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# Kinase Cascades Regulating Entry into Apoptosis

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## EVOLUTIONARY ORIGINS OF APOPTOSIS

In unicellular organisms, proliferation is controlled by the availability of exogenous nutrients. As long as nutrients are available, individual cells divide at a rate set by the biochemical reactions that comprise the cell cycle. Although a programmed series of biochemical events leading to cell division is essential for the survival of individual cells, the survival of individual species further requires the ability to cope with periods of nutrient deprivation. In some unicellular organisms (e.g., yeasts), nutrient deprivation induces sporulation, a genetic program leading to a reversible, nonproliferative state of dormancy. The ability of spores to survive prolonged periods of nutrient deprivation allows yeasts to weather inhospitable environments. Another way that a unicellular species can survive nutrient deprivation is to reduce the number of individual organisms, allowing a few members of the species to utilize scarce nutrients. Remarkably, this altruistic response appears to be invoked in selected unicellular organisms (e.g., trypanosomes and *Dictyostelium*) that respond to nutrient deprivation either by entering a $G_0/G_1$ arrest or, in cells that continue cycling, by activating programmed cell death (1, 26). Activation of programmed cell death in response to nutrient deprivation would have disastrous consequences if it were a hardwired genetic program, but if such a response were regulated by a stochastic genetic switch, it could allow the species to survive environmental hardship by allowing a few cells to survive on limited nutrients. Such a genetic switch may control whether trypanosomes arrest in $G_0/G_1$ or continue cycling in response to nutrient deprivation. It is likely that the evolution of programmed cell death preceded, and was essential for, the subsequent evolution of multicellular organisms. Whereas the rate of cell division in unicellular organisms is a simple function of nutrient availability, multicellular organisms are subject to additional constraints introduced by the need to deliver nutrients to each individual cell. These constraints effectively limit the size and shape of multicellular organisms. In primitive multicellular organisms, nutrients are delivered to individual cells by diffusion, imposing a selective pressure for shapes and sizes that allow all cells access to environmental nutrients. In more complex multicellular organisms, nutrients are delivered to specialized organs by a circulatory system, but similar constraints on size and shape are applicable. In both cases, cell death becomes essential to mold the shape of the organism and limit its size to optimize nutrient delivery. For these reasons, unregulated growth of individual cells in multicellular organisms is antithetical to survival. In multicellular organisms, cell division must be balanced by cell death.

## MORPHOLOGY OF APOPTOSIS

In the adult human, millions of cells divide into identical daughter cells every hour. It follows from the above discussion that this impressive renewal must be accompanied by an equally impressive elimination of existing cells. Where then is the carnage? If one examines histologic sections derived from individual tissues (including tissues undergoing massive cellular turnover such as the thymus gland, in which positive and negative selection eliminates more than 97% of newly formed thymocytes) (149), one finds relatively few (in most tissues $<1\%$) dead cells (25). The reason for this apparent anomaly is that the process of apoptosis, refined over evolutionary millennia, is neat, tidy, and extraordinarily rapid. Initiation of programmed cell death results in shrinkage of the cytoplasm and condensation of the nuclear chromatin (2, 83). Cytoplasmic changes also include the clustering of organelles and formation of crystalline arrays of ribosomes. In addition, endoplasmic reticulum-derived vacuoles are commonly observed, producing a characteristic "bubbling" of the cytoplasm (22, 176). The condensed nucleus is fragmented into membrane-enclosed "apoptotic bodies" (2, 83). The cell surface expresses opsonic receptors that allow neighboring parenchymal cells to rapidly phagocytose and digest the corpse (140, 141). All this occurs without leakage of cellular constituents, without inflammation, and without damage to surrounding cells. In the thymus gland,

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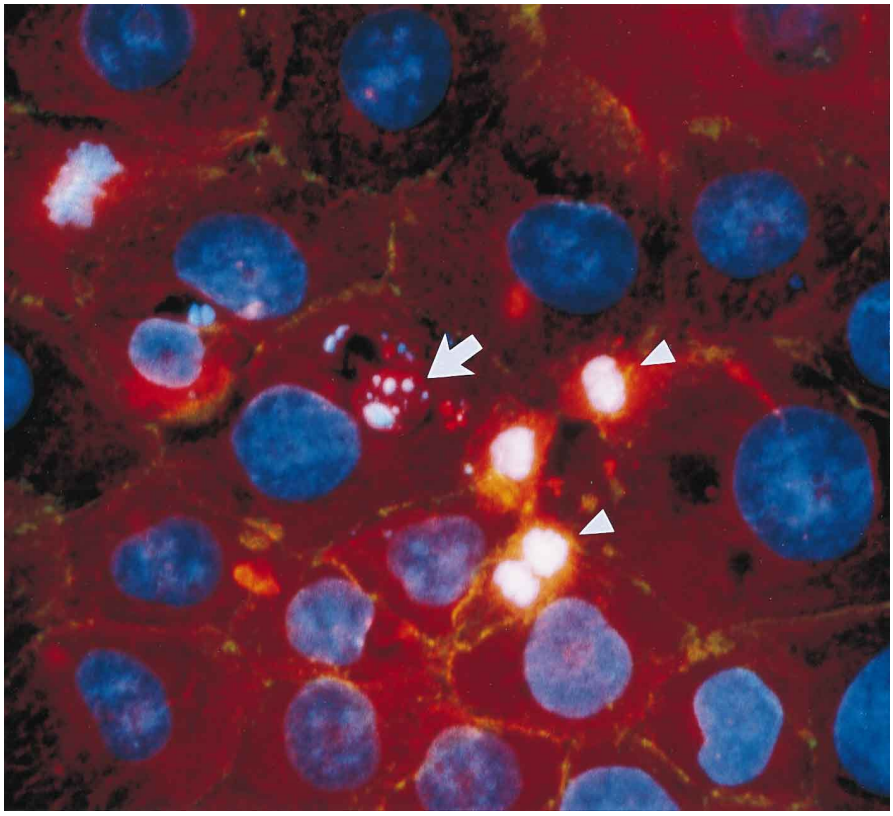

FIG. 1. Morphology of apoptosis and mitosis in human squamous skin carcinoma cells in culture stained with fluorescence-tagged antibodies reactive with myosin (red) and cadherin (green) and counterstained with Hoechst dye. The arrow indicates an apoptotic cell being engulfed by a neighboring cell. Arrowheads indicate cells undergoing mitosis. The photomicrograph was provided by Nancy Kedersha, Immunogen, Inc., Cambridge, Mass.

this entire process is completed within an hour. It is the rapidity of the process that accounts for the rarity of apoptotic cells in healthy tissues. In Fig. 1, the processes of cell division and cell death are captured in a photomicrograph of human squamous skin carcinoma cells in culture. Arrowheads point out cells undergoing mitosis, and the arrow points out the single cell undergoing apoptosis. This cell exhibits characteristically condensed cytoplasmic and nuclear compartments. The nucleus has fragmented into several brightly staining apoptotic bodies, and the entire cell has been phagocytosed by a neighboring tissue culture cell.

