CELL DEATH PROTEINS

Edited by Gerald Litwack

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CELL DEATH PROTEINS

Edited by

GERALD LITWACK
Department of Biochemistry and Molecular Pharmacology
Jefferson Medical College
Thomas Jefferson University
Philadelphia, Pennsylvania

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Preface

This volume on Cell Death Proteins is timely in view of the explosive interest in programmed cell death or apoptosis, the obvious counterpart to cell proliferation. Although the field is progressing rapidly, this volume is a snapshot in time of several topics important to our knowledge of apoptosis.

Of the six contributions included here, some focus on apoptotic proteins and others on the inhibitors of apoptosis. The first paper is from the laboratory of R. Muschel and collaborators, who discourse on cell cycle checkpoints and apoptosis. M. Tocci reviews the structure and function of interleukin-1β-converting enzyme, the prototypical protease involved in the mammalian cell death machine. R. Baserga and collaborators discuss the role of IGF-I receptor in apoptosis. J. Reed reviews the Bcl-2 family of proteins, and L. Grasso and W. E. Mercer review p53-dependent apoptosis. Finally, A. Uren and D. Vaux inform us about viral inhibitors of apoptosis.

Thus, the contents of this book provide an excellent overview of some of the major players in the cell death cascade and its controls. Interested readers will be found in every sector of modern biological research, including cancer research, signal transduction, and biochemistry.

GERALD LITWACK

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Cell Cycle Checkpoints and Apoptosis: Potential for Improving Radiation Therapy

RUTH J. MUSCHEL,* W. GILLIES MCKENNA,† AND ERIC J. BERNHARD†

*Department of Pathology and Laboratory Medicine
and †Department of Radiation Oncology
University of Pennsylvania
Philadelphia, Pennsylvania 19104

I. Introduction

Current understanding of the events leading to cell death, including the induction of apoptosis, has suggested new approaches for enhancing the efficacy of cancer therapy. The thrust of this review is to consider some of the current information available about apoptosis and to assess its potential use to enhance the efficacy of radiation therapy. Radiation is used effectively for Hodgkin's lymphoma (Hoppe, 1990) and other lymphomas (Jacobs *et al.*, 1993), squamous cell carcinomas of the head and neck (Harari and Kinsella, 1995) and cervix (Perez, 1993a,b), and prostate (Perez *et al.*, 1993) and breast (Mansfield *et al.*, 1991) cancer, but, unfortunately, in many cases treatment does not eliminate the tumor. There are two different avenues through which augmentation of apoptosis might improve cancer therapy. Since cancer cells may die by apoptotic processes after exposure to radiation (or to various chemotherapeutic agents), targeting the apoptotic process might be expected to alter therapeutic success. If it were possible to increase apoptosis in the tumor after treatment, it might be possible to greatly enhance the death of the cancer cells after exposure to therapeutic agents. It is also

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possible to contemplate therapies that might directly induce apoptosis in tumor cells without the application of toxic agents, although we do not discuss this strategy here. This approach would include the application of various hormonal agents or blockage of autocrine growth factor loops. In this review we discuss the induction of apoptosis in cancer cells by therapeutic agents, especially ionizing radiation, and consider some of the genetic mechanisms known to control that induction and their link to cell proliferation. We also consider whether the data in fact support the idea that manipulation of apoptosis or of cellular proliferation checkpoints will affect survival of tumor cells after treatment with toxic agents such as radiation.

II. Apoptosis

In 1972, Kerr and colleagues distinguished between two forms of cell death, necrosis and apoptosis. Necrosis is the form of death that results from cellular metabolic collapse, when a cell can no longer maintain ionic homeostatis. Necrosis is not seen in normal development, but is a response to injury or toxic damage. Apoptosis, or programmed cell death, as it is also known, is a central part of normal development. It is a genetically mediated form of cell death that is involved in organogenesis, tissue homeostasis, and the editing of the immune system to remove autoreactive clones (Clarke *et al.*, 1993; Kerr *et al.*, 1972). In tissues, apoptosis characteristically affects scattered individual cells, in contrast to necrosis, which usually involves tracts of contiguous cells. Inflammation is typically absent in areas of tissue undergoing apoptosis. Apoptotic cells lose contact with their neighbors, decrease in size, and show condensation of chromatin (Kerr *et al.*, 1972). In the past, many of the cells undergoing apoptosis were called pyknotic by pathologists on the basis of their morphology. In apoptotic cells, the DNA is often degraded at the internucleosomal linker sites, yielding DNA fragments in multiples of 180 bp and resulting in a nucleosomal ladder, although this does not always occur (Arends *et al.*, 1990); sometimes the DNA is degraded into larger fragments (Walker and Sikorska, 1994). The cell membrane proteins are cross-linked, making the membrane more rigid (Dive *et al.*, 1992), and the apoptotic cells are typically phagocytosed by adjacent cells or macrophages. Under some circumstances apoptosis can also be seen in response to abnormal stimuli, including hormonal or growth factor manipulations; aberrant gene expression, particularly of oncogenes or antioncogenes; and a number of toxic agents, including chemotherapeutic drugs and X-rays (D'Amico and McKenna, 1994).

It is for the latter reasons that apoptosis has generated great interest among oncologists and cancer biologists, both because of the potential insights it may yield into carcinogenesis and in the hope of generating new strategies for cancer treatment (see additional reviews by D'Amico and McKenna, 1994; Lane, 1992; Reed, 1994; Wyllie *et al.*, 1990).

While cells that are irradiated may undergo apoptosis, they may also die via other modes, sometimes called mitotic death (Hurwitz and Tolmach, 1969), or they may die by necrosis, although this is uncommon at lower doses. Primary lymphocytes or thymocytes readily undergo apoptosis within a relatively short time after irradiation, but primary fibroblasts die with little evidence of apoptosis. Tumor cells also may die after irradiation by an apoptotic process, but this varies depending upon the particular cell line (Meyn *et al.*, 1993). At the present time, the determinants leading to apoptosis are not known. In the case of irradiated lymphocytes, apoptosis occurs rapidly after irradiation, within 6–8 hr. In some of the radiobiological literature this is called an interphase death (Hurwitz and Tolmach, 1969; Yamada and Ohyama, 1988). In contrast, radiation of tumor cells in culture may lead to apoptosis, but, when apoptosis occurs, it is usually seen after a longer time course, with initial evidence of apoptosis seen only after 12–24 hr and peaking at 48 hr (McKenna *et al.*, 1996). Radiation of tumors *in vivo,* however, sometimes leads to rapid apoptosis (Meyn *et al.*, 1993). Thus there appear to be kinetic distinctions in the induction of apoptosis reflecting differences in cell type and cellular milieu.

In terms of cancer therapy, the loss of replicative potential is the most important determinant of therapeutic outcome; loss of immediate viability is not a prerequisite for successful treatment. If cells do not undergo apoptosis, they may remain viable, as judged by trypan blue exclusion or maintenance of metabolic capabilities, but be unable to form colonies. Their nuclei do not fragment and they do not show other morphological features of apoptosis. This form of cell death is manifested not by an immediate loss of viability, but instead by the failure of clonogenic survival (Puck and Markus, 1956). The ultimate defect in the ability to replicate is not seen immediately in that several rounds of replication will often occur after irradiation. Thus clonogenic assays, in which the ability of each cell to continue successive rounds of replication is determined, are frequently used to evaluate the lethal potential of irradiation or treatment with chemotherapeutic drugs. Loss of viability will of course be accompanied by loss of clonogenic capacity, but loss of viability is in itself not sufficient to indicate whether cells have the capacity to continue to proliferate over many generations. For example, after treatment of cells with doses likely to lead to loss of 50–99%

of clonogenic survival, the vast majority of the cells will complete one cell division and at least 70% will complete several more divisions yet will not be able to replicate sufficiently to form colonies (O'Reilly *et al.*, 1994). Thus the results of clonogenic survival assays may give different results from the immediate assessment of viability. There is now accumulating evidence that eventually some of the cells that stop dividing will undergo an apoptotic cell death many days after their first exposure to the toxic agent (Tauchi and Sawada, 1994).

