation and for maintaining cell viability. In contrast, Fas ligand, tumor necrosis factor (TNF)-α, interleukin-6, and gonadotropin-releasing hormone-like peptides act as intraovarian apoptotic factors through specific apoptotic pathways (17, 110, 184, 185, 201, 259). Furthermore, in vivo studies have implicated estrogens as antiapoptotic hormones in ovarian follicles, whereas androgens are proapoptotic (15, 16). It has been clearly demonstrated that the majority of follicles undergo apoptosis when they reach the early antral (in rodents) or antral (in human) stages unless rescued by gonadotropins (mainly follicle-stimulating hormone) (15, 37–39). However, the exact mechanisms underlying this programmed follicle demise and role of granulosa and thecal cells are not clear. Future studies on the potential role of various intraovarian apoptotic factors during follicle development are of interest.

The diverse hormones function through endocrine, paracrine, or autocrine mechanisms. Pituitary gonadotropins appear to be the most important survival factors for ovarian follicle cells because gonadotropin treatment increases the expression of local survival factors in ovarian follicles. Specifically, the gonadotropins suppress ovarian cell apoptosis by activating the cAMP-dependent pathway and increasing the production of paracrine and autocrine factors such as estrogens, interleukin-1, nitric oxide, and insulin-like growth factor I (IGF-I) (Fig. 4). These factors in turn promote cell survival through the activation of nuclear estrogen receptor, the cGMP-dependent pathway, and protein tyrosine phosphorylation, respectively (91). Because the progression of apoptosis in ovarian follicles is dependent on the cooperative regulation of different paracrine and autocrine factors, it is likely that none of these factors is singularly obligatory in the control of follicle growth or demise. Instead, a balance of these different survival and apoptotic factors may decide whether a follicle will continue development or undergo apoptosis. How the various extracellular hormones and their intracellular signal transduction mediators are linked to the intracellular decision step of apoptosis in ovarian follicles remains, however, largely undefined.

To elucidate the hormonal regulation of ovarian follicle atresia, we first tested whether the Bcl-2 system is important in the regulation of ovarian cell apoptosis in vivo. Transgenic mice overexpressing Bcl-2 in the ovary were generated by using the mouse inhibin-α gene promoter/enhancer to drive the Bcl-2 transgene (88, 89). In these mice, a suppression of follicular cell apoptosis, enhancement of folliculogenesis, and an increased incidence of benign ovarian teratoma development were found, indicating that Bcl-2 associated regulatory system is operating in the ovary. However, endogenous expression of Bcl-2 in follicle cells was not evident based on in situ and immunostaining approaches, suggesting Bcl-2 family proteins other than Bcl-2 are involved in the regulation of ovarian cell apoptosis.

Taking advantage of the heterodimerization property of Bcl-2-related proteins, we constructed an ovarian fusion cDNA library and used Bcl-2 as bait to search for endogenous Bcl-2-interacting preys in the yeast two-hybrid protein-protein interaction screen. After each Bcl-2-
interacting protein obtained from the ovarian library was characterized, new baits were chosen and, in turn, used for second round screens for additional interacting molecules. Repeating this procedure multiple times allowed the identification of several known and novel Bcl-2 family proteins as well as their interacting regulators in the ovary. First, a Bcl-2-interacting protein, BAD, was found as an important proapoptotic ligand in the ovary that bridges the upstream signaling proteins, 14–3–3 and P11, to the channel-forming antiapoptotic Bcl-2 family proteins (87) (Fig. 5). Of interest, 14–3–3 proteins preferentially bind phosphorylated BAD, whereas the use of an underphosphorylated BAD mutant as bait allowed the isolation of P11 and Mcl-1 as BAD-interacting proteins (130a). It appears that the antiapoptotic Mcl-1 is a mammalian ced-9 homolog in the ovary which, in turn, dimerizes with two novel ovarian proapoptotic Bcl-2 members, Bok and BOD, as well as the known proapoptotic Bax in the decision step of apoptosis (86, 90). The antiapoptotic protein Mcl-1 may prevent apoptosis by interacting with unknown downstream ovarian ced-4/Apaf-1 homolog capable of activating caspases in the execution step of apoptosis. The antiapoptotic activity of Mcl-1 could also be modulated by the proapoptotic family proteins including Bok, BOD, BAD, and Bax. Studies on ovarian Mcl-1 mRNA levels indicated that the expression of this antiapoptotic protein is induced by survival hormones in the ovary (130a).