## MOLECULAR COMPONENTS OF THE CORE APOPTOTIC PROGRAM

Although the morphologic features of apoptosis have been appreciated for several decades (83), the biochemical pathways responsible for apoptotic cell death are only beginning to be elucidated. Just as genetic analysis of cell division cycle mutants in unicellular eukaryotes (e.g., *Saccharomyces cerevisiae*) has proven invaluable for the identification of mitotic effector molecules (120, 146), genetic analysis of cell death mutants in a simple multicellular eukaryote (*Caenorhabditis elegans*) has begun to identify key apoptotic effector molecules (63, 65, 182). In *C. elegans*, *ced-3*, *ced-4*, and *ced-9* have been identified as genetic cornerstones of the death program that are required to promote (*ced-3* and *ced-4*) (183, 184) or prevent (*ced-9*) (64) apoptosis (Fig. 2). Remarkably, human homologs of *ced-3* and *ced-9* were known to be apoptotic effectors even before their genetic identification as *C. elegans* death genes (a human homolog of *ced-4* has not yet been identified). Thus, interleukin-1β (IL-1β)-converting enzyme (ICE), a structural and functional homolog of CED-3 (186), was first identified as the cysteine protease responsible for proteolytic activation of

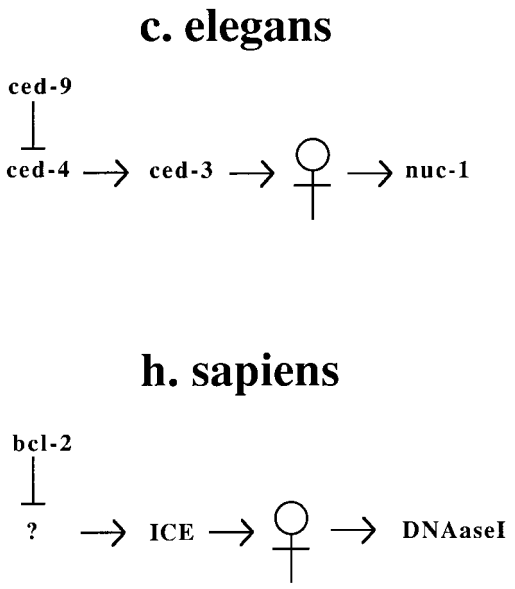


FIG. 2. Phylogenic conservation of a putative "core death pathway."

TABLE 1. Homologs of *ced-3* and *ced-9* comprise multigene families in

| *C. elegans* gene | Homologs[a] | Apoptosis |
|---|---|---|
| *ced-3* | ICE, Nedd-2/ICH-$1_L$, TX/ICH2/ICE rel-II, ICE rel-III, Mch2, apopain/CPP32/Yama, Mch3/ICE-LAP3/CMH-1, Mch4, FLICE/MACH1/Mch5 | +++ |
| | ICH-$1_S$ | – |
| *ced-9* | bcl-2, bcl-$x_L$, Mcl-1, ASFV HMW5-HL, EBV BHRF1, E1B19K, A1/BFL-1, BRAG-1, herpesvirus saimiri ORF16 | – |
| | bax-α, bak, bcl-$x_S$, bik1 and Nbk, bad | +++ |

[a] ASFV, African swine fever virus; EBV, Epstein-Barr virus.

pro-IL-1β in macrophages undergoing apoptosis (68). Subsequent research has identified additional substrates for ICE, some of which may be critical targets for initiation of the death program. *bcl-2*, a structural and functional homolog of *ced-9*, is an oncogene that is transcriptionally activated by the t(14;18) (5, 23) chromosome translocation found in B-cell follicular lymphomas. Overexpression of *bcl-2* inhibits apoptosis in these cells (160), an essential component of lymphomatous growth. The realization that CED-3/ICE and CED-9/BCL-2 are essential, evolutionarily conserved regulators of programmed cell death produced an explosion of research designed to determine the function of these death effectors. One outcome of this research is the realization that in higher eukaryotes, BCL-2 and *ice* are members of multigene families (Table 1). Remarkably, individual members of each family, some of which are produced by alternative splicing, have been shown to be functional antagonists (alternatively spliced versions of *ced-4* similarly promote or inhibit apoptosis in *C. elegans* [145]). Thus, structural homologs such as *bcl-2* and *bcl-xL* inhibit cell death, whereas *bax* and *bak* promote cell death. The further observation that BCL-2 family members tend to dimerize has led to the hypothesis that homodimerization of death agonists (i.e., BAX and BAL) somehow triggers apoptosis, a process that can be prevented by death antagonists (i.e., BCL-2 and BCL-xL) (91, 142, 179). Individual members of the ICE family can similarly promote (e.g., ICE and ICH-1L) or inhibit (e.g., ICH-1S) apoptosis (169). ICE family members have further been divided into functional subgroups based on their location in a putative proteolytic cascade that leads to activation of effector proteases (46). Molecular characterization of the two cytolytic effector mechanisms used by cytotoxic lymphocytes in the elimination of target cells (66) has uncovered two related proteolytic cascades centered on ICE family proteases. Thus, granule-mediated killing involves the introduction of granzyme B into the cytoplasm of target cells, triggering proteolytic activation of apopain/CPP32/Yama (30, 105, 132) and subsequent proteolysis of apoptotic substrates including PARP, DNA-PK, etc. (20) (Fig. 3). Alternatively, aggregation of FAS antigen on the surface of target cells triggers an intrinsic protease cascade that similarly activates apopain/CPP32/Yama. The cytoplasmic domain of FAS (or, analogously, the tumor necrosis factor alpha [TNF-α] receptor) binds to the adapter molecule FADD (the cytoplasmic domain of the TNF receptor binds to TRADD, which then binds to FADD), which interacts with an ICE family member designated FLICE/MACH1 (13, 115). It appears that ligand-induced trimerization of FAS/TNF receptor serves to aggregate FLICE/MACH1, resulting in its proteolytic activation. Activated FLICE/MACH1 could then activate downstream proteases, culminating in the activation of apopain/CPP32/Yama and proteolytic cleavage of death substrates. Although the details of such a proteolytic cascade are not yet known, this putative mechanism is well suited for amplification of minimal exogenous stimuli. According to this scheme (46), ICE family proteases have been designated initiator proteases (e.g., FLICE/MACH1), amplifier proteases (e.g., ICE), and machinery proteases (e.g., apopain/CPP32/Yama). The ability of different ICE family members to promote or inhibit apoptosis also allows such a mechanism to integrate conflicting exogenous signals. Detailed descriptions of the molecular characterization of BCL-2 and ICE family members, an area of intensive investigation, are topics of several recent reviews (59, 161, 172).

All cells can be induced to die by apoptosis. Even transformed cell lines grown for many years in culture remain susceptible to programmed cell death. This observation implies that the essential components of the death pathway are, paradoxically, required for survival. It has been argued that the retention of the death program indicates that mitotic and apoptotic pathways have common effector molecules (112). This appears to be true in the case of cyclin-dependent kinases which are activated during mitosis and apoptosis (111, 112). The argument requires, however, that each essential component of the death program also be an essential component of the mitotic program. This requirement is not met in *C. elegans ced-3* mutants, in which mitosis proceeds normally under conditions in which developmentally programmed cell deaths do not occur. Similarly, *Drosophila* mutants lacking the expression of reaper undergo mitosis under conditions in which the death program is severely impaired (173). If the mitotic program does not require essential elements of the apoptotic program in lower eukaryotes, it seems unlikely that this will be the case in higher eukaryotes, where key apoptotic effectors are potentially redundant members of multigene families. Indeed, targeted disruption of *bcl-2* (78, 116, 117, 162) or *ice* (92, 98) in mice affects apoptosis in some cells but not in others. Even in cells rendered deficient in apoptosis, however, mitosis appears to proceed normally. It therefore appears unlikely that all of the essential components of apoptosis are also required for mitosis. The failure to identify tumor cells that have disabled the apoptotic program suggests that key effectors of apoptosis are either required for transformation or are essential for other cellular processes. It is possible, for example, that BCL-2 family members are essential for some aspect of protein or nucleic acid transport across membranes (111, 138). Similarly, ICE family members might be essential for the regulation of repair enzymes in normal cells (20).