III. Linkage of Cell Cycle Checkpoints to Apoptosis

After irradiation, normal cells greatly increase their transit time through the cell cycle due primarily to arrests at G1 and G2. These arrests were described in the first paper accurately defining the phases of the cell cycle (Howard and Pelc, 1953). This classic work described the G1, S, G2, and M components of the cell cycle and noted that both G1 and G2, but not S or M, were prolonged in irradiated cells. It was subsequently noted that all eukaryotic cells undergo a G2 delay after irradiation, but that only some cells undergo a G1 arrest (Yamada and Puck, 1961). It should also be noted that at higher doses S-phase perturbations can also be detected (Painter, 1962; Painter and Young, 1975; Watanabe, 1974). It is now known that expression of wild-type p53 is required for the G1 arrest (Kastan *et al.*, 1991). It is interesting to note that many of the genes that affect the G1 arrest also influence whether cells undergo apoptosis. For example, overexpression of Myc leads cells to bypass the usual G1 arrest that is induced by serum starvation. Correlating with the loss of the G1 checkpoint is the induction of apoptosis (Evan *et al.*, 1992). Cells with absent or mutated p53 do not undergo a G1 arrest, but the loss of the G1 arrest does not necessarily lead to apoptosis (Hermeking and Eick, 1994). In contrast, the expression of wild-type p53 can lead to apoptosis, but this is not always a consequence of overexpression of p53. In some cases cells with increased levels of wild-type p53 will undergo a G1 arrest, but in other cases the presence of wild-type p53 lead to apoptosis. For example, irradiated fibroblasts with wild-type p53 undergo a G1 arrest but do not die, at least over the first 72 hr after irradiation, by apoptotic mechanisms (Di Leonardo *et al.*, 1994; McKenna *et al.*, 1996), whereas thymocytes rapidly undergo apoptosis that is p53 dependent (Lowe *et al.*, 1993). An important question under investigation is what determinants regulate whether a cell will arrest in G1 or will instead undergo apoptosis. Kastan has postulated in one system, that of a cell line dependent upon

interleukin-3, that the absolute level of p21 (WAF1/CIP1) might contribute to this determination (Canman *et al.,* 1995), but, while intriguing, there is as yet no general support of this observation.

In many normal tissues there is a counterbalance between cell proliferation and programmed cell death, or apoptosis. A characteristic feature of many tumors is the loss of G1-specific regulators of proliferation (Hartwell and Kastan, 1994). However, if tumor cells are to continue to proliferate and to increase in cell number, then they must also lose the apoptotic response. Mutation of p53 appears to confer both properties during ultraviolet light-induced skin carcinogenesis. Thus cells with DNA damage may die by apoptosis after irradiation due to induction of wild-type p53, but cells that have p53 mutations will continue to proliferate and actually establish sectors of skin bearing the mutation (Zeigler *et al.,* 1994). Zarbl's group has described similar sectors of cells with *ras* mutations in the mammary glands of mice that give rise to tumors upon *N*-nitroso-*N*-methylurea exposure (Cha *et al.,* 1994). These mutations may also act to suppress apoptosis during mammary carcinogenesis, as Ras can inhibit apoptosis in some settings (McKenna *et al.,* 1996; Nooter *et al.,* 1995). These observations have led to the hypothesis that the loss of G1-specific regulation must be accompanied by suppression of apoptosis as a necessary step in carcinogenesis. Similar selection for p53 mutations may occur during severe hypoxia in tumors. Graeber *et al.* (1996) showed that cells with wild-type p53 undergo apoptosis at very low oxygen tensions, while cells with mutations in p53 do not, leading to a selection for cells with mutations in p53 under hypoxic conditions both *in vitro* and *in vivo.*

The loss of the G1 checkpoint in itself does not lead to the inability of cells to undergo apoptosis. In tumor cells the G1 checkpoint is frequently missing, perhaps because of ablation of antioncogene activity, such as loss of *Rb* of p53, or because it is overridden by the effects of other oncogenes such as *myc.* Many tumor cells that have lost components of G1 regulation, including loss of G1 arrest after serum starvation or irradiation, can nonetheless be induced to undergo apoptosis after treatment with a variety of other stimuli, including radiation and some chemotherapeutic drugs (Bernhard *et al.,* 1996; Lock *et al.,* 1994; McKenna *et al.,* 1996). The study of human tumor cells in tissue culture and oncogene transfection of cells allows modeling of some of these situations. We have studied oncogene-transfected rat embryo fibroblasts and found that *myc*-transfected rat embryo fibroblasts lack a radiation-induced G1 block but are induced to undergo apoptosis by radiation or by serum withdrawal (McKenna *et al.,* 1996). Co-transfection of *ras* with *myc* overrides the apoptotic effect of X-rays but not of serum with-

drawal. Transfection of *ras* does not restore the G1 checkpoint but greatly increases the radiation-induced G2 delay (McKenna *et al.*, 1991). A similar situation is seen in HeLa cells, which are very resistant to radiation-induced apoptosis and lack a G1 checkpoint but have a pronounced G2 checkpoint after X-irradiation (Yamada and Puck, 1961). Thus, effects at the G1 checkpoint cannot be the sole determinants of apoptosis in tumor cells, and we have proposed the hypothesis that events controlling the G2 checkpoint also can impact the induction of apoptosis similar to the effects at G1 (McKenna *et al.*, 1996). This checkpoint, however, will only come into play in those situations when a G2 checkpoint is induced.

Finally, it should be noted that apoptosis is clearly independent of cell cycle effects in some cases. Treatment of thymocytes with steroids occurs while the cells are in G_0, and the resulting apoptosis is a p53-independent process (Clarke *et al.*, 1993; Donehower *et al.*, 1992; Lowe *et al.*, 1993). There is also no evidence that apoptosis induced by hormone withdrawal from prostate or breast tissue is related to the cell cycle (Berges *et al.*, 1993; Kyprianou *et al.*, 1991).

IV. Regulation of Cell Proliferation and the Control of Apoptosis

There are several instances in which control of apoptosis seems to be linked to some of the genes that also regulate cell cycle progression. Aberrant regulation of cell proliferation, both by subversion of tumor suppressor gene activity and by the action of dominant oncogenes, is now recognized to be the hallmark of most if not all malignancies. These changes might represent useful targets for therapeutic manipulation of apoptosis. This linkage has been recognized both for the tumor suppressor genes p53 and *Rb* and for the dominant oncogene *myc* (Hermeking and Eick, 1994; Morgenbesser *et al.*, 1994; White, 1994). Additionally, it has been recognized that many transforming viruses exert their transforming function in part by abrogating apoptotic functions in the cell (Scheffner *et al.*, 1990; White *et al.*, 1992). This has been most fully studied for simian virus 40 and the herpes and adenoviruses, but similar mechanisms clearly are also found in polyomavirus, Epstein–Barr virus, and the baculoviruses (Shen and Shenk, 1995). In viruses these antiapoptotic functions presumably evolved to permit viral replication, which could be subverted by apoptosis, but they in turn promote the uncontrolled cellular proliferation that is similarly seen in both virally induced and spontaneous tumors (Nevins, 1995; White, 1993). We discuss some of this evidence for a few oncogenes, using them

as examples of the evidence relating apoptosis to cell proliferation rather than as an exhaustive review of the relationship of oncogenes to apoptosis.

A. p53 AND APOPTOSIS

Mutations in the p53 gene are strongly implicated in human and animal carcinogenesis. The p53 protein can also be a significant regulator of the process of apoptosis. Thymocytes undergo apoptosis at the G_0 to G1 transition in response to glucocorticoids of X-rays (Telford *et al.*, 1991). For X-ray-induced apoptosis, but not for glucocorticoid-induced apoptosis, p53 expression is necessary (Lowe *et al.*, 1993). Thymocytes from p53-null mice are extremely resistant to induction of apoptosis in response to X-rays. After irradiation, the crypt cells of the large and small intestine are highly susceptible to apoptosis. Homozygous p53-null mice fail to show apoptosis in the colonic crypts after receiving a dose of 8 Gy, while the $p53^{-}/p53^{WT}$ heterozygous mice undergo substantial apoptosis after the same dose (Merritt *et al.*, 1994).

Mutations of p53 are now recognized to be the most common genetic changes in human cancer (Hollstein *et al.*, 1991). Mutant p53 was first isolated as what was believed to be a dominant cooperating oncogene that behaved in many ways like *myc* or the E1A gene of adenovirus in that it would cooperate with *ras* in transformation assays of 3T3 cells (Parada *et al.*, 1984). Subsequently it was realized that the form of p53 that was first isolated yielded a mutant, inactive, but long-lived form of the protein that displaced the wild-type but short-lived native form from its binding site, and because of this appeared to act dominantly. Thus p53 is now more properly thought of as a tumor suppressor gene.