Because the phosphorylation status of BAD plays an essential role in its proapoptotic activity, gonadotropins and other upstream survival factors likely enhance the phosphorylation of BAD to allow the binding of 14–3–3 proteins, leading to the dampening of BAD-induced cell killing (49, 65, 87, 264). Likewise, some survival factors could induce the synthesis of P11 that also binds to BAD and decreases its proapoptotic action. Thus these findings allowed one to construct a network map of Bcl-2-interacting proteins in the ovary to understand the intracellular mechanisms of ovarian follicle cell apoptosis and its regulation by upstream survival and/or apoptotic factors. Although the exact link between ovarian Bcl-2 proteins and the ovarian ced-4/Apaf-1 proteins remains to be elucidated, recent reports indicated that Apaf-1 is expressed in human and mouse granulosa cells (105, 197), and treatment with gonadotropins suppresses Apaf-1 expression. Furthermore, Diva/Boo, a recently discovered, ovary-specific Bcl-2 protein (100, 214), is capable of binding to Apaf-1. Future studies on the role of Diva/Boo in the ovary could allow the identification of additional ovarian Apaf-1 homolog and the exact ovarian Bcl-2 protein partner for Apaf-1 and Diva/Boo.

It is becoming clear that a given tissue has a set of Bcl-2 proteins, and apoptosis is regulated through selective dimerization of channel-forming antiapoptotic Bcl-2 proteins, channel-forming proapoptotic Bcl-2 proteins, and the BH3-only apoptotic ligands (69, 191). The present approach could serve as an experimental paradigm for the construction of Bcl-2 network maps in diverse tissues. Several useful points have been gained from these studies: 1) the protein-protein interaction network map constructed is tissue specific, 2) novel Bcl-2 and interacting genes in the apoptosis pathway can be identified, and 3) posttranslational modification of proteins such as the phosphorylation of BAD plays an important role in protein dimerization.

Although the two-hybrid system is a powerful tool, it has limitations: 1) Gal-4 fusion proteins used in the yeast cell screen may behave differently from native proteins, 2) observed interactions in yeast may not take place inside mammalian cells due to differential cellular localization or cell type-specific expression, and 3) some fu-
sion proteins encoded by apoptosis-inducing genes may be lethal to yeast cells, thus preventing the identification of such genes. To avoid these potential pitfalls, it is important to perform the following steps to further characterize each candidate gene: 1) confirmation of direct protein-protein interactions by coprecipitation using endogenous or recombinant proteins, 2) expression of protein pairs in mammalian cells to identify their putative roles in apoptosis, 3) examination of temporal and spatial expression pattern of candidate genes in the tissues of interest, 4) mutations of putative phosphorylation sites to control the degree of protein phosphorylation in yeast cells, and 5) the use of selective domains of genes as bait to avoid a lethal phenotype or potential stereochemical hindrance.

V. PROAPOPTOTIC BAD AND BOD ARE LIGANDS FOR ANTIAPOPTOTIC BCL-2 PROTEINS AND CAPABLE OF TRANSMITTING UPSTREAM SIGNALS

BAD and BOD were isolated from the ovarian fusion cDNA library based on their binding to antiapoptotic Bcl-2 proteins (87, 90, 111). Both BAD and BOD belong to the subgroup of proapoptotic ligands with only the BH3 domain. After overexpression in transfected cells, these ligands induce apoptosis. Because both BAD and BOD lack the transmembrane region important for anchoring to intracellular membranes, they function by serving as cytoplasmic proapoptotic ligands for membrane-bound channel-forming Bcl-2 proteins through heterodimerization. Indeed, coexpression with specific antiapoptotic Bcl-2 proteins abolishes the cell killing activity of BAD or BOD.

BOD, also known as Bim (171), is expressed in multiple tissues. Analysis of protein-protein interactions in the yeast two-hybrid system demonstrated that BOD interacts with diverse antiapoptotic Bcl-2 proteins including Mcl-1, Bcl-2, Bcl-xL, Bcl-w, Bfl-1, and the Epstein-Barr viral-derived BHRF-1 (90). Thus the widely distributed BOD could serve as an apoptosis mediator in diverse cell lineages through dimerization with a variety of antiapoptotic proteins. There are several splicing variants of BOD that vary in their NH2-terminal sequences (BOD-L, BOD-M, and BOD-S) in ovarian tissues; all three forms contain the BH3 domain and promote cell death in transfected cells. Similar to several BH3-only proapoptotic ligands, mutation analysis demonstrated that the BH3 domain of BOD is essential for its heterodimerization with antiapoptotic Bcl-2 members and for its cell-killing ability (76, 98, 115).