## SIGNALING CASCADES LINKING ENVIRONMENTAL STIMULI TO THE CORE APOPTOTIC PROGRAM

The profound phenotypes of *ced-3*, *ced-4*, and *ced-9* mutants of *C. elegans* suggest that these molecules are part of, or regulate entry into, a core death pathway. In mammalian cells, the universal ability of pharmacological agents such as staurosporine, which inhibits growth-promoting signaling pathways, to trigger apoptosis in the absence of RNA or protein synthesis suggests that the molecular effectors of the core death pathway are present in all cells (10, 71). Although some physiologically important apoptotic stimuli (e.g., TNF-α- and FAS ligand-induced death of lymphocytes) also do not require RNA or protein synthesis, many others (e.g., calcium-induced [109, 110], ATP-induced [189], glucocorticoid-induced [24, 186], or radiation-induced [144] thymocyte death, some programmed deaths due to growth factor withdrawal [8], and death due to

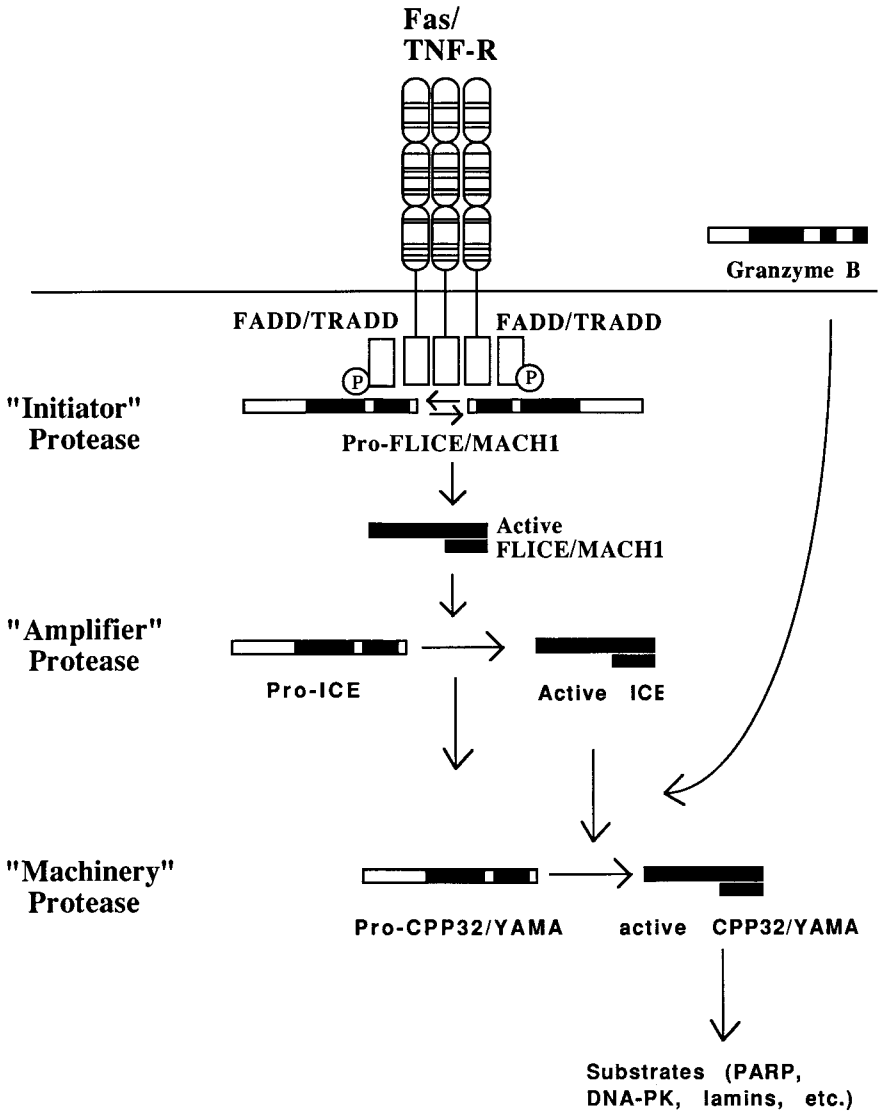


FIG. 3. Proteolytic cascades involved in the effector phase of apoptosis. This simplified diagram is not meant to imply direct links between the designated proteases. Intermediate proteases and parallel protease cascades are probably involved in this signaling cascade. The proforms of individual proteases are thought to exist as dimers, whereas the active forms are thought to exist as tetramers.

oxidative stress) clearly do. It follows that the signaling pathways used by these two types of apoptotic stimuli must be fundamentally different. It is reasonable to assume that stimuli that trigger apoptosis in the absence of macromolecular synthesis must engage the core apoptotic program relatively directly whereas stimuli that require macromolecular synthesis trigger the core apoptotic program more indirectly. The direct linkage of FAS and TNF receptor to an ICE family protease as schematized in Fig. 3 is consistent with the ability of FAS ligand and TNF-α to trigger apoptosis in the absence of RNA or protein synthesis.

An instructive contrast is the activation-induced death of T cells and B cells triggered by ligation of the T-cell receptor complex or surface immunoglobulin, respectively. Whereas antigen receptor ligation initiates the immune response by inducing the proliferation of resting, primed lymphocytes, this stimulus can also terminate an ongoing immune response by inducing apoptosis in activated lymphocytes. Activation-induced lymphocyte death is blocked by inhibitors of RNA or protein synthesis, an effect that can be explained by the requirement for the autocrine or paracrine action of cytokine-receptor pairs including TNF-α/TNF receptor and FAS ligand/FAS in this process (16, 36, 77, 188). Transcriptional regulation of these cytokines controls entry into the death pathway (16, 36, 77, 188). In this specialized case, it may be desirable for these stimuli to directly engage the core death pathway, as the decision to die has already been made at the transcriptional level. In contrast to the activation-induced death of lymphocytes, in which an exogenous stimulus is designed to lead inexorably to death, all cells are constantly subjected to exogenous stimuli that give conflicting instructions regarding life or death. It appears, for example, that all cells require continuous exposure to autocrine or paracrine growth factors for survival (6, 7, 25, 71, 72, 134–136, 154). Survival factors come in multiple flavors. Some are secreted polypeptides (134) or steroid hormones (50) that bind to specific surface receptors, others are components of the cell matrix that interact with cellular adhesion molecules (49, 113, 137), and

still others are intracellular molecules that monitor cellular damage, triggering apoptotic checkpoints when damage exceeds the possibility of repair (61, 79). Thus, multiple environmental cues determine the survival and proliferative fate of individual cells.

In addition to the proliferative signals transmitted by survival factors, cells are subject to antiproliferative signals transmitted by certain cytokines, as well as by environmental stress. Examples of stress conditions that counter proliferative signals include heat, hyperosmolarity, UV light, and gamma irradiation. The cellular response to these stimuli must be evolutionarily ancient, since survival in the face of these conditions would be required for the survival of any species. Just as cells learned to respond to nutrient deprivation by inducing cell cycle arrest and/or cell death, cells have developed a similar response to these noxious stimuli. It is clear, then, that cells constantly monitor their environment for the presence or absence of factors that signal survival and/or proliferation and for stresses that signal growth arrest and/or cell death. It then follows that cells must be able to integrate these exogenous signals to make decisions regarding survival, proliferation, and death. One of the ways that cells have addressed this challenge is to develop systems for the reversible modification of cellular constituents that can serve to integrate conflicting exogenous stimuli. Phosphorylation of cellular components is commonly used for this purpose. Phosphate can be reversibly added to serine, threonine, tyrosine, or histidine residues of proteins, as well as to hydroxyl groups of sugars. Hundreds of protein and lipid kinases and phosphatases provide specificity to these reactions, and cross talk between individual kinase cascades appears to integrate conflicting exogenous stimuli. Recent evidence suggests that protein phosphorylation plays an important role in signaling apoptotic cell death (53). I will review recent evidence linking protein kinase activation to the induction of apoptosis. I will also review the evidence for cross talk between individual kinase cascades that might serve to integrate exogenous triggers of mitosis and apoptosis. Finally, I will speculate on the possible connection between kinase cascades and triggering of the core apoptotic program.