The action of p53 is complex. It binds to many important cellular proteins and is involved in the control of gene expression (Miyashita *et al.*, 1994). The last several years have seen an intense focus on its roles in cell cycle delay in G1 phase and in apoptosis. It is now recognized that p53 can both regulate cell proliferation and induce apoptosis depending on the circumstances and cellular background (DiLeonardo *et al.*, 1994; Fan *et al.*, 1994; Oren, 1992). In some cases merely the introduction of an expression vector for wild-type p53 is sufficient to induce apoptosis (Liu *et al.*, 1994; Wang *et al.*, 1993), although, as noted later, in other cases overexpression of p53 leads to a G1 arrest.

An insight into the complex role of p53 can be derived from studies of its interaction with the *Rb* gene product. Two studies have demonstrated that, in the absence of retinoblastoma function, the apoptotic action of p53 can compensate for this loss and thus prevent malignant transformation (Morgenbesser *et al.*, 1994; Pan and Griep, 1994). The

Rb protein prevents cell cycle progression at G1–S by inhibiting members of the E2F family of transcription factors, thereby inhibiting the expression of many genes implicated in S phase, including TK, *myc, myb, DHFR,* and DNA polymerase α (Almasan *et al.,* 1995; Chen *et al.,* 1995). Normal expression of the *Rb* gene product in the developing lens causes the cells to undergo growth arrest and to terminally differentiate. Studies in *Rb* knockout mice have shown that, in the absence of Rb, the cells continue to enter S phase; however, an active p53 gene product causes the cells to undergo apoptosis. Such mice show abnormal eye development (micropthalmia) (Morgenbesser *et al.,* 1994). Studies in p53 knockout mice show normal eye development since in these mice Rb functions normally (Donehower *et al.,* 1992). Double-knockout mice cannot be studied since they die in midgestation, but an insight into the effect of the absence of both genes can be gained from the study of mice transgenic for the human papillomavirus E6 and E7 genes, which target and inactivate p53 (Scheffner *et al.,* 1990) and Rb (Munger *et al.,* 1989). Such transgenic mice develop eye tumors (Pan and Griep, 1994). In this case, therefore, the apoptotic action of p53 is essential for its tumor suppressor activity in the absence of Rb. It is of note that the functions of both gene products are tightly linked to cell cycle events in the G1 phase of the cell cycle. The *Rb* gene product is linked to events at the G0–G1 transition, while p53 is able to induce G1 arrest as well as apoptosis.

X-rays or other DNA-damaging agents have been shown to increase the levels of p53 by a posttranslational mechanism. Increased p53 is known to result in a G1 arrest (Kastan *et al.,* 1991; Kuerbitz *et al.,* 1992), and this arrest is mediated by WAF1/CIP1 (El-Diery *et al.,* 1994). Increases in the level of p53 lead to transcriptional activation of promoters that contain a p53 consensus site (Kern *et al.,* 1992). Increased levels of WAF1/CIP1 are induced via this mechanism, as well as being controlled by p53-independent pathways (Macleod *et al.,* 1995). WAF1/CIP1 binds to cyclin–cyclin-dependent kinase (cdk) complexes, including cyclin D1–cdk 4 and cyclin E–cdk 1, resulting in inhibition of kinase activity and hence a G1 arrest (Xiong *et al.,* 1993). Whether physiologically increased levels of WAF1/CIP1 lead to S-phase arrests has not been reported but might be expected since recombinant WAF1/CIP1 inhibits DNA replication in an *in vitro* system (Li *et al.,* 1994). In that same *in vitro* system, while DNA replication was inhibited, DNA excision repair was unaltered, suggesting that repair could continue unabated in the presence of a DNA replication block, although there is one report indicating some conditions under which WAF1/CIP1 might also inhibit DNA excision repair (Hurwitz, 1995). The effect of WAF1/CIP1 on DNA replication may well be mediated by its ability to

interact with proliferating cell nuclear antigen (PCNA), a component of DNA polymerase δ (Li *et al.,* 1994; Luo *et al.,* 1995).

Also, p53 has the ability to induce transcription of GADD45, another protein capable of binding to PCNA (El-Deiry *et al.,* 1992; Fornace *et al.,* 1989; Kastan *et al.,* 1992). GADD45 in *in vitro* systems actually stimulated excision repair, suggesting a mechanism through which activation of p53 might lead to increased DNA repair and hence increased survival. However, because p53 expression may also lead to apoptosis, in many systems expression of wild-type p53 actually leads to lessened survival, not increased survival.

A model is emerging to synthesize these data into an explanation of the role of p53 in response to DNA damage. After DNA damage occurs, the levels of p53 increase through posttranslational mechanisms. The increased levels of p53 activate WAF1/CIP1 transcription through direct action of p53 on the WAF1/CIP1 promoter, resulting in a G1 arrest. During this arrest the cell attempts to repair the damage, perhaps with enhanced repair capacity due to the induction of GADD45 and activation of other repair processes. If unable to repair the damage, the cell then undergoes apoptosis, and the organism thereby eliminates a damaged and potentially mutated cell. The role of the G1 arrest in this pathway is still not fully understood. Wang *et al.* (1995) showed that Bcl-2 inhibited the p53-induced apoptosis but not the G1 arrest, showing that the two processes are not obligatorily linked. This conclusion was also demonstrated by the properties displayed by WAF1/CIP1 knockout mice (Brugarolas *et al.,* 1995; Deng *et al.,* 1995). The cells from these mice have a complete lack of the G1 arrest after exposure to various DNA-damaging agents and an impaired G1 arrest after irradiation, demonstrating the importance of WAF1/CIP1 induction in the G1 arrest. After irradiation, these cells undergo levels of apoptosis comparable to the amount found in cells from the parental mice. It has not yet been reported whether other DNA-damaging agents induce apoptosis in this system. Cells in which WAF1/CIP1 has been eliminated by homologous recombination in tissue culture also display the loss of the DNA damage-induced G1 checkpoint (Waldman *et al.,* 1995). These experiments also separate the p53-dependent pathways leading to G1 arrest and apoptosis.

B. *Myc* AND APOPTOSIS

The *myc* oncogene has also been intensively studied for its roles in both cell proliferation and apoptosis. In nontransformed cells, Myc expression is tightly linked to mitogenic stimuli and is a prerequisite for cell growth. Myc expression has been shown to be both necessary and

sufficient to cause G_0 fibroblasts to enter the cell cycle. Conversely, immortalized fibroblasts that constitutively express Myc are unable to exit the cycle upon serum withdrawal, and they then undergo apoptosis. Similar results are seen in lymphocytes expressing *myc* (Harrington *et al.,* 1994a). The relationship of Myc-induced apoptosis to cell cycle progression is somewhat unclear. The *myc* oncogene is an early response gene whose expression rises rapidly at the G_0–G1 transition and whose currently known functions are largely linked to G1 and early S. Unlike most early response genes, however, Myc expression is sustained throughout the cell cycle. In addition to its proliferative function, Myc can be shown to induce apoptosis in G_0 and G1, and Evan has argued that it can also do so in S phase (Evan *et al.,* 1992). In the experiment he performed, cells carrying an estrogen–Myc chimeric protein, in which estrogen induces Myc activity, were held for 48 hr in high concentrations of thymidine to induce an S-phase arrest. When estrogen was added after 48 hr, 100% of the cells in the culture underwent apoptosis, from which Evan concluded that Myc could induce apoptosis in S phase. From this experiment Evan has argued that the apoptotic action of Myc is not cell cycle specific.

Evan has argued also that *myc* is a gene whose proliferative function is not linked to any specific phase of the cell cycle. Its continued, unregulated expression throughout the cycle would tend to support this point of view. However, multiple experiments point to roles for Myc at very specific points in the cell cycle. As was previously stated, Myc expression can cause G_0 cells to enter the cycle (Eilers *et al.,* 1991). In continuously proliferating cells, a role for Myc can also be demonstrated in G1. Serum withdrawal from proliferating fibroblasts leads to rapid disappearance of Myc from all cells. Nevertheless, cells in S, G2, and M at the time of serum withdrawal continue to move through the cycle and the entire population accumulates in G1. Interferon-γ and transforming growth factor-β (TGF-β) lead to rapid downregulation of Myc, and these agents also lead to a G1 arrest (Pietenpol *et al.,* 1990). [TGF-β also causes cell cycle arrest by inducing inhibitors of cdks (Hannon and Beach, 1994; Polyak *et al.,* 1994; Toyoshima and Hunter, 1994).] These effects are not restricted to fibroblasts. Treatment of proliferating keratinocytes or hematopoietic cells with antisense oligonucleotides to c-Myc leads to an accumulation of cells in G1 (Heikkila *et al.,* 1987). There is thus an abundance of evidence that Myc plays an essential role in G1. Evidence for its role in other parts of the cycle is much more limited. Shibuya *et al.* (1992) studied BAF-B03 pro-B cells expressing a transfected epidermal growth factor (EGF) receptor. These cells did not express c-Myc in response to EGF but entered S normally, only to block before entering G2. This led the authors to conclude that Myc was nec-

essary for the S–G2 transition in these cells, although S–G2 blocks were not noted in the other experiments described earlier (Shibuya *et al.*, 1992). These experiments, taken together, provide a somewhat confusing view of the role of Myc in relationship to the cell cycle. They do, however, suggest that the actions of the *myc* oncogene are certainly not random with respect to the cell cycle, but they may be very specific with regard to the system under study.