Similar to BOD, BAD has been shown to heterodimerize with the antiapoptotic Bcl-2 proteins through its BH3 domain (115, 263). In addition to its role as a ligand for antiapoptotic Bcl-2 proteins, BAD has been found to interact with upstream signaling molecules capable of regulating its proapoptotic activity. With the use of the yeast two-hybrid system to search for BAD-binding proteins in the ovary, multiple isoforms of 14–3–3 were identified. The highly conserved ubiquitous 14–3–3 proteins are expressed in perhaps all eukaryotic organisms including yeast, plants, insects, and mammals with at least seven different isoforms found in mammalian cells (139, 247, 258). Proteins of the 14–3–3 family bind diverse enzymes and signaling molecules including Raf-1 kinase, B-Raf, phosphatidylinositol 3-kinase, CDC25 phosphatases, Bcr, Cbl, and polyoma middle-T antigen (4, 144, 193). They are important adaptors in intracellular signaling, cell cycle control, oncogenesis, and neurotransmitter biosynthesis. Crystallographic studies further indicated that different isoforms of 14–3–3 dimerize to generate a complex with two ligand-binding sites (139, 258), thus allowing the assembly or anchoring of different signaling proteins and cytoskeletal elements.

In transfected cells, overexpression of 14–3–3 suppresses apoptosis induced by BAD, indicating that interactions between them may allow coordination of cell death and other 14–3–3-regulated intracellular signaling pathways (87, 264). The 14–3–3 proteins have been shown to bind to their interacting proteins through a consensus serine phosphorylation sites, and serine phosphorylation of BAD is important for 14–3–3 binding and cell survival. Point mutation of BAD in one (S137A), but not the other (S113A), putative binding site found in diverse 14–3–3 interacting proteins abolished the interaction between BAD and 14–3–3 without affecting interactions between BAD and Bcl-2. In mammalian cells, cotransfection with 14–3–3 suppressed apoptosis induced by wild-type BAD or the S113A BAD mutant, but not by the S137A mutant that was incapable of binding 14–3–3 (87). The 14–3–3 proteins could suppress BAD activity by sequestering the phosphorylated BAD in an inactive state or by bridging the phosphorylated BAD with a putative inhibitor of BAD through the consensus 14–3–3 phosphorylation site.

Because IGF-I and insulin activate the Akt/PKB kinase to phosphorylate BAD, BAD phosphorylation has been suggested to be an important mechanism by which upstream survival factors suppress apoptosis (47, 49, 128). Thus gonadotropin and other survival factors could increase the serine phosphorylation of BAD in ovarian cells to enhance the binding of BAD to the constitutively expressed 14–3–3, thus maintaining BAD in an inactive state. The proapoptotic BAD is functional only after withdrawal of the survival signals followed by the dephosphorylation of BAD (Fig. 5).

Because the S137A BAD mutant presumably resembles an underphosphorylated form of BAD incapable of binding 14–3–3 proteins, this mutant was used to screen for additional BAD-interacting proteins in the ovary.
using the yeast two-hybrid system. P11, a nerve growth factor (NGF)-induced neurite extension factor, was found to be a BAD interacting protein. P11 is also known as 42C or calpain I light chain and belongs to the S100 family of calcium binding proteins (64, 149, 150). It was originally identified as an early response gene following NGF stimulation of a rat pheochromocytoma (PC12) cell line. In addition, P11 was also increased after transformation induced by viral oncopgenes in diverse cell lines (43, 175). Of interest, overexpression of P11 induces neurite outgrowth and enhances PC12 cell survival in the absence of NGF (150).

P11 interacted preferably with the mutant BAD but less effectively with the wild-type protein (87). In transfected cells, coexpression of P11 attenuated the proapoptotic effect of both wild-type BAD and S137A BAD mutant. In addition, direct P11 binding to BAD was also demonstrated in vitro. Like 14–3-3, P11 may also function as a BAD binding protein to dampen the apoptotic activity of BAD. Because the 14–3-3 family of proteins could interact with key signaling proteins, whereas P11 is an early response gene induced by survival factors, extracellular hormonal signals could modulate BAD-induced apoptosis through induction of this BAD-binding protein in addition to regulating the interaction between BAD and 14–3-3 via BAD phosphorylation (87, 264). BAD appears to play an important role in mediating communication from different upstream signal transduction pathways to the Bcl-2-regulated apoptotic decision step. Although how BOD activity is regulated by upstream signaling molecules has not been reported, BOD and other BH3-only proapoptotic ligands could also function as ligands that bridge upstream signals to membrane-bound channel-forming Bcl-2 receptor proteins.