## SERINE/THREONINE PROTEIN KINASES

### Stress-Activated Kinases

Parallel cascades of structurally related serine/threonine kinases serve to integrate extracellular growth-promoting and growth inhibitory signals (17, 175). Growth-promoting signals transmitted by extracellular mitogens activate a cascade comprising the RAS guanine nucleotide binding protein, the RAF-1 serine protein kinase, dual-specificity kinases designated MAP kinase kinase (MKK1 and MKK2), and the mitogen-activated protein (MAP) kinases p42 and p44 (also known as extracellular signal-regulated kinases [ERK1 and ERK2]). Activated ERK1 and ERK2 induce the production of proteins required for cell growth by phosphorylating transcription and translation factors. The RAS/RAF/MKK/ERK signaling pathway is engaged by a variety of mitogenic receptors, particularly those whose cytoplasmic domains are tyrosine kinases (e.g., receptors for epidermal growth factor [EGF], insulin, insulin-like growth factors, and platelet-derived growth factor [PDGF]). In other cases (e.g., immune receptors for antigens and Fc receptors), Src-family tyrosine kinases are recruited to the cytoplasmic domain of the receptor in response to ligand binding (41). In both cases, tyrosine kinase activation results in (i) recruitment of guanine nucleotide exchange factors that are required for the activation of RAS and (ii) activation of phospholipase C by phosphorylation, with the consequent liberation of lipid second messengers (i.e., diacyglycerol) that trigger lipid kinase cascades as described below (53, 103, 104, 143).

Two additional members of the MAP/ERK family are stress-activated protein kinase (also known as Jun N-terminal kinase and henceforth designated SAPK/JNK) and p38 kinase, both of which are activated by environmental stress (e.g., UV light, gamma irradiation, hyperosmolarity, and heat shock), and inflammatory cytokines (including IL-1β, TNF-α, and FAS ligand) (29, 32, 93). SAPK/JNK and p38 are substrates for and are activated by MKK3 and MKK4, respectively. Activated SAPK/JNK appears to regulate cell proliferation by phosphorylating transcription factors. Candidate substrates for these kinases are c-JUN, ATF2, and ELK-1, all of which are phosphorylated under stress conditions (174). Overexpression of a dominant negative c-*Jun* mutant lacking the N terminus inhibits stress-induced apoptosis, suggesting that phosphorylated c-JUN may be required for activation of downstream events in the apoptotic program (163). Although phosphorylated c-JUN activates transcription of c-*Jun* mRNA, the ability of dominant interfering c-*Jun* mutants to inhibit TNF-α-mediated apoptosis, a process which is potentiated by actinomycin D, indicates that the ability of phosphorylated c-JUN to induce apoptosis is not a function of transcriptional activation. Although phosphorylated c-JUN might function to trigger apoptosis directly, it is also possible that dominant interfering c-*Jun* mutants prevent interactions between JNK and unidentified substrates whose phosphorylation leads to apoptosis.

Functional antagonism between ERK1/ERK2 and SAPK/JNK-p38 kinase cascades in transducing growth-promoting and growth inhibitory signals is further suggested by studies of rat PC-12 pheochromocytoma cells grown in the presence or absence of nerve growth factor (NGF) (177). PC-12 cells rapidly undergo apoptosis following NGF withdrawal. In the presence of NGF, MAP kinases are phosphorylated and catalytically active in cycling PC-12 cells. Following NGF withdrawal, ERK1 and ERK2 are dephosphorylated and rendered catalytically inactive, suggesting a role for the RAS/RAF/MKK/ERK cascade in growth factor-mediated survival. Although NGF withdrawal has not been shown to directly activate the SAPK/JNK-p38 kinase cascade, coexpression of p38 and its activating kinase, MKK3, triggers apoptosis in PC-12 cells, and overexpression of a dominant negative MKK3 mutant inhibits apoptosis induced by NGF withdrawal. Dominant interfering mutants of c-JUN also inhibit apoptosis induced by NGF withdrawal, again suggesting that phosphorylation of JNK-p38 substrates is required for some forms of apoptosis.

A common feature of stress-induced apoptosis is the rapid generation of ceramide, a lipid mediator liberated by sphingomyelin hydrolysis (89, 177). Ceramide has been shown to directly activate a heterotrimeric protein serine/threonine phosphatase called protein phosphatase 2A (37), as well as a 97-kDa proline-directed serine/threonine kinase (106), suggesting a role in regulating phosphotransferase reactions. C2-ceramide directly induces apoptosis in a variety of cell types, an effect that is accompanied by the activation of SAPK/JNK (163). A requisite role for active SAPK/JNK is suggested by the ability of dominant inhibitory N-terminal deletion mutants of c-JUN to abrogate ceramide-mediated apoptosis (163). This conclusion is supported by the ability of dominant inhibitory mutants of MKK4 (the upstream activator of JNK) to also inhibit ceramide-mediated apoptosis (177). Importantly, ceramide does not activate ERK1 or ERK2, consistent with a role in signaling growth inhibition and/or apoptosis (163, 177).

The requirement for distinct lipid second messengers for the activation of growth-promoting and growth inhibitory kinase

cascades may provide the cell with an opportunity to integrate the response to conflicting stimuli soon after receptor engagement. Indeed, it appears that the presence of diacylglycerol can profoundly influence the cellular response to ceramide (131). Whereas ceramide analogs are potent inducers of apoptosis in susceptible cells and elevation of the ceramide concentration can be detected in cells undergoing stress-induced apoptosis, elevations of the ceramide concentration can also be measured in response to stimuli that trigger growth and differentiation (89). The further observation that diacylglycerol mimetics can inhibit TNF-α- and ceramide-induced apoptosis suggests a role for cross talk between these antagonistic lipid mediators (74, 122). The possibility that diacylglycerol and ceramide serve to integrate growth signals within the cell is further supported by observations showing that although Molt-4 leukemia cells are exquisitely sensitive to C6-ceramide-induced apoptosis, they are relatively resistant to apoptosis induced by serum withdrawal, a stimulus that induces the production of both diacylglycerol and ceramide (75).

BCL-2, a potent inhibitor of apoptosis, has been reported to interact with both R-RAS (43) and RAF-1 (166), suggesting that interactions between CED-9/BCL-2 family members and the RAS/RAF/MKK/ERK signaling cascade might influence susceptibility to apoptosis. Surprisingly, overexpression of activated R-RAS in IL-3-dependent cell lines was found to increase their susceptibility to apoptosis triggered by IL-3 withdrawal (165). Overexpression of activated R-RAS in NIH 3T3 cells has been shown to result in phosphorylation of ERK1 and ERK2 (28), an effect that might be expected to inhibit apoptosis. It is possible, however, that R-RAS also activates SAPK/JNK and/or p38 to promote apoptosis following growth factor withdrawal. It is also possible that R-RAS has other unknown functions that might regulate entry into the apoptotic pathway. In this regard, it is of interest that the RAS signaling pathway is also activated during FAS-mediated apoptosis (55). Although recombinant BCL-2 and RAF-1 can be coprecipitated from Sf9 insect cells (166), recent results indicate that their association may require additional proteins, one of which may be BAG-1 (167), a BCL-2-interacting protein that is also an apoptotic antagonist (154). BAG-1 binds to and activates RAF-1, suggesting that it might act upstream of raf. Since BCL-2 family members are membrane associated, it is possible that their interaction with kinases in the RAS/RAF/MKK/ERK cascade is important for their activation or substrate specificity. If this were the case, one would predict that BCL-2 family members that facilitate apoptosis (e.g., BAX and BAK) might interact with kinases in the SAPK/JNK-p38 family. Further analysis of interactions between BCL-2 family members and serine/threonine kinases is clearly indicated.