Several models have been proposed to explain the apparently contradictory roles of Myc in both proliferation and apoptosis. Evan originally proposed what he termed the "conflict model." Myc is viewed in this model as promoting proliferation. In the presence of mitogens there is no conflict between the action of Myc and the other signals the cells are receiving via other mitogens. When mitogens are *absent,* however, as in serum withdrawal or when a cytostatic drug is added, there is a "conflict" between the signal from Myc to proliferate and the other signals the cells are receiving to cease proliferation. This "conflict" is viewed, in an unspecified manner, as leading to apoptosis. However, it is now thought that the functions of Myc in promoting proliferation and apoptosis may be inseparable. Myc is a transcriptional regulator acting via an amino-terminal transactivating domain that can be shown to be involved in both proliferation and apoptosis (Evan and Littlewood, 1993). Levels of Myc expression correlate with both proliferative capacity and susceptibility to apoptosis (Evan and Littlewood, 1993). Finally, Myc is known to form an active complex by heterologous dimerization with a partner protein named Max. Myc–Max dimerization has been shown to be necessary for both proliferation and apoptosis (Amati *et al.,* 1993; Amati and Land, 1994; Evan and Littlewood, 1993). As evidence has accumulated that Myc is itself directly involved in the control of apoptosis, Evan has more recently modified the conflict model by proposing what he now terms the "dual signal" model. In this model, Myc simultaneously activates *both* a proliferative and an apoptotic pathway (Harrington *et al.,* 1994b). Mitogens then stimulate the proliferative pathway while the apoptotic pathway is actively held in check by other antiapoptotic cellular factors. The most widely studied of these antiapoptotic factors is the oncogene *bcl-2.*

C. *Bcl-2* AND APOPTOSIS

The *Bcl-2* oncogence was discovered as a gene whose expression was increased by chromosomal translocations in B-cell malignancies (this subject has been extensively reviewed by Reed, 1994). It is found to be activated in the majority of follicular non-Hodgkin's lymphomas. It has also been noted less commonly in other malignancies, such as prostate

cancer and breast cancer. Its activation has also been seen in some benign conditions such as follicular hypertrophy of lymph nodes and tonsils. For several years the function of *bcl-2* was viewed, like that of many other oncogenes, as promoting proliferation or cooperating with other oncogenes in doing so. More recent years have seen attention focused on its function as an opponent of apoptosis or of cell death (Korsmeyer, 1992). In multiple systems—lymphocytes, fibroblasts, neurons, hematopoietic cells, and the like—expression of Bcl-2 can be shown to delay or even prevent apoptosis after most but not all stimuli (Sentman *et al.,* 1991; Vanhaesebroek *et al.,* 1993). Conversely, downregulation of Bcl-2 in many of these same systems can be shown to promote apoptosis. Its mechanism of action in this regard is poorly understood at this time. Bcl-2 is a membrane-associated protein that in the intact cell is largely found in the nuclear envelope, endoplasmic reticulum, and mitochondria (Akao *et al.,* 1994; de Jong *et al.,* 1994; Hockenbery *et al.,* 1990). The *bcl-2* gene is a member of a family of genes including *bax, bcl-X* (which gives rise to two protein products, Bcl-X_L and Bcl-X_S), *mcl-1,* and *Al.* It is also similar to the *ced-9* gene of *Caenorhabditis elegans* (Craig, 1995), and its gene product is related in function, and to some extent in sequence, to a number of viral proteins, such as p19-E1B of adenovirus, p30 of baculovirus, and BHFR-1 of Epstein–Barr virus (Oltvai *et al.,* 1993; Williams and Smith, 1993). Some of the products of these genes function like Bcl-2 in preventing apoptosis (p19-E1B, Bcl-X_L); others (Bax, Bcl-X_S) oppose Bcl-2 action and hence promote apoptosis. It has been suggested that apoptosis is regulated not by Bcl-2 alone but by the ratio of Bcl-2 to Bax in the cells where both are found. Korsmeyer has suggested that the Bcl-2–Bax heterodimer constitutes a "pre-set rheostat within cells" determining the extent of apoptosis (Oltvai *et al.,* 1993). Another Bcl-2 binding protein, BAD, may act in a similar way to Bax. Korsmeyer's group and others have suggested that Bcl-2 may be exerting its antiapoptotic action as a scavenger for oxidative free radicals (Hockenbery *et al.,* 1993; Kane *et al.,* 1993). Our own data (Muschel *et al.,* 1995) and those of Jacobsen and Raff (1995) and of Shimizu *et al.* (1995) on the induction of apoptosis under hypoxia were not consistent with this model. Thus the mechanism of action of Bcl-2 remains unclear, but most data would place it downstream of p53, Myc, or Ras in the pathways of apoptosis. While Bcl-2 can block or at least delay the induction of apoptosis, in many cases its expression does not oppose all forms of apoptosis.

The effects of Bcl-2 on apoptosis appear to be independent of proliferation checkpoints. Bcl-2 specifically blocks the ability of c-Myc to induce apoptosis but does not affect its mitogenic properties (Bissonnette

et al., 1992; Fanidi *et al.,* 1992; Wagner *et al.,* 1993). Bcl-2 also blocks adenovirus E1A-induced apoptosis and can thus replace the antiapoptotic function of the adenovirus E1B protein (Rao *et al.,* 1992). Expression of Bcl-2 can block Myc-induced apoptosis after serum withdrawal (Fanidi *et al.,* 1992). Transgenic mice engineered to express Bcl-2 and Myc develop B-cell lymphomas at a much higher rate than mice engineered to express only one of the two proteins, perhaps because of the ability of Bcl-2 to block the Myc-induced apoptosis, thereby leading to unabated proliferation (Marin *et al.,* 1995). Two things should be noted, however. Myc deregulation appears to be a more frequent event than Bcl-2 overexpression in human malignancies of many kinds. Deregulation of both genes together has not so far been commonly seen in human cancers. There may be many reasons for this. Bcl-2 expression is common in many tissues in the embryo but is uncommon in adult tissues, with a few exceptions such as B cells or intestinal crypt cells. Second, while *myc* and *bcl-2* have been described as cooperating oncogenes based on experiments such as that described in transgenic mice, the nature of their cooperation is different from that seen between *myc* and *ras, raf,* and some of the kinase oncogenes, such as *src.* In fibroblasts, coexpression of Myc and Ras results in colonies that express morphological transformation, are focus forming, have lost contact inhibition, and are tumorigenic. Cells expressing Myc and Bcl-2 are not tumorigenic and have a flat, nontransformed morphology. These cells are resistant to the induction of apoptosis by serum deprivation. Of course, the expression of the other proteins that interact with Bcl-2 may be the critical element, and these studies are only beginning.