VI. PROAPOPTOTIC BOK AND BAX WITH A CHANNEL-FORMING DOMAIN REGULATE APOPTOSIS BY RELEASING APAF-1 FROM SUPPRESSION BY ANTIAPOPTOTIC PROTEINS AND BY PROMOTING CYTOCHROME C RELEASE

The Bok gene was first identified as a Mcl-1 binding protein in the ovary using the yeast two-hybrid system (86). Unlike other channel-forming proapoptotic Bcl-2 proteins including Bax and Bak, Bok preferentially dimerizes with antiapoptotic proteins Mcl-1, Bfl-1, and BHRF1 but does not interact with Bcl-2, Bcl-w, and Bcl-xL. Although low levels of mRNA for Bok, also named as Mtd, are present in diverse tissues (99), it is expressed at high levels in several reproductive organs, including ovary, testis, and uterus (86). The distinct heterodimerization property and tissue distribution pattern of Bok suggested that it is a tissue-specific mediator of cell death and could promote apoptosis by interacting with selective antiapoptotic protein. This notion is consistent with the hypothesis that apoptosis is regulated through the dimerization of Bcl-2 family proteins in a tissue-specific manner.

Functional analysis indicated that Bok is a proapoptotic protein, and its cell killing ability can be dampened following coexpression with its dimerization partners Mcl-1 and BHRF-1 (86). Bok, together with Bax and Bak, belong to the subgroup of proapoptotic channel-forming Bcl-2 proteins containing BH1, BH2, BH3, and a COOH-terminal transmembrane region (2). During the analysis of Bok mRNA and gene structure, we found a Bok splicing variant in which the region encoded by exon 3 is absent, suggesting the existence of a truncated short form (Bok-S) of the full-length Bok protein (Bok-L) (85). The skipping of exon three encoding 43 amino acids maintained the original reading frame and retained the BH2 and the COOH-terminal membrane anchoring domains; however, parts of the BH3 and BH1 domains were deleted. Of interest, functional analysis indicated that Bok-S is still capable of inducing apoptosis while the truncated Bok has lost its ability to heterodimerize with Mcl-1, BHRF-1, and Bfl-1, suggesting that an intact BH3 domain of Bok is not essential for apoptosis regulation and the short splicing variant induces apoptosis through a heterodimerization-independent mechanism (85).

Although the BH3 domain was originally shown to be essential for the heterodimerization property of different proapoptotic Bcl-2 proteins and for their cell killing property, recent studies indicated that the BH3 domain and associated heterodimerization property are dispensable for apoptosis induction by the channel-forming proapoptotic protein Bax and Bak (108, 191). Likewise, mutation analysis indicated that the BH3 domain in Bok is not essential for apoptosis induction and substitution of conserved residues in the BH3 domain of Bok also abates its ability to dimerize with antiapoptotic proteins. Similar to Bok-S, mutants of related proapoptotic proteins Bax and Bak with their homologous BH3-BH1 region deleted also retained proapoptotic activity, thus suggesting a common mechanism of action is shared by this subgroup of channel-forming proapoptotic proteins.

Although proapoptotic Bcl-2 proteins could function by antagonizing the activity of antiapoptotic proteins mediated by intact BH3 domains, structure-functional characterization indicated that the α5- and α6-helices spanning BH1 and BH2 domains are important for channel formation in the mitochondria and for the regulation of cell death by these proteins. Deletion of the channel-forming domain of Bax completely abolished its proapoptotic activity in yeast while substitution of the Bcl-2 channel-forming domain with Bax sequences converts it into a proapoptotic protein (152). Of interest, truncation of the region between BH3 and BH1 from Bok-L does not affect the homologous α5- and α6-regions proposed to be impor-
tant for channel formation of Bax (191). In addition, the hydropathicity property between the 5′-end of the BH1 region and the COOH-terminal transmembrane domain is not altered by the truncation found in the 5′-end of the channel formation region of Bok-S (85).

It is likely that the α5- and α6-regions found in Bok-S comprise a module sufficient for channel formation to promote apoptosis, thus mediating apoptosis through a heterodimerization-independent pathway (210). Findings that Bok-L and Bok-S could also promote apoptosis independent of heterodimerization further suggest that this subgroup of proapoptotic proteins could induce apoptosis through channel formation in addition to their role as “ligands” for antiapoptotic Bcl-2 proteins. Thus Bok-S represents a new form of proapoptotic protein consisting of only minimal functional modules and manifests its proapoptotic action without direct interactions with antiapoptotic proteins. Bok-S expression in the ovary and uterus could provide a short circuit to promote cell demise in hormone-dependent cell populations that express abundant antiapoptotic proteins (such as Mcl-1), thus allowing swift removal of superfluous cells during reproductive cycles.