Another stress-activated serine/threonine kinase involved in signaling apoptosis is FAS-activated serine/threonine kinase (FAST) (157). Comparison of the deduced amino acid sequence of FAST with the MAP kinases reveals little sequence similarity. Unlike the ERKs, which are activated by phosphorylation, FAST is constitutively phosphorylated on serine and threonine residues and is activated by dephosphorylation. FAST is rapidly dephosphorylated and concomitantly activated to phosphorylate TIA-1, an RNA-binding protein involved in signaling apoptosis (155, 157), in response to FAS ligation. Activation of FAST and phosphorylation of TIA-1 precede the onset of DNA fragmentation, suggesting that phosphorylated TIA-1 might signal downstream events in the apoptotic program (155, 157). The observation that FAST is activated by TNF-α, UV irradiation, heat shock, and ceramide, but not by mitogenic stimuli transmitted via the T-cell receptor complex, indicates that FAST, like the SAPK/JNK-p38 kinases, is a stress-activated serine/threonine kinase.

RNA-binding proteins such as TIA-1 and its structural homolog TIAR are components of a signaling cascade involved in stress-induced apoptosis. Like many RNA-binding proteins, TIA-1 and TIAR shuttle between the nucleus and the cytoplasm in viable cells. At steady state, both TIA-1 and TIAR are concentrated in the nucleus, where they decorate RNA tracts that are sites of active RNA transcription. In Jurkat cells undergoing apoptosis triggered by environmental stress or inflammatory cytokines, TIA-1 and TIAR accumulate in the cytoplasm (155), where they associate with ribosomes lining the rough endoplasmic reticulum (81). Both TIA-1 and TIAR bind specifically to a "CUUUUC" motif (35) that resembles a *cis* element found at the 5′ untranslated region of mRNAs encoding proteins required for cellular proliferation (e.g., ribosomal proteins) (15, 76, 97). mRNAs possessing this regulatory motif are subject to translational arrest in response to environmental stress. Taken together, these results suggest that TIA-1 and TIAR may be involved in the translational regulation of this class of mRNAs. It is therefore possible that FAST-mediated phosphorylation of TIA-1 regulates the translation of mRNAs encoding proteins essential for survival and/or proliferation. The possibility that FAST and TIA-1 regulate translation is reminiscent of the role of ERKs in facilitating translational initiation. Activated ERKs phosphorylate PHAS-I (99), a protein that inhibits translation by binding to the cap binding protein eIF-4E (127). ERK-mediated phosphorylation of PHAS-I liberates eIF-4E, resulting in a global enhancement of cap-dependent translation initiation. The possibility that phosphorylated TIA-1 inhibits translation of selected mRNAs would be consistent with the known functional antagonism between ERK kinase cascades and stress-activated kinase cascades.

FAST is structurally related to a serine/threonine kinase domain at the amino terminus of ICP10, the herpes simplex virus type 2 ribonucleotide reductase (157). The ICP10 kinase domain is essential for virus replication, and its overexpression induces cellular transformation (150). ICP10 is a transmembrane protein that is localized to the plasma membrane (69, 150). Remarkably, overexpression of the isolated ICP10 kinase domain activates the RAS/RAF/ERK kinase cascade (69, 150). These observations raise the possibility that ICP10 is derived evolutionarily from FAST or a FAST-related kinase. Its ability to activate the RAS/RAF/ERK survival cascade suggests a possible role in preventing apoptosis during herpesvirus infection. This might result from direct activation of the RAS/RAF/ERK cascade or inhibition of an antagonistic, stress-induced FAST cascade. A schematic depiction of the parallel kinase cascades involved in the integration of growth regulatory stimuli is shown in Fig. 4.

## Cyclin-Dependent Kinases

Genetic analysis of cell division cycle mutants of yeasts has identified a large family of serine/threonine kinases that regulate cell cycle progression (46, 78, 95, 112, 120). The catalytic subunit of cyclin-dependent kinases (cdks) is regulated by phosphorylation, subcellular localization, and, most importantly, their association with regulatory subunits known as cyclins (46, 78, 94, 112, 120). Progression through the cell cycle requires the temporal activation of selected cdks at defined intervals. For example, tyrosine phosphorylation of CDC2 on Tyr15 renders it catalytically inactive despite its association with cyclin B during the S and $G_2$ phases. Dephosphorylation

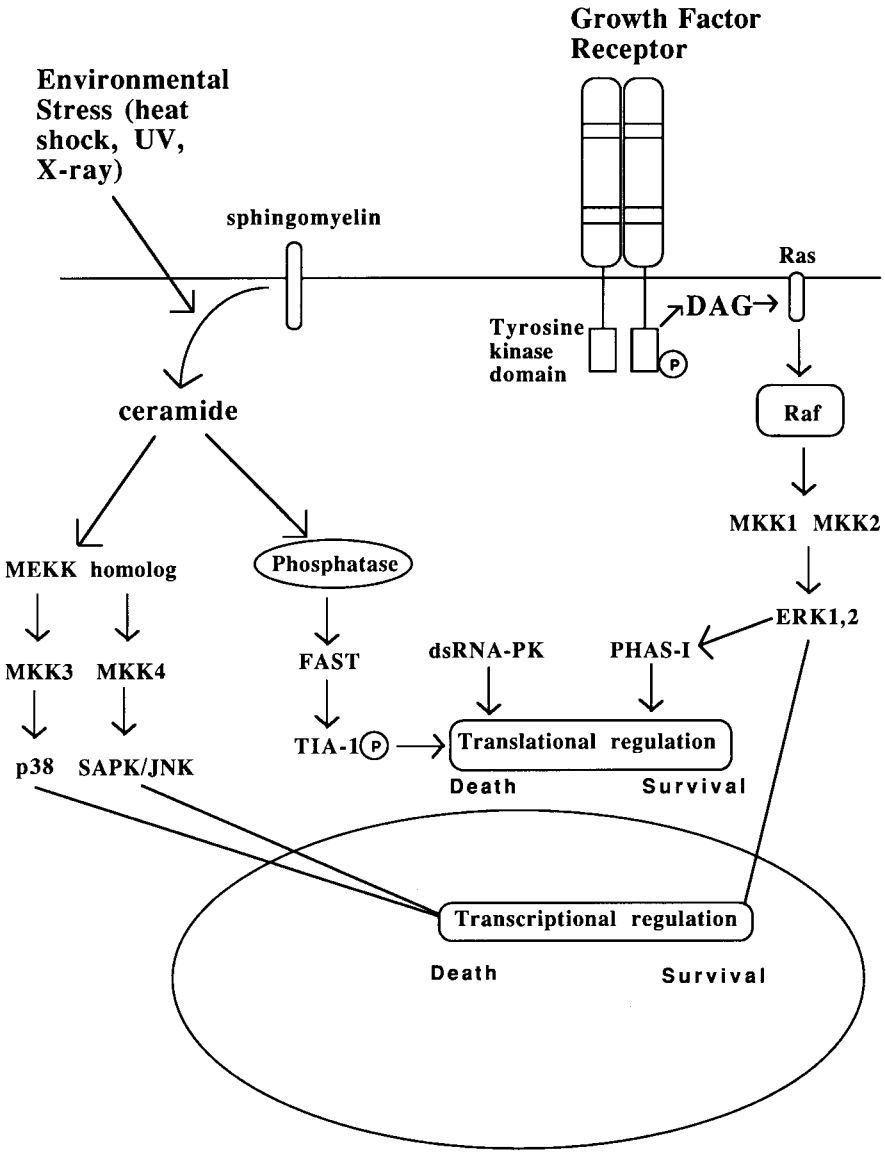


FIG. 4. Kinase cascades involved in the integration of exogenous triggers of proliferation and death. DAG, diacylglycerol; dsRNA, double-stranded RNA.