D. E1A and Apoptosis

The adenovirus system has provided support for the interpretation that blocking apoptosis is important for tumorigenicity and is linked to expression of genes deregulating cell cycle controls. White (1993, 1994) proposed a model to explain the actions of the E1A and E1B oncogenes of adenovirus in apoptosis and transformation. The E1A gene product of adenovirus targets the *Rb* gene product and inactivates, it thereby leading to disruption of the G1 checkpoint. By White's model this leads, in the presence of normal p53 function, to apoptosis. Mymryk *et al.* (1994) have shown that E1A does not induce apoptosis in proliferating cells, suggesting that the disruption of a proliferation block in quiescent cells is a necessary component for the induction of apoptosis in this system. This finding is analogous to Evan's results using *myc* (Evan *et al.,* 1992). In similar experiments, Gibson *et al.* (1995) showed that Chinese

hamster ovary cells overexpressing c-Myc underwent apoptosis during serum withdrawal, as expected from the experiments of Evan, but serum withdrawal did not induce apoptosis if the cells were arrested by confluence. These observations led to the prediction that E1A action as a cooperating oncogene in transformation requires the action of another gene that will alter the proliferation status of the cells; otherwise the E1A effect alone would kill the cells. The same is true in viral infections in which E1A action is supplemented by E1B, which acts to suppress the apoptosis that would be induced by the unopposed action of E1A (White *et al.*, 1992). Lowe and Ruley (1993) have shown that expression of E1A can stabilize the p53 protein and so lead to increased levels of p53, and this may be a contributing mechanism to the induction of apoptosis. The E1B gene gives rise to two protein products, p55 and p19. The p55 protein targets and inactivates p53. However, the genes of adenovirus demonstrate an additional level of control over apoptosis. The second product of the E1B gene, p19, opposes apoptosis but does not target p53. It appears to oppose the apoptotic process directly in a manner analogous, and perhaps identical, to the effects of the *bcl-2* oncogene. The *ras* oncogene can also oppose the apoptosis-inducing capacity of E1A (Lin *et al.*, 1995), and this may account for the ability of E1A to cooperate with the *ras* oncogene in transformation of primary rodent fibroblasts.

V. Effect of Oncogenes on Radiation-Induced Apoptosis

As was noted earlier, fibroblasts generally do not undergo apoptosis after irradiation. However, irradiation of Myc-immortalized fibroblasts leads to apoptosis in a substantial number of the cells. We and others have shown that, at any tested dose of X-rays, there was substantially more apoptosis in the cells overexpressing Myc than in the cells transfected with the ras^{H} oncogene plus *myc* (Ling *et al.*, 1994a,b; McKenna *et al.*, 1996). This result contrasts with the induction of apoptosis by serum withdrawal, where apoptosis occurred at high levels equally in *myc*- and *ras* plus *myc*-transfected cells (McKenna *et al.*, 1996). Other workers have evaluated the induction of apoptosis in H-*ras*-transformed cells. Evan *et al.* cite unpublished data that *ras* does not have an antiapoptotic effect after serum withdrawal (Harrington *et al.*, 1994b). Arends *et al.* (1993) studied three cell lines derived from rat lung fibroblasts transfected with the *myc* and *ras* oncogenes after serum withdrawal. Two of the *myc* plus *ras* cell lines they examined showed significant apoptosis upon serum withdrawal while one did not. They related the effects on apoptosis to the level of available endonu-

clease in the cells, which was low in the cell line that did not undergo apoptosis. These data indicate that, while it can suppress induction of apoptosis after ionizing irradiation, Ras does not seem to inhibit apoptosis induction by serum withdrawal. Ras also suppresses apoptosis after the expression of E1A, as noted previously.

Neither *myc*-transfected fibroblasts nor ras^H plus *myc*-transformed cells undergo a G1 arrest after irradiation. However, after irradiation the ras^H plus *myc* cells undergo a substantial G2 delay while the *myc*-immortalized cells do not (McKenna *et al.*, 1991). The result is a greater induction of apoptosis in the cells with the lesser G2 delay. These results have led us to suggest that the induction of a G2 block by radiation can also serve an antiapoptotic function and that this is influenced by the ras^H oncogene. When the G2 delay was reduced in the ras^H plus *myc*-transformed cells by caffeine, the effect was an increase in apoptosis, as would be predicted by this model. In contrast, after serum withdrawal both cell types fail to arrest in G1 or G2 and both are subject to equivalent induction of apoptosis (McKenna *et al.*, 1996).

Other experiments also provide evidence that abrogation of the G2 delay induced by either radiation or other DNA-damaging agents may lead to increased apoptosis. Lock *et al.* (1994) found that high doses of caffeine potentiated the toxicity of etoposide and that, at high doses of caffeine, increased apoptosis was seen. Demarcq *et al.* (1994) found that treatment of cells with cisplatin led to a G2 delay that caffeine reduced, and that increased apoptosis and decreased survival were the result. Palayoor *et al.* (1995) also found that drugs that reduced the G2 delay resulted in increased amounts of apoptosis in EL4 cells. In TK6 lymphoma cells, Zhen and Vaughan (1995) found caffeine to diminish the G2 delay but also diminish the extent of apoptosis induced by ionizing radiation, but not by serum withdrawal. Bernhard *et al.* (1996) showed that HeLa cells that do not undergo apoptosis after irradiation or after treatment with caffeine or staurosporine alone do die via apoptosis after irradiation plus treatment with caffeine, a treatment that abolishes the G2 delay.

As mentioned earlier, one difference between v-*myc* and H-*ras* plus *myc* embryo cell lines is that radiation induces a much more pronounced G2 delay in the ras^H-transfected cells (McKenna *et al.*, 1991). We have also shown that the radiation-induced G2 block in HeLa cells and in ras^H plus v-*myc*-transformed cells is accompanied by a depression in cyclin B1 accumulation, even though cyclin A levels rise at the expected times (Muschel *et al.*, 1991, 1993). Furthermore, drugs such as caffeine, which are known to diminish the radiation-induced G2 block, also decrease the delay in cyclin B1 accumulation in HeLa cells (Bernhard *et al.*, 1994). Datta *et al.* (1992) also found that cyclin B1 mRNA levels

were decreased after exposure of U937 cells to radiation. These data suggest that lowered levels of cyclin B1 contribute to the G2 delay that is induced by irradiation. In contrast, v-*myc*-immortalized rat embryo fibroblasts (REF) undergo a relatively short G2 delay after irradiation and show no depression in cyclin B1 mRNA accumulation after irradiation. This suggests that the prolongation of the G2 block can be influenced by cyclin B1 mRNA levels. Overexpression of cyclin B1 can reduce the G2 block after irradiation, confirming this suggestion (Kao *et al.*, 1997).

There are multiple mechanisms contributing to controlling the G2 delay after DNA damage, with several targets having being identified in addition to cyclin B1. Lock and Ross (1990) initially showed that $p34^{cdc2}$ H1 kinase activity is depressed in HeLa cells after irradiation or treatment with etoposide (a topoisomerase II inhibitor), another DNA-damaging agent that results in a G2 delay. In the case of cells treated with etoposide, Lock (1992) found increased phosphorylation of cdc2 kinase during G2 in the arrested cells. O'Connor *et al.* (1993), using nitrogen mustard, another DNA-damaging agent, subsequently showed that this was an effect on the cdc2–cyclin B1 complexes. The cdc2–cyclin A complexes also showed decreased activity, but cdk2–cyclin A complexes were not inhibited (O'Connor *et al.*, 1993). Further evidence that the phosphorylation of cdc2 might be one target comes from experiments in *Schizosaccaromyces pombe,* where mutants in Wee-1, a kinase capable of phosphorylating cdc2, eliminate the radiation-induced G2 delay at lower doses, suggesting that phosphorylation of cdc2 is a component of the block in this case (Rowley *et al.*, 1992a), although other experiments implicated additional regulatory factors (Enoch *et al.*, 1992). Jin *et al.* (1996) overexpressed cdc2 that could not be phosphorylated and shortened the G2 delay, a result supporting the involvement of phosphorylation. In lymphoma cells, O'Conner *et al.* (1994) found that increased phosphorylation occurs after treatment with nitrogen mustard and provided evidence that this increase in phosphorylation was mediated by inhibition of cdc25C, the phosphatase responsible for dephosphorylating both Thr-14 and Tyr-15. Karbanda *et al.* (1994) have shown that lyn kinase is activated in irradiated cells and have suggested that lyn may phosphorylate cdc2. In addition to mechanisms that lead to decreased cdc2–cyclin B1 kinase activity, a series of highly radiosensitive mutants in yeast, termed rad or MEC, have been identified in which the DNA-damage-induced G2 delay is eliminated. Many of these mutants, but not all, have proven to affect genes involved in DNA repair (Carr, 1995; Rowley, 1992, Rowley *et al.*, 1992b; Weinert, 1992; Weinert and Hartwell, 1988; Weinert *et al.*, 1994). Thus there appear to be several targets for control of the G2 delay.