At least three mechanisms could be proposed for the action of proapoptotic Bcl-2 proteins (Fig. 6): 1) the subgroup of proapoptotic Bcl-2 proteins with only the BH3 domain (e.g., the BAD and BOD proteins) heterodimerizes with membrane-bound antiapoptotic proteins to regulate apoptosis; 2) the subgroup of channel-forming proapoptotic Bcl-2 proteins with BH1, BH2, BH3, and TM domains, represented by Bok-L, heterodimerizes with antiapoptotic proteins (Mcl-1/Bfl-1) or functions as mitochondrial channels to regulate apoptosis; and 3) the unique Bok-S variant does not dimerize with antiapoptotic proteins but probably forms mitochondrial channels to regulate apoptosis. Although multiple reports suggested a role for several Bcl-2 proteins (e.g., Bax, Bak, Bok) in the formation of mitochondria channels, it is important to note that no direct in vivo evidence for such channel is presently available, and additional studies are needed to confirm this hypothesis.

VII. CELL DEATH EXECUTION BY CASPASES: CONVERGING PATHWAYS FOR EXTRACELLULAR AND INTRACELLULAR SIGNALS

It is becoming apparent that mitochondrial changes play a central role in apoptosis regulation in a three-step model of apoptosis: 1) a premitochondrial decision step during which upstream signal transduction pathways are activated to regulate Bcl-2 proteins; 2) a mitochondrial phase, during which mitochondrial homeostasis is lost resulting in the release of proteins that activate caspases as well as the disruption of mitochondria electron transport, oxidative phosphorylation, and ATP production; and 3) a postmitochondrial phase, during which proteins released from mitochondria cause the activation of Apaf-1 and initiator caspases followed by sequential activation of a cascade of effector caspase zymogens.

In the mitochondria phase, the channel-forming Bcl-2 family proteins could alter mitochondrial inner transmembrane potential through the opening of a large-conductance channel known as the mitochondrial permeability transition (PT) pore (146, 147). Pharmacological
studies indicated that antiapoptotic Bcl-2 proteins prevent PT pore opening whereas proapoptotic ones induce it, perhaps through direct interaction with inner membrane proteins such as the adenine nucleotide translocator. In the postmitochondria execution phase of apoptosis, the array of activated caspases amplifies the apoptotic process through proteolytic degradation of vital cellular components as well as the activation of additional catabolic enzymes such as the nucleases (DFF40/CAD) involved in internucleosomal DNA fragmentation (140, 141).

The regulation of mitochondrial homeostasis by channel-forming anti- and proapoptotic Bcl-2 proteins controls the release of cytochrome c (and other uncharacterized mitochondrial apoptosis-inducing factors) from the outer surface of the inner mitochondrial membrane to the cytoplasm. The liberated cytochrome c becomes associated with Apaf-1 and procaspase-9 to form the active apoptosome complex (93, 137, 178, 217) (Fig. 7). Caspase-9 and Apaf-1 bind to each other via their respective NH2-terminal CARD domain in the presence of cytochrome c and dATP, an event that leads to the conversion of the latent procaspase-9 to its active form (81). Activated caspase-9, in turn, cleaves and activates downstream caspases (e.g., caspase-3). Caspases and mitochondria can engage in a circular self-amplification loop to accelerate the apoptotic process. An increase in mitochondrial membrane permeability would cause the release of caspase activators, and caspases, once activated, could in turn increase the mitochondrial membrane permeability through conversion of antiapoptotic Bcl-2 family proteins to proapoptotic ones (27, 260) and the activation of proapoptotic ligands (71, 133, 142).

At least 14 mammalian homologs of the ced-3 caspase gene have been isolated (3, 92, 168, 231). These cysteine proteases require an Asp residue at the P1 site for cleavage (69, 231). In addition, the pentameric peptide signature, QAC(R/Q/G)/G, surrounding the putative active site cysteine, is highly conserved in all caspase family proteins. Unlike most other posttranslational modifications, the caspase cascade is irreversible. Once the caspases in the execution step are activated, the apoptotic process normally proceeds to completion. Most caspases are synthesized as precursors, and their activities are tightly regulated at the activation step and/or through interaction with specific inhibitors. Although the role of cytochrome c in the activation of caspases is gaining support, the potential role of different ions (such as K+ and Na+) in this process should also be considered (20, 95).