of cdc2 at M phase activates the kinase, allowing cell division to proceed. Premature activation of CDC2 triggers a "mitotic catastrophe" consisting of chromatin condensation and nuclear fragmentation resembling that induced during apoptosis (62). In combination, the cytotoxic granule constituents perforin and granzyme B have been shown to induce premature activation of CDC2 that is coincident with the induction of apoptosis (147). Activation of CDC2 is a consequence of dephosphorylation of Tyr15 and requires the serine protease activity of granzyme B. The further observation that a cell line possessing a temperature-sensitive mutation in CDC2 is resistant to staurosporine-induced apoptosis at the nonpermissive temperature suggests that CDC2 activation may be required for some forms of apoptosis (147). Further evidence for the importance of CDC2 in triggering granzyme B-induced apoptosis comes from the observation that overexpression of WEE1 the negative regulatory kinase that phosphorylates CDC2 on Tyr15, inhibits perforin/granzyme B-mediated apoptosis (21).

The activity of CDC2/cyclin B is also increased in lymphocytes undergoing T-cell receptor-mediated activation-induced death (45) and in PC12 cells following NGF withdrawal (52). Cyclin A-associated protein kinases CDC2 and CDK2 are similarly activated in S phase-arrested HeLa cells induced to undergo apoptosis by pharmacologic triggers such as staurosporine, caffeine, and okadaic acid (111). However, activation of CDC2 is not essential for some forms of apoptosis. Postmitotic neurons undergo apoptosis in the absence of detectable CDC2 (47), and thymocyte apoptosis triggered by etoposide or dexamethasone is not accompanied by CDC2 activation (119). It is possible, however, that other members of the CDC2 kinase family can substitute for CDC2 or CDK2 in the induction of apoptosis. p58-PITSLRE, a CDC2-related serine/threonine kinase, directly triggers apoptotic cell death when overexpressed in CHO cells (94). p58-PITSLRE is also proteolytically activated during apoptosis induced by FAS ligation or dexamethasone treatment of CEM T cells (94), suggesting that it may be

involved in signaling apoptosis triggered by multiple exogenous stimuli. Taken together, these results strongly implicate cdks as regulators of apoptotic cell death.

cdks are negatively regulated by inhibitors such as p21, p27, p16INK4a, and related proteins (146). Recent reports showing that p21 is required for the survival of differentiating neuroblastoma cells support a role for cdks in the regulation of apoptosis. Thus, NGF-induced neuronal differentiation was found to be accompanied by the expression of p21 (129). Antisense oligonucleotides complementary to p21 mRNA triggered apoptotic cell death in this system (129). In an in vitro system used to study myogenesis, withdrawal of growth factors from C2C12 myoblasts induces terminal differentiation of some myocytes and programmed cell death of others (56). Cells that undergo terminal differentiation express p21, whereas cells that die by apoptosis do not, implying that inhibition of cdks can prevent apoptosis (168). This conclusion is supported by the observation that overexpression of p21 (or p16INK4A) inhibits apoptosis during myocyte differentiation (168). Taken together, these results suggest that the regulation of cyclin-dependent kinases affects not only entry into mitosis but also entry into apoptosis.

Another cell cycle protein that regulates entry into apoptosis is CDC25, a tyrosine phosphatase that is phosphorylated and concomitantly activated as cells enter mitosis (85). CDC25 dephosphorylates an inhibitory tyrosine on CDC2 to promote mitosis. Consequently, *cdc25* functions as a proto-oncogene, cooperating with Ha-*Ras* in the transformation of primary fibroblasts. Overexpression of *cdc25* also potentiates the induction of apoptosis in response to growth factor withdrawal (51). Thus, *cdc25* resembles c-*myc* in its ability to cooperate with Ha-*Ras* in the transformation of primary fibroblasts and its ability to potentiate the induction of apoptosis. Remarkably, *cdc25* is transcriptionally activated by myc-max heterodimers, and it appears to be essential for myc-induced apoptosis.

E2F-1 is another transcription factor that regulates entry into both mitosis and apoptosis. E2F-1 is found in an inactive complex with dephosphorylated retinoblastoma protein (Rb) during $G_0$ and $G_1$ (170). Its liberation following phosphorylation of Rb by cdks prior to entry into S phase allows the transcriptional activation of several genes required for S phase entry, including c-*myc* (67). Mutant mice that lack E2F-1 exhibit defective apoptosis in a variety of tissues, resulting in lymphoproliferation and in the development of a range of tumors (44, 178). Whether this phenotype results from reduced expression of c-*myc* and *cdc25* is not yet known.

### Lipid-Activated Kinases

Enzymatic degradation of membrane phospholipids can liberate second-messenger molecules that activate serine/threonine protein kinases. The protein kinase C (PKC) family includes several isoforms that are activated by lipid cofactors (34, 118). Thus, PKCα, PKCβ, and PKCγ are $Ca^{2+}$-dependent kinases that are activated by diacylglycerol, a phosphatidylcholine metabolite liberated by phospholipase C, or by pharmacologic agents such as phorbol esters (34, 118). The ability of phorbol esters to either potentiate (87, 102, 133) or inhibit (9, 57, 108, 124) apoptosis in a variety of experimental systems supports a role for PKC isoforms in signaling cell death. Because phorbol esters activate several PKC isoforms and probably affect other intracellular enzymes as well, it is difficult to compare the effects of these compounds in different experimental systems. Analysis of individual PKC isoforms has implicated PKCβ and PKCδ in apoptotic signaling cascades. Thus, expression of PKCβ has been correlated with susceptibility to apoptosis (88, 101, 130), and proteolytic activation of PKCδ has been demonstrated during stress-activated apoptosis in myeloid cells (40). Proteolytic activation of PKCδ appears to involve an ICE-like protease, as the ICE inhibitor YVAD blocks both apoptosis and PKCδ cleavage (40). Although a requirement for PKCδ activation in stress-induced apoptosis has not been established, and downstream substrates have yet to be identified, the correlation between PKCδ activation and apoptosis strongly suggests a role in signaling the death program.

Although PKC substrates involved in regulating apoptosis have not been identified, one candidate substrate is the anti-death protein BCL-2. BCL-2 is phosphorylated by purified PKC in vitro and in cells treated with bryostatin-1, a PKC agonist (107). Serine phosphorylation of BCL-2 is also induced by survival factors such as IL-3 and erythropoietin in a factor-dependent cell line (107). These results suggest that activation of PKC and phosphorylation of BCL-2 result in suppression of apoptosis. In contrast, serine phosphorylation of BCL-2 is induced in lymphoid cells by okadaic acid and taxol, agents that trigger apoptosis (58). It is therefore possible that phosphorylation of BCL-2 can either enhance or inhibit its antiapoptotic effects, depending upon the cell type and the exogenous apoptotic trigger. It will be important to determine whether other members of the BCL-2 family are similarly phosphorylated during apoptosis.