VI. Therapeutic Implications

We now return to the questions of whether it might be possible to increase the induction of apoptosis in tumor cells after radiation therapy and, second, of whether this might enhance therapeutic efficacy. In spite of the great interest in apoptosis as a mode of death in cancer cells, it is perhaps surprising that the data are still not available to decide whether enhancing apoptosis after treatment with a DNA-damaging agent will enhance its toxicity. Many of the genes discussed here in the context of their ability to regulate the induction of apoptosis are found to be altered in malignancy, and in some instances ways to manipulate these genes are beginning to be developed. The presence of high levels of Myc or mutations in *Rb* tend to lead to enhanced apoptosis, and in many systems it can be shown that this tendency must be opposed to lead to tumorigenesis. If in some cases apoptosis is blocked by Bcl-2, then perhaps drugs that block Bcl-2 action could be efficacious when used in combination with radiation or other DNA-damaging agents. Antisense Bcl-2 polynucleotides will block its expression (Reed *et al.*, 1994), and these could potentially increase apoptosis in the tumor cells after radiation treatment. The normal host cells that express little Bcl-2 should be unaffected. However, while Bcl-2 blocks apoptosis and enhances viability, there are only a few studies examining the effect of Bcl-2 expression on clonogenic survival. While survival is prolonged in cells expressing Bcl-2 treated with certain cytotoxic drugs (Miyashita and Reed, 1992, 1993), it is not prolonged after irradiation (Ruholtz *et al.*, unpublished data) or after aphidicolin treatment (Yin and Schimke, 1995). Activity of the *ras* oncogene has also been shown to oppose apoptosis after irradiation (McKenna *et al.*, 1996). Blockade of *ras* action (e.g., by farnesylation inhibitors) could be predicted to enhance toxicity (Bernhard *et al.*, 1996). Hematopoietic cells from mice lacking p53 do not undergo apoptosis after irradiation, but those cells from the mice with p53 do (Clarke *et al.*, 1993; Lowe *et al.*, 1993). This information might suggest that p53 would determine apoptosis in cancer cells. In this case clonogenic survival assays indicate that survival is prolonged in the cells without apoptosis (Lee and Bernstein, 1993). However, tumor cells with mutant p53 also undergo apoptosis. The extent to which p53 status can affect the response to therapy is under intensive investigation. Caffeine or a similarly acting drug, pentoxyphylline, has been shown to enhance apoptosis after irradiation of cells (Bernhard *et al.*, 1996); Lock *et al.*, 1994) or after treatment with chemotherapeutic agents (Shinomiya *et al.*, 1994). Interestingly, caffeine only reduces the G2 delay after treatment with X-rays or with DNA-damaging chemotherapeutic agents if the cells lack p53 (Fan *et al.*, 1995; Powell *et al.*,

1995; Russell *et al.*, 1995). Caffeine or pentoxyphylline does not appear to alter the cell cycle after DNA damage in cells with wild-type p53. While the mechanism for this effect is not understood, it raises the possibility of pharmacologically enhancing apoptosis in cancer cells with p53 mutations while not affecting the normal host cells. Although none of these approaches is close to actual clinical trials at present, they suggest at least the theoretical possibility of greatly enhancing the therapeutic effect of radiation or chemotherapeutic agents.

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Structure and Function of Interleukin-1β Converting Enzyme

MICHAEL J. TOCCI

Department of Molecular Immunology
Merck Research Laboratories
Rahway, New Jersey 07065

I. Introduction

Cells employ specific processing enzymes to control a number of important physiological and pathophysiological processes. These enzymes modulate the biological activities of individual polypeptides by converting them from one form to another, thereby generating an active, an inactive, or an altered form of the original protein. Interleukin-1β (IL-1β) is an extremely potent and broad-acting proinflammatory cytokine whose production is regulated, in part, by proteolytic processing (Black *et al.*, 1989; Kostura *et al.*, 1989; Dinarello, 1994). Initially, IL-1β is synthesized as an inactive 31-kDa precursor protein (pIL-1β). The precursor is cleaved by a highly specific proteinase, termed IL-1β converting enzyme (ICE; EC 3.4.22.36), as it is released from the cell, generating the mature biologically active cytokine. ICE is a cysteine proteinase that cleaves pIL-1β twice within the prodomain at aspartic acid residues (Thornberry, 1994; Tocci and Schmidt, 1996). The requirement for aspartic acid in the P_1 position of the substrate cleavage site is highly unusual, as is the finding that ICE is unrelated to all previously known cysteine proteinases (Black *et al.*, 1989; Kostura *et al.*, 1989;

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Sleath *et al.*, 1990; Howard *et al.*, 1991; Cerretti *et al.*, 1992; Thornberry *et al.*, 1992). Recent findings reveal that ICE is the prototype of a now rapidly growing family of novel cysteine proteinases that all appear to cleave at aspartic acid residues (Miller *et al.*, 1996). The development of potent inhibitors based on the unique substrate specificity of ICE and genetically engineered mice deficient in the enzyme confirmed the importance of the enzyme in pIL-1β processing and suggested that inhibitors of the enzyme may be useful in the treatment of inflammatory disease. This article reviews our knowledge of the structure and function of this intriguing new proteinase based on its purification, molecular cloning, three-dimensional structure, and functional deletion in mice.

II. The IL-1 System

Two members of the *IL1* gene family, designated IL-1α and IL-1β, are produced by mammalian cells. Each is encoded by a separate gene that produces a 31-kDa polypeptide (Auron *et al.*, 1984; March *et al.*, 1985). The active forms of IL-1α and IL-1β are alike in several respects. Both bind with similar affinities to the same cell surface receptors and elicit virtually indistinguishable biological responses (Sims and Dower, 1994). The COOH-terminal portions of the proteins that bind to IL-1 receptors (IL-1R) are less than 30% identical in amino acid sequence yet possess nearly superimpossible three-dimensional structures (Priestle *et al.*, 1988; Graves *et al.*, 1990; Clore *et al.*, 1991; Murzin *et al.*, 1992). The need for two *IL1* genes is not readily apparent since on first approximation IL-1α and IL-1β appear functionally redundant. However, subtle differences in their subcellular localization and cellular distribution suggest that there is a division of labor among these two members of the *IL1* gene family. IL-1α appears to remain cell associated, whereas IL-1β is released from the cell (Tocci and Schmidt, 1996). This difference suggests that the two molecules serve different biological functions. Indeed, certain inflammatory responses are absent in IL-1β-deficient knockout mice, confirming this idea (Kozak *et al.*, 1995; Zheng *et al.*, 1995).

Most stimuli induce the coordinate expression of both *IL1* genes. Their expression is transcriptionally, translationally, and posttranslationally controlled (Fenton *et al.*, 1987, 1988; Schindler *et al.*, 1990a,b; Fenton, 1992). The *IL1* genes are activated transcriptionally in response to external stress or inflammatory stimuli and then translated into 31-kDa polypeptides on free polysomes in the cytosol of the cell. Many cell types are capable of producing IL-1α and IL-1β, but activated monocytes and macrophages are the major producers (Arend, 1993a;

Tocci and Schmidt, 1996). In activated monocytes, the mRNAs for IL-1α and IL-1β can reach as much as 5% of the total polyadenylated RNA; however, under certain conditions not all of the mRNA is translated (Fenton *et al.*, 1987, 1988; Schindler *et al.*, 1990a,b; Fenton, 1992). Like many cytokines and early-phase response genes, the 3′ untranslated regions (3′UTR) of the mRNAs for IL-1α and IL-1β contain AU-rich sequence elements that reportedly regulate mRNA half-life, transport, and translation (Caput *et al.*, 1986; Cosman, 1987; Decker and Parker, 1995).

In activated monocytes, the 31-kDa primary translation products are abundant in the cytoplasm and can be detected on the plasma membrane (Bayne *et al.*, 1986; Singer *et al.*, 1988, 1995). IL-1 proteins are not secreted by the conventional secretory pathway. Transport from the cytosol to the plasma membrane occurs by a poorly understood mechanism (Kuchler, 1993). Neither IL-1α nor IL-1β possesses typical hydrophobic secretory signal sequences, nor are they detected in classical secretory organelles, such as endoplasmic reticulum, Golgi vesicles, or secretory granules (Bayne *et al.*, 1986; Bakouche *et al.*, 1987; Bursten *et al.*, 1988; Hazuda *et al.*, 1988; Singer *et al.*, 1988, 1995; Rubartelli *et al.*, 1990; Stevenson *et al.*, 1992). In addition, fibroblasts transfected with the full-length IL-1β cDNA are unable to process pIL-1β and secrete mature IL-1β (mIL-1β) (Young *et al.*, 1988).