Active caspases are formed by cleavage of a proprotein precursor at internal aspartate residues (231). These residues divide the proprotein into NH2-terminal, middle, and COOH-terminal domains. After proprotein processing, the middle and COOH-terminal domains associate to become an active protease. The NH2-terminal domain, which is highly variable in sequence and length, is involved in the regulation of proprotein activation. After the initial activation, two units of the middle and COOH-terminal domains associate to form an active tetramer complex.

Based on their positions in the apoptosis protease cascade, caspases can be divided into two subgroups. The “initiator” caspases, such as caspase-6, -8, and -9, initiate the disassembly process directed by upstream apoptotic signals. The downstream “effector” caspases, such as caspase-3 and -7, are activated by initiators and function in the subsequent degradation of cellular components, thereby precipitating morphological and biochemical

![FIG. 7. Formation of the active complex of apoptosome to initiate the caspase cascade. The execution step of apoptosis depends on the formation of an active apoptosome complex consisting of Apaf-1, cytochrome c released by mitochondria, and procaspase-9. Once the procaspase-9 is cleaved in the active apoptosome complex, the active enzyme, in turn, activates downstream caspases such as caspase-3, leading to the degradation of multiple substrates important for the integrity of the cellular organelles. When Apaf-1 is bound by antiapoptotic Bcl-2 proteins, they are present as an inactive complex, and Apaf-1 is not capable of activating caspases. The suppressing action of antiapoptotic Bcl-2 "receptors" on caspase activation can be relieved after binding of the proapoptotic ligands (BAD and BOD). In addition, the levels of cytochrome c in the apoptosome also determine the activation of caspase.](http://physrev.physiology.org/)
changes characteristic of apoptosis (69). Although effector caspases are perhaps activated in every apoptotic cell, only selective initiator caspases are activated by specific signaling pathways in a given dying cell after binding to pathway-specific adaptor proteins.

It appears that death caspases are present in all cells, ready to be activated by upstream apoptotic signals. The interaction between initiator caspases and upstream signaling molecules is mediated through their NH2-terminal regulatory sequences such as death effector domain (DED) and CARD domains (13, 81, 131, 211, 219). The NH2-terminal CARD domain of procaspase-9 is important for its association with Apaf-1 in the apoptosome complex and for its subsequent conversion to the active enzyme (Fig. 8). Similarly, the NH2-terminal DED domain of the caspase-8 proprotein binds to DED domain-containing adaptor proteins, which, in turn, interact with upstream death domain-containing receptors including Fas antigen, TNF receptor I, DR3, DR4, DR5, and DR6 (32, 163, 176, 207, 212). Activation of these receptors after ligand occupancy leads to the formation of a death-inducing signaling complex (DISC) containing oligomers of receptors, receptor-associated DED domain-containing adapters (such as TRADD and FADD), and procaspase-8, followed by the autoactivation of caspase-8 (123, 157). These diverse structure-functional characteristics of procaspases suggest that caspases play tissue-, signal-, and pathway-specific roles.

The effector caspases cleave cellular proteins to disable critical homeostatic and repair processes essential for cell survival; they also disassemble key structural components including cytoskeleton and nuclear DNA (69). The DNA repair enzyme poly(ADP)-ribose polymerase (PARP) is one of the first cellular substrates shown to be cleaved by caspase-3 during apoptosis (46, 72, 129, 167). Subsequently, fragmentation leads to the formation of a death-inducing signaling complex (DISC) containing oligomers of receptors, receptor-associated DED domain-containing adapters (such as TRADD and FADD), and procaspase-8, followed by the autoactivation of caspase-8 (123, 157). These diverse structure-functional characteristics of procaspases suggest that caspases play tissue-, signal-, and pathway-specific roles.

In addition to cleaving proteins essential for cell integrity, caspases also act on upstream Bcl-2 proteins to further amplify the progression of apoptosis. Of interest, the NH2-terminal BH4 domain of the antiapoptotic Bcl-xL proteins can be cleaved by caspase-3, and the liberated COOH-terminal fragment of Bcl-2 lacking the BH4 domain potently induces apoptosis (27, 260). Thus cleavage of Bcl-xL during the execution phase of cell death converts Bcl-xL from a protective to a lethal protein. Likewise, the cleavage of proapoptotic ligand BID by caspase-8 allows the translocation of BID from cytoplasm to the mitochondria and further amplification of the apoptotic signals through destruction of mitochondria homeostasis (71, 161, 236, 253).