As mentioned above, the sphingomyelin metabolite ceramide is liberated during stress-induced apoptosis. A variety of cell types undergo apoptosis when cultured in the presence of C2-ceramide (121, 122, 131, 156), implicating this lipid metabolite in a signaling cascade leading to cell death. Although ceramide-induced apoptosis is accompanied by the activation of SAPK/JNK and FAST, the effects of ceramide are probably indirect. Several proximal targets for ceramide have been proposed, including a serine/threonine phosphatase, a proline-directed protein kinase, and PKCζ (100). Because FAST is activated by dephosphorylation, a ceramide-activated phosphatase might be involved in FAST activation.

### Nucleic Acid-Dependent Kinases

Several serine/threonine kinases that integrate exogenous growth-promoting and growth-inhibitory signals phosphorylate substrates involved in the regulation of mRNA translation. As mentioned above, phosphorylation of PHAS-I by activated ERKs increases the general rate of translational initiation, an effect that is consistent with the growth-promoting effects of activated ERKs (99, 128). In contrast, the double-stranded RNA-dependent kinase (PKR) negatively regulates translational initiation by phosphorylating eIF-2α (27, 90). PKR is an interferon-inducible kinase that contributes to the antiviral response by inhibiting protein translation in virus-infected cells (73). PKR also functions as a tumor suppressor protein, limiting the ability of NIH 3T3 transformants to form tumors in mice (114). Like the tumor suppressor protein p53, overexpression of PKR induces apoptosis (96). In contrast, overexpression of a dominant inhibitory, kinase-dead PKR mutant induces malignant transformation (90). Taken together, these results suggest the existence of an apoptotic checkpoint monitoring translational initiation. It is possible that the stress-activated FAST kinase also regulates the translation of a subpopulation of mRNAs. The RNA-binding protein substrate of FAST (i.e., TIA-1) accumulates in the cytoplasm of cells during stress-induced apoptosis (155). Cytoplasmic TIA-1 has been shown to associate with ribosomes at the rough endoplasmic reticulum (81), suggesting that it might regulate translation

of mRNAs encoding transmembrane or secretory proteins (unpublished results). It is possible that phosphorylation of TIA-1 by FAST results in translational arrest, triggering the same apoptotic checkpoint that is guarded by PKR.

Another nucleic acid-dependent kinase that has been proposed to regulate apoptosis is DNA-PK, a nuclear serine/threonine kinase that is a substrate for ICE-like proteases activated during apoptosis (3, 19, 20, 82). DNA-PK is a multisubunit enzyme comprised of a 350-kDa catalytic subunit (DNA-PKcs) joined to a heterodimeric regulatory subunit (Ku-p70 and Ku-p80). All three subunits are common targets of autoantibodies found in the serum of patients with systemic lupus erythematosus (153). Substrates for apoptotic proteases are commonly identified as autoantigens in patients with autoimmune disease (19, 20). Whether this is a consequence of the production of neoepitopes following proteolysis, the presentation of these antigens on the surface of apoptotic cells, or both has not been determined. DNA-PK is an essential component of V(D)J recombination. Deletion of DNA-PKcs produces severe combined immunodeficiency (scid) in mice (12). Although it is not known whether proteolysis of DNA-PK is required for apoptosis, the observation that proteolytic fragments retain kinase activity (20) suggests the possibility that substrate specificity is altered following cleavage. Because several enzymes essential for DNA repair processes (e.g., PARP, DNA-PK, and topoisomerases I and II) are cleaved by ICE family proteases during apoptosis, it has been proposed that cleavage of DNA-PK ensures the irreversibility of apoptotic cell death by preventing DNA repair (20).

### cAMP-Dependent Kinases

Cyclic AMP (cAMP) is an important intracellular signaling molecule that exerts its effects primarily through the actions of cAMP-dependent protein kinases. Activation of cAMP-dependent protein kinases produces different effects in different cell types. These effects are determined, in part, by the specific substrates that are targets for phosphorylation. Elevation of cAMP levels induces programmed cell death some cell lines. Thus, cAMP induces S49 lymphoma cells to undergo $G_1$ arrest followed by cell death (48) and induces IPC-81 myeloid leukemia cells to undergo apoptosis (38), an effect that requires de novo RNA and protein synthesis. Microinjection of the catalytic subunit of cAMP-dependent protein kinase into IPC-81 cells induces apoptosis (164), establishing a direct role for the kinase in cAMP-mediated apoptosis. This conclusion is supported by the ability of a kinase-dead mutant of cAMP-dependent kinase to inhibit cAMP-mediated apoptosis in these cells (38). Elevations in cAMP levels can also trigger apoptosis in untransformed T cells (86), indicating that this effect is not peculiar to tumor cells. Since cAMP is a second-messenger molecule used in all cells, it is clear that its ability to trigger apoptosis will be limited to specialized cells in particular circumstances. When the specific substrates phosphorylated by cAMP-dependent protein kinase during apoptosis are identified, the specific link between this ubiquitous kinase cascade and the core apoptotic pathway may be revealed.

### Miscellaneous Serine/Threonine Kinases

It is clear from the above discussion that multiple serine/threonine kinases function as molecular gatekeepers guarding entry into the core death pathway. As a general principle, kinases targeting substrates that are several steps removed from the core death pathway (e.g., PKC and cdks) can either enhance or inhibit susceptibility to apoptosis, depending on the cell type and the experimental system. It is possible that kinases exhibiting a more consistent functional phenotype (e.g., stress kinases and dsRNA-PK) target substrates that are closer to the core death program. Given the ability of kinase cascades to integrate multiple exogenous signals in a matrix-like fashion, it is likely that many kinases that may not be primarily involved in regulating apoptosis will, when aberrantly expressed, affect entry into the death program. Examples may include casein kinase I (CK-I), which influences a variety of physiologic processes by phosphorylation of multiple substrates (e.g., insulin receptor, progesterone receptor, and protein phosphatase 1). CK-I also phosphorylates the 75-kDa subunit of the TNF receptor, a modification that inhibits TNF-α-mediated apoptosis (11). In a similar manner, a 160-kDa calmodulin-dependent serine/threonine kinase (DAP-1) has been implicated as an effector of gamma interferon-mediated apoptosis in HeLa cells (33). Expression of DAP-1 antisense RNA was found to reduce the expression of DAP-1 kinase and inhibit interferon-mediated apoptosis. Further investigation is required to determine whether these kinases regulate entry into the death pathway in other experimental systems.

## TYROSINE PROTEIN KINASES

The cytoplasmic domains of several survival factor receptors are protein tyrosine kinases (e.g., insulin-like growth factor I receptor, EGF receptor, and PDGF receptor). Several other cell surface receptors (e.g., the T-cell receptor complex, membrane immunoglobulin, and Fc receptors for immunoglobulin) capable of transmitting mitogenic signals are linked to nonreceptor protein tyrosine kinases (e.g., FYN, LCK, and ZAP-70), which are activated in response to receptor ligation (41). In this manner, protein tyrosine kinases play an essential role in receptor-proximal signaling events during mitogenesis. Activation of tyrosine protein kinases precedes the activation of RAS-dependent serine/threonine kinase cascades that directly activate transcription of genes required for cell proliferation. It is therefore not surprising that tyrosine protein kinases can contribute to the induction of, or susceptibility to, apoptotic cell death.

As is often the case in studies of signaling cascades that are some distance from the final molecular effectors, tyrosine kinases have been shown to either facilitate or inhibit apoptosis in different experimental systems. Consistent with the notion that tyrosine phosphorylation prevents cell death is the observation that inhibitors of protein tyrosine kinases trigger apoptosis in some experimental systems (18, 181). Supporting such a model is the observation that tyrosine phosphorylation of receptors for EGF and basic fibroblast growth factor inhibits apoptosis in cultured ovarian granulosa cells (158). The correlation between tyrosine kinase activation and inhibition of apoptosis is also observed in cells transformed with v-*abl* or *bcr*/*abl* (42, 126). In the case of c-*abl*, these effects may result from the formation of c-ABL/p53 complexes that down-regulate CDK2 (185).