The targeting of IL-1α to the plasma membrane is thought to be achieved by myristoylation on internal lysine residues (Bursten *et al.*, 1988; Stevenson *et al.*, 1992). The 31-kDa membrane-associated form of IL-1α is biologically active and appears to remain associated with the intact cell (Mosley *et al.*, 1987). A 17.5-kDa COOH-terminal fragment of IL-1α, which retains full biological activity, can be released from cells when their integrity is compromised, such as occurs during necrosis, apoptosis, or cell permeation (Hogquist *et al.*, 1991a,b; Haecker and Vaux, 1994; Watanabe and Kobayashi, 1994). The NH_2 termini of 17.5-kDa IL-1α released from human monocytes have been identified as Leu^{119} and Ser^{121}, consistent with cleavage by calpain and other "bystander" proteases that are present in biological fluids (Cameron *et al.*, 1985; Kobayashi *et al.*, 1990; Watanabe and Kobayashi, 1994).

Unlike IL-1α, IL-1β is synthesized as an inactive 31-kDa precursor protein (pIL-1β) and is processed to its active form by a specific processing enzyme (ICE) (Howard *et al.*, 1991, 1995). Processing is temporally coordinated with release from the cell (Chin and Kostura, 1993). Several observations support this conclusion. Mature 17.5-kDa IL-1β is not detected inside intact cells. Immunoelectron microscopic studies of human monocytes show that IL-1β is present in the cytosolic ground substance, but not on the plasma membrane of stimulated cells prior to secretion, and then translocates to the plasma membrane during the

TABLE I
EVOLUTIONARY CONSERVATION OF ICE-LIKE CLEAVAGE SITES IN pIL-1β[a]

Species	Site 1	Site 2
Human	EDDLFFEAD GPKQMK	YVHD APVRSL
Cow	ENELLFEAD DPKQMK	FLCD APVQSI
Rabbit	ENDLFFEAD GPNYMK	LECD A-VRSL
Rat	ENDLFFEAD RPQKIK	LVCD VPIRQL
Mouse	ENDLFFEVD GPQKMK	LVCD VPIRQL

[a]Data from Kostura *et al.* (1989) and Howard *et al.* (1991).

secretory phase (Singer *et al.*, 1995). The mechanism of targeting IL-1β to the plasma membrane by posttranslational modifications such as myristoylation on lysine residues remains unknown at this time (Bursten *et al.*, 1988; Stevenson *et al.*, 1992). Human monocytes secrete the processed propiece as well as the mature COOH-terminal fragment of IL-1β, indicating that the entire molecule transits through the membrane (Higgins *et al.*, 1994; Singer *et al.*, 1995). While temporally coordinated, the processing and release of mature IL-1β are not mechanistically coupled. In human monocytes, inhibition of mIL-1β production by a tetrapeptide ICE inhibitor results in the corresponding release of intact pIL-1β (Thornberry *et al.*, 1992; Chin and Kostura, 1993). Thus the entire precursor can thread across the membrane if processing is blocked by a low-molecular-weight ICE inhibitor.

ICE catalyzes the cleavage of pIL-1β at two sites, Asp^{27}-Gly^{28} and Asp^{116}-Ala^{117}, which have been conserved evolutionarily (Table I). Cleavage at the latter site generates the mature biologically active cytokine with Ala^{117} at its NH_2 terminus (Black *et al.*, 1989; Kostura *et al.*, 1989). The purpose of the upstream site is unknown; however, expression of a pIL-1β Ala^{116} cleavage site mutant in cells revealed that the upstream site is cleaved by ICE and that the 26-kDa product resulting from cleavage of the mutant precursor is released from the cell (Howard *et al.*, 1991). This observation suggests that processing of pIL-1β in cells may occur via a processive mechanism.

III. MOLECULAR AND BIOCHEMICAL CHARACTERIZATION OF ICE

A. SUBSTRATE SPECIFICITY

One of the most distinguishing features of ICE is its requirement for aspartic acid in the P_1 position of the substrate cleavage site. Except for

ICE gene family members, the serine proteinase granzyme B from T lymphocytes is the only other mammalian enzyme that cleaves at aspartic acid residues, although it does not exhibit an absolute requirement for aspartic acid in P_1 (Odake *et al.*, 1991; Poe *et al.*, 1991). ICE cleaves pIL-1β at Asp^{27} and Asp^{116}. Amino acid substitutions in sequences corresponding to either site showed that aspartic acid is required for cleavage of both protein and peptide substrates (Black *et al.*, 1989; Kostura *et al.*, 1989; Sleath *et al.*, 1990; Howard *et al.*, 1991). Mutation of one site does not affect cleavage at the other site (Howard *et al.*, 1991, 1995). Substitution of aspartic acid with any other amino acid reduces k_{cat}/K_m by more than 100-fold regardless of whether the substrate is a peptide or protein (Thornberry *et al.*, 1992).

The minimum peptide substrate for ICE consists of four amino acids NH_2-terminal to the cleavage site (P), Ac-Tyr-Val-Ala-Asp, and is similar to the sequence in the natural human substrate, Tyr^{113}-Val-His-Asp^{116}. Truncation at the P_4 position leads to >100-fold reduction in k_{cat}/K_m. Unlike other cysteine proteinases, ICE does not utilize important substrate recognition sites on the COOH-terminal side of the cleavage site (P′). Amino acid substitutions and deletions are tolerated on the P′ side of the cleavage site in both peptide and pIL-1β substrates. The best peptide substrate known, Ac-Tyr-Val-Ala-Asp-$NHCH_3$ ($k_{cat}/K_m = 4 \times 10^5\ M^{-1}\ s^{-1}$), is comparable to pIL-1β ($k_{cat}/K_m = 1.5 \times 10^5\ M^{-1}\ s^{-1}$) and does not contain an amino acid on the P′ side (Thornberry *et al.*, 1992). Several enzymatic assays have been developed based on this minimal peptide substrate (Thornberry and Molineaux, 1995). A continuous fluorometric assay based on the tetrapeptide substrate (Ac-Tyr-Val-Ala-Asp-aminomethylcoumarin has been particularly useful in characterizing the enzyme (Thornberry *et al.*, 1992). This elegant assay is described in detail elsewhere (Thornberry *et al.*, 1994).

The enzyme prefers hydrophobic residues in the P_4 position, whereas broad substitutions are tolerated in both the P_2 and P_3 positions. As mentioned later, recent X-ray crystallographic studies of tetrapeptide-based inhibitors complexed with the enzyme indicate that the side chains of the residues in P_2 and P_3 are solvent exposed and do not play a significant role in substrate binding. The ability to make broad substitutions at these positions has been used to great advantage in the design of inhibitors and molecular probes of the enzyme.

B. Purification

Two very different approaches have been used to purify native human ICE from THP.1 cell cytosolic extracts: ion exchange–reverse-phase chromatography and ligand affinity chromatography (Thornber-

TABLE II
ICE SUBUNITS

Subunit	Mass[a]	M_r
p22	21,456	24,000
p20	19,866	22,000
p10	10,248	12,000

[a]In atomic mass units (amu).

ry *et al.*, 1992; Miller *et al.*, 1993). The latter is an extraordinarily powerful technique that employs a potent reversible tetrapeptide aldehyde inhibitor, Ac-Tyr-Val-Lys-Asp-CHO (K_i = 3 n*M*) as an affinity chromatography ligand (Thornberry *et al.*, 1992). This approach achieves >100,000-fold enrichment of catalytically active enzyme, enabling purification to homogeneity from crude cell lysates in a single step.

Both chromatographic approaches result in the isolation of three polypeptides that consistently track with ICE activity. The three polypeptides have been designated p22, p20, and p10 based on their respective mass values of 21,465, 19,866, and 10,248 atomic mass units as determined by liquid capillary electrospray ionization mass spectrometry. Amino acid sequences derived from each of the purified proteins proved to be novel at the time of their isolation. Additional sequence analysis showed that p20 was an NH_2-terminally truncated version of p22 that was missing the first 16 amino acids. On sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), these three proteins exhibit corresponding apparent migration rates (M_r) of 24,000, 22,000, and 12,000 (Table II). These proteins were eventually shown to be the individual subunits of the active enzyme, which consisted of a 1:1 stoichiometric complex of either p22–p10 or p20–p10 (Thornberry *et al.*, 1992; Miller *et al.*, 1993). The p20–p10 complex is the predominant form of the human enzyme isolated from THP.1 cell lysates.