![Diagram](Image 54x57 to 390x231)

**FIG. 8.** Converging pathways of apoptosis are shown. Apoptosis can be initiated by diverse mechanisms. The withdrawal of tissue-specific survival factors could regulate anti- and proapoptotic Bcl-2 proteins and trigger caspase activation by dissociating Apaf-1 from antiapoptotic Bcl-2 proteins and by increasing the release of cytochrome c from the mitochondria. As the result of genome instability, intracellular death signals could increase the levels of p53, which, in turn, enhance apoptosis by increasing the expression of proapoptotic Bcl-2 proteins (such as Bax), leading to caspase activation. In addition, extracellular death ligands could bind to death receptors and activate death effector proteins through death domain adapters, also leading to caspase activation and apoptosis.
Furthermore, caspase activity has been directly linked to the formation of ladderlike DNA fragments in apoptotic cells. During apoptosis, the cleavage of an inhibitor of nuclease (DDF45/CAD) relieved the inhibition of nuclease DFF40/caspase-activated deoxyribonuclease (CAD), thus allowing DFF40/CAD to induce nuclear DNA fragmentation (101, 140, 141, 159, 228).

Two additional families of proteins important for the regulation of caspase activity have been identified. One group, including XIAP, cIAP1, cIAP2, x-IAP, and survivin, acts as substrate inhibitors of caspases to block the activation of different effector caspases (caspase-3 and caspase-7) (50–52, 57, 58). Overexpression of these inhibitor proteins leads to the suppression of apoptosis triggered by diverse proapoptotic signals in both mammalian and insect cells. The other group of caspase regulators includes FLICE inhibitory protein (FLIP) and death effector protein in testis (DEFT) (13, 102, 103, 113, 131, 218, 219, 229, 233). These proteins regulate apoptosis through direct interaction with initiator caspases such as procaspase-8 and procaspase-10 through their death effector domains (103, 168, 212). Caspase-9 has also been shown to be regulated by mitogen-regulated PKB/Akt kinase through phosphorylation (22). Because the phosphorylated form of caspase-9 is catalytically inactive, these findings suggest that upstream survival factors could promote cell survival through direct phosphorylation and inactivation of caspases. It is now becoming clear that the intricate mechanisms involved in caspase regulation are not only important to ensure against accidental activation of these death enzymes but also for coordinating signals triggered by diverse extracellular survival and apoptotic factors. Although the role of these proteins in gonads has not been specifically investigated, DEFT, FLIP, caspase-9, and inhibitor of apoptosis proteins (IAP) are expressed at high levels in gonads (50, 52, 107, 131, 199, 226, 229, 233), suggesting that these caspase regulators could play important roles in the regulation ovarian cell death as well. Of interest, recent studies showed that apoptosis of granulosa cells from preantral and early antral follicles is associated with reduced protein levels of IAP2 and xIAP while gonadotropin treatment increases IAP2 and xIAP content (134).

Mutant mice with deletion of caspase genes have been generated. Mutation of caspase-9 results in embryonic lethality and defective brain development (126). In addition, fibroblasts isolated from caspase-9 null mice showed defects in cytochrome c-mediated cleavage of procaspase-3. Likewise, mutation of caspase-3 causes perinatal death and all mutant mice die at 1–3 wk of age (127). The major morphological changes were found in the brain and include a variety of hyperplasia and disorganized cell deployment. Targeted disruption of caspase-8 also leads to embryonic death, and the caspase-8 null embryos exhibit defects in heart muscle development (238).

In contrast, mice deficient in caspase-1, caspase-2, or caspase-11 develop normally and show limited functional impairments in specific organs (12, 21, 135, 136, 246). In caspase-2 null mice, the major apoptosis defect was found in the developing motor neurons (12). In caspase-1 and caspase-11 null mice, the production of interleukin-1α and -1β was impaired; however, no aberrant apoptosis could be observed (21, 135, 136, 246). These findings are consistent with the hypothesis that apoptosis in most tissues is regulated by a coordinated array of tissue-specific factors at the decision as well as execution phases of apoptosis. Although the important role of the caspase cascade in the execution phase of apoptosis is clearly documented, it is important to note that there are multiple examples of apoptosis induction without the apparent involvement of known caspases (1, 30, 183).

After an explosion of literature on apoptosis regulation in recent years, converging pathways of apoptosis regulation have become apparent (Fig. 8). In addition to the regulation of apoptosis by extracellular survival signals mediated through anti- and proapoptotic Bcl-2 proteins, intracellular death signals including genomic instability and the excessive accumulation of free oxygen radicals could also trigger apoptosis. The tumor suppressor protein p53 is a “gatekeeper” of genome integrity (124, 153, 190) and increases in p53 levels as the result of cell cycle imbalance or DNA damage induce the synthesis of Bax, leading to apoptosis (160, 192). In addition, mitochondrial dysfunction as the result of the collapse of the mitochondrial inner transmembrane potential, uncoupling of the respiratory chain, hyperproduction of superoxide anions and outflow of matrix calcium and glutathione could also lead to cytochrome c release and activation of the apoptosis cascade (190, 192). These direct connections between genomic instability, cellular aberration, and different steps of apoptosis could have evolved to ensure the proper removal of cells that are damaged or harmful to the whole organism.