In contrast, some experimental systems require protein tyrosine kinases for the induction of apoptosis. Consistent with a role for tyrosine phosphorylation in signaling apoptosis is the observation that radiation-induced apoptosis in B cells requires the activation of tyrosine kinases (159). Similarly, activation-induced B-cell death triggered by ligation of membrane immunoglobulin is dependent upon the SRC family kinase BLK (180). In addition, Fas-mediated apoptosis is blocked by tyrosine kinase inhibitors (39), and a physical association between FAS and the SRC-family kinase FYN appears to contribute to FAS-mediated apoptosis (4). Specific substrates for tyrosine phosphorylation have not yet been identified in these

experimental systems. It is therefore possible that differential effects of tyrosine kinases on the induction of apoptosis reflect the expression of different classes of substrates in different cell types.

## PROTEIN PHOSPHATASES

The integration of exogenous growth-promoting and growth inhibitory signals by protein phosphorylation involves an interplay between protein kinases and protein phosphatases. Inhibitors of serine/threonine phosphatases profoundly affect the susceptibility to programmed cell death in a variety of cell types. Okadaic acid, an inhibitor of protein phosphatases 1 and 2A, induces apoptosis in cultured leukemia cells (80), retinoblastoma cells (70), T cells (171), kidney epithelial cells (31), and breast cell lines (84). Similarly, calyculin A, another inhibitor of protein phosphatases 1 and 2A, induces apoptosis in IL-2-activated T cells (171) and breast carcinoma cell lines (84). Paradoxically, these compounds inhibit glucocorticoid-induced apoptosis in murine T-cell hybridomas (123), as well as stress-induced apoptosis in a Burkitt's lymphoma cell line (151). The opposing effects of these drugs on different cell types exposed to different apoptotic triggers suggest that substrates for the involved phosphatases are significantly upstream of the core death pathway and are capable of affecting more than one signaling cascade.

Calcineurin, a calcium- and calmodulin-dependent protein phosphatase, has also been reported to affect susceptibility to programmed cell death. Thus, cyclosporine, a specific inhibitor of calcineurin, inhibits calcium-induced apoptosis in Burkitt's lymphoma-derived cell lines (14). Consistent with this result, overexpression of a constitutively active calcineurin catalytic subunit in T-cell hybridoma cells inhibited glucocorticoid-induced apoptosis (187). In contrast, expression of constitutively active calcineurin in BHK cells directly induces cell death, an effect that is dependent upon its phosphatase activity (148). Once again, the calcineurin substrates responsible for these effects have not been identified. It is likely that calcineurin can affect more than one phosphorylation cascade that is involved in signaling apoptosis.

Protein tyrosine phosphatases can also affect susceptibility to programmed cell death. Thus, overexpression of CD45 in transgenic mice leads to an enhancement of activation-induced death in T lymphocytes (125). Whether this is due to inhibition of growth signals transmitted by receptors whose cytoplasmic domains are tyrosine kinases has not been determined. Monoclonal antibodies reactive with distinct CD45 isoforms have been reported to inhibit apoptosis in T cells and macrophages (60). Specific CD45 substrates involved in these processes have not been identified. Although CD45 does not appear to affect FAS-mediated apoptosis, two different protein tyrosine phosphatases have been reported to affect FAS-induced cell death. FAP-1, a protein tyrosine phosphatase that interacts with the cytoplasmic domain of FAS, appears to have an inhibitory effect on FAS-mediated apoptosis (139). In contrast, the hematopoietic cell protein tyrosine phosphatase appears to be required for optimal FAS-mediated apoptosis (152). Lymphocytes derived from hematopoietic cell protein tyrosine phosphatase-deficient mice are relatively resistant to FAS-mediated apoptosis.

## GENERAL CONCLUSIONS

Given the finality of cell death, it is hardly surprising that the entrance to the core death pathway is tightly regulated. This is especially true given the evidence that the death program is (i) constitutively present in all cells and (ii) under control of a "dead man's switch," which must be actively repressed to allow cell survival. It is therefore reasonable to assume that regulation of cell death will be at least as complex as regulation of cell proliferation. Just as multiple kinase cascades and regulatory mechanisms guard entry into the mitotic program, it is clear that multiple kinase cascades and regulatory mechanisms regulate entry into the apoptotic program. Furthermore, just as cell division can be influenced by multiple factors that are not directly involved in mitosis, cell death can be influenced by multiple factors that are not directly involved in the core death program. Given this scenario, caution must be exercised when evaluating the effects of a given gene, protein, or pharmacologic agent on cell death since many cellular processes can influence entry into apoptosis. Although all of the kinases cataloged above clearly influence entry into apoptosis, it will be important to distinguish kinases that are required to execute cell death, those that are required to signal cell death in certain circumstances, and those that influence but are not required for either execution or signaling of the death program.

There is currently no evidence indicating that kinases are required for execution of the death program. Most evidence implicates ICE family proteases as executioners of the death program, none of which have been shown to be regulated by phosphorylation. It is more likely that kinases and phosphatases play important roles in signaling cascades linking exogenous stimuli to the core death program. In this context, kinase cascades are well suited to amplify exogenous stimuli and integrate conflicting signals prior to entry into apoptosis. There is good evidence that parallel cascades of MAP/ERK family kinases serve to regulate cell proliferation and cell death. Thus, growth factor receptors commonly activate the RAS/RAF/MKK/ERK proliferative cascade by stimulating the release of the PKC agonist diacylglycerol by the action of phospholipase C. In contrast, activation of SAPK/JNK-p38 antiproliferative kinases correlates with the release of ceramide by the action of sphingomyelinase. Although the precise nature of the lipid-activated kinase that is engaged by ceramide is not yet known, these results suggest an important role for lipid second messengers in integrating signals leading to cell growth or cell death.

The ability of parallel kinase cascades to integrate proliferative and antiproliferative signals requires that some kinases will have effects on both cell cycle progression and apoptosis. Clearly, this is the case regarding MAP/ERK kinase cascades, in which phosphorylation or dephosphorylation of transcription and translation factors determines whether an individual cell will divide, arrest in $G_0/G_1$, or die by apoptosis. It is therefore not surprising that cyclin-dependent kinases and their regulators affect not only entry into mitosis but also entry into apoptosis. It is clear that future research designed to understand the molecular effectors of mitosis will have to take into account concomitant effects on apoptosis.

There are many unanswered questions regarding the regulation of apoptotic cell death. There is a need to know the precise nature of the "core death pathway." Are proteases central to this pathway, and, if so, what are the key proteolytic substrates? Are CED-9/BCL-2 family members part of the core death pathway, or do they regulate entry into apoptosis? How are the kinase cascades involved in signaling apoptosis linked to the core death pathway? It is clear that the answers to these questions will come from many different lines of research. There is much to be learned from the continued genetic analysis of simple multicellular organisms such as *C. elegans*. There are certainly additional members of the *ced-3*/*ice* and *ced-9*/*bcl-2* families to be identified in higher eu-

karyotes, and the human homolog of *ced-4* has yet to be identified. The molecular biology of apoptosis is considerably more advanced than the cell biology of apoptosis. It will be important to learn the subcellular localization of the known apoptotic effectors and determine how they interact with one another in the cell. Regarding the link between kinase cascades and death effector pathways, it is likely that the production of mutant mice lacking individual kinases will distinguish kinases that are essential for apoptotic triggering from those whose activation is merely correlated with apoptosis. In the end, of course, the most exciting advances in our understanding of apoptotic signaling will come from unexpected directions that are impossible to predict.

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