C. ICE ENZYMOLOGY

1. *Protease Classification*

ICE was classified as a cysteine proteinase based on its sensitivity to known nonspecific competitive thiol aklylating agents such as iodoacetate and *N*-ethylmaleimide and its inhibition by a tetrapeptide dia-

zomethylketone specific for ICE. Diazomethylketones are classical mechanism-based inhibitors of cysteine proteinases. The lack of inhibition by the epoxide E64, which inhibits other cysteine proteinases, is the one notable exception in this profile (Black *et al.,* 1989; Kostura *et al.,* 1989). As described later, this classification was subsequently confirmed by active site labeling and the identification of the catalytic cysteine residue (Thornberry *et al.,* 1992; Walker *et al.,* 1994; Wilson *et al.,* 1994).

2. *Active Site Labeling*

The catalytic cysteine of ICE was identified by competitive active site labeling using either a nonselective agent, [^{125}]iodoacetic acid (IAA), or a highly selective tetrapeptide acyloxymethylketone inhibitor, Ac-Tyr-Val-Ala-Asp-$CH_2OC(O)$-[2,6(CF_3)$_2$]Ph (Thornberry *et al.,* 1992, 1994). IAA reacts 10 times faster with the catalytic cysteine than with other cysteine residues, while the tetrapeptide inhibitor has a second-order inactivation rate constant of $9.5 \times 10^{-5}\ M^{-1}\ s^{-1}$. Both are competitive with substrate. IAA and the inhibitor both label the p20 subunit under competitive reaction conditions. Digestion of the p20 subunit with trypsin and purification of the labeled peptide show that the modified cysteine was contained within the tryptic peptide Val-Ile-Ile-Ile-Gln-Ala-Cys. This sequence has since been confirmed in the ICE cDNA sequence (Thornberry *et al.,* 1992).

3. *Concentration Dependence of Catalytic Activity*

The catalytic activity of ICE is a function of enzyme concentration. Dilution of the enzyme 1000-fold results in complete loss of enzyme activity within 3 hr. Activity is fully restored upon reconcentration of the enzyme. The enzyme is stabilized by saturating amounts of substrate or competitive inhibitors. Agents such as 10% sucrose, high glycerol concentrations, glutathione, and 0.1% CHAPS also stabilize ICE (Thornberry *et al.,* 1992). The loss of enzymatic activity is believed to result from dissociation of the individual subunits or higher order multimers of the heteromeric enzyme complex.

4. *Catalytic Mechanism*

In spite of its unique characteristics, ICE is catalytically a typical cysteine proteinase (Black *et al.,* 1989; Kostura *et al.,* 1989; Sleath *et al.,* 1990; Howard *et al.,* 1991; Thornberry *et al.,* 1992; Walker *et al.,* 1994; Wilson *et al.,* 1994). The crystal structure of ICE indicates that the active site of the enzyme contains a standard catalytic dyad, consisting of Cys^{285} and His^{237}, that mechanistically perform a general acid–base

function. As in other cysteine proteinases, Cys^{285} and Gly^{231} act to stabilize the oxyanion during catalysis (Walker *et al.*, 1994; Wilson *et al.*, 1994).

5. *ICE Inhibitors*

a. Synthetic Inhibitors. Inhibitors of cysteine proteinases employ two general chemical schemes involving reversible or irreversible inactivation of the catalytic cysteine. For reversible inhibitors the catalytic cysteine undergoes nucleophilic addition, forming a thiol adduct with the reactive group of the inhibitor. Inhibitors based on this mechanism are transition state analogs that appear to mimic the tetrahedral and acyl-enzyme intermediates formed during substrate hydrolysis. Peptide aldehydes, nitriles, and ketones, which form thiohemiacetals, thioimidates, and thiohemiketals, respectively, are examples of reversible inhibitors. Irreversible inhibitors typically employ an α-substituted ketone core structure of the type R-CO-CH_2-X, where X is a leaving group. X can be a halogen, a diazonium ion, or a carboxylate, corresponding to halomethylketones, diazomethylketones, and acyloxymethylketones, respectively. These inhibitors form a thiomethylketone with the active site cysteine through expulsion of the leaving group (X^-), thereby irreversibly inactivating the enzyme.

Consistent with the substrate specificity of ICE, selective peptide-based inhibitors contain the sequence Ac-Tyr-Val-Ala-Asp. Inhibitors containing this sequence have relatively slow rates of association with the enzyme ($1 \times 10^6\, M^{-1}\, s^{-1}$) (Thornberry *et al.*, 1994). It has been suggested based on recent results that binding of the inhibitor to the enzyme is diffusion controlled and requires a viscosity-dependent conformational change in either the enzyme or the inhibitor (Thornberry *et al.*, 1994). The topology of the active site determined from the crystal structure of ICE complexed with a tetrapeptide based inhibitor suggests that charge–charge interactions between the P_1 aspartic acid of the inhibitor and Arg^{179} and Arg^{341} in the S_1 binding pocket may also play a role in this process (Walker *et al.*, 1994; Wilson *et al.*, 1994). One unusual curiosity revealed by the structure of the inhibited enzyme was that the tetrapeptide aldehyde Ac-Tyr-Val-Ala-Asp-CHO was bound in the opposite configuration.

ICE inhibitors based on either of the mechanisms described here have been extremely useful tools. The reversible tetrapeptide aldehyde described earlier is an extremely powerful affinity ligand, while an irreversible acyloxymethylketone is an extraordinarily selective active site probe (Thornberry *et al.*, 1992, 1994; Singer *et al.*, 1995). Inhibitors from these two general classes have also been shown to prevent, dose

dependently, the processing of pIL-1β in both human monocytes *in vitro* (IC_{50} ~1 μ*M*) and murine macrophages *in vivo* (ED_{50} ~1 mg/kg, ip) (Thornberry *et al.*, 1992; Molineaux *et al.*, 1993; Fletcher *et al.*, 1995; Miller *et al.*, 1995).

b. CrmA: the Vaccinia Virus Serpin Gene. Viruses have evolved a variety of ways to evade cellular responses to infection and thereby assure their propagation. Certain poxviruses (e.g., vaccinia virus) encode a 38-kDa serpin protein known as the cytokine response modifier A (*crmA*) gene product, which is an extremely potent inhibitor ($K_i \leq 7$ p*M*) of ICE (Ray *et al.*, 1992; Komiyama *et al.*, 1994). This finding is highly unusual since serpins were believed to interact only with serine proteinases. The *crmA* gene is the only serpin gene superfamily member known to inhibit a cysteine proteinase. Evolutionarily, viruses containing *crmA* possess a selective advantage by virtue of their ability to shut down the host's inflammatory response to infection (Ray *et al.*, 1992). More recently, it has been shown that cells transfected with *crmA* do not exhibit the nuclear changes associated with apoptotic cell death, suggesting that the protein may also provide an advantage for the virus by prolonging the life of the infected cell. CrmA expression is first detected during the early phase of virus replication and continues throughout the late phase of replication. The protein is not secreted from infected cells and appears to act as an intracellular inhibitor. CrmA (1 μ*M*), when added to the medium of cultured human monocytes, has no effect on the processing and release of IL-1β (Singer *et al.*, 1995). Coexpression studies in insect *Sf9* cells indicate that CrmA has the ability to inhibit activation of the proenzyme as well as the activity of the p20–p10–ICE complex (Howard *et al.*, 1995).

The *crmA* gene has been an important tool for exploring the role of ICE–Ced-3-related proteases in cellular processes. Transfection of the *crmA* gene or microinjection of the protein blocks apoptosis in a variety of cell types (Gagliardini *et al.*, 1994). With the exception of ICE itself, though, the specificity of *crmA* for other members of the ICE–*ced-3* gene family is largely unknown. Interactions with other family members are likely since CrmA blocks apoptosis in a variety of cells, including some that do not contain detectable expression of ICE (Thornberry *et al.*, 1996). The *crmA* gene has been shown to be a weak inhibitor of apopain, also known as CPP32β (Nicholson *et al.*, 1995; Tewari *et al.*, 1995).

The 38-kDa CrmA protein strongly resembles other members of the serpin gene family (Ray *et al.*, 1992). The region homologous to the reactive stress loop (RSL) contains the sequence Ala-Leu-Val-Asp-Cys-Ala-Ser-Thr, which has not been found in known serpins (Ray *et al.*, 1992). Amino acid substitutions at aspartic acid or cysteine elimi-

nate the ability of CrmA to inhibit ICE. Mechanistically, the RSL of serpins acts as a trap for the active site of the enzyme and enables the formation of an enzyme–serpin complex. The parameters for binding to ICE ($k_{on} = 1.7 \times 10^7\ M^{-1}\ s^{-1}$) are among the fastest reported for a serpin interacting with a proteinase, whereas