In addition to extracellular survival factors, apoptosis in many cells is also regulated by extracellular death factors. These death factors are members of the TNF family of cytokines and include Fas ligand (also known as Apo-1/CD95 ligand), TNF-α, TRAIL/Apo-2 ligand, and TWEAK. These membrane-bound or soluble proteins induce apoptosis in target cells by binding to specific members of the TNF receptor superfamily called death receptors (31, 53, 82, 143, 177, 205, 206, 212). The stimulation of death receptors by their respective ligands induces the formation of a death-inducing signaling complex (DISC) (123, 212). The intracellular domain of the death receptor binds to the adaptor molecules FADD, which in turn recruits catalytically inactive procaspase-8 to allow their intermolecular autocatalytic cleavage, thus releasing ac-
tive caspase-8 to activate downstream procaspases. The induction of apoptosis mediated by cell surface death receptors is important for the removal of unwanted cells (e.g., autoreactive lymphoid cells), cells that have been infected by foreign agents (e.g., virus) or cells no longer supported by survival factors. The exact role of the death ligands and their receptors in ovarian follicle atresia is under investigation. However, different components of the receptor-mediated proapoptotic pathways (Fas ligand, TNF-α and death receptors, TNF-α receptor I, Fas antigen, p75 NGF receptor, DR3, and DR4) have all been found in the ovary (23, 32, 110, 143, 154, 184, 185, 201, 207, 259). It is likely that apoptosis of specific ovarian cells is triggered by these pathways.

VIII. CONCLUSION

It is clear that active cell suicide plays an important role in the elimination of superfluous, damaged, or malignant cells to allow the general well-being of the whole organism. In a normal organism, cells are alive because they are constantly stimulated by diverse survival factors. If survival signals are removed, apoptosis is initiated to ensure that superfluous cells would die. Alternatively, death signals could also actively target unwanted cells to undergo apoptosis.

The signaling mechanism for apoptosis is modulated by a diverse array of intracellular regulators. Studies using the yeast two-hybrid system provide an experimental paradigm to identify tissue-specific Bcl-2 proteins and their binding proteins in the decision step of apoptosis. In the ovary, the McI-1 and other antiapoptotic proteins are believed to heterodimerize with proapoptotic Bcl-2 proteins (BAD, BOD, Bok, and Bax), and the ratio of these protein pairs could affect downstream events including the release of cytochrome c and binding to ovarian Apaf-1 in the regulation of caspase activation. The proapoptotic ligands, BAD and BOD, likely interact with upstream binding proteins to mediate the action of survival factors. In addition, mechanisms both dependent and independent of heterodimerization mediated by Bok variants may be important for cell death regulation for reproductive tissues that exhibit constant or cyclic cell turnover during the reproductive life. Further characterization of these Bcl-2 proteins and the recently discovered DIVA/Boo would allow a better understanding of the intracellular decision step of apoptosis, particularly for hormone-regulated cell death.

In addition to the maintenance of cell survival by diverse survival factors mediated through the Bcl-2 decision step of apoptosis, death ligands also enhance apoptosis by acting through death receptors. In addition, corpses for apoptotic cells are removed by phagocytosis mediated by conserved cell surface markers. Because aberrant apoptosis regulation is the basis of tumorigenesis and various degenerative diseases, these pathological conditions can be corrected after pharmacological manipulation of apoptosis. Tumor cells can be induced to undergo apoptosis, whereas inhibitors of caspases could reduce myocardial reperfusion injury in vivo by attenuating cardiomyocyte apoptosis within the ischemic area at risk. Future studies on specific Bcl-2 proteins and their binding proteins in individual tissues could allow manipulation of cell fate in a tissue-specific manner. Elucidation of the regulatory mechanisms regarding follicular cell death may provide new therapeutic modalities for ovarian diseases including premature ovarian failure and polycystic ovarian syndrome.

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Address for reprint requests and other correspondence: A. J. W. Hsueh, Div. of Reproductive Biology, Dept. of Gynecology and Obstetrics, Stanford University School of Medicine, Stanford, CA 94305-5317.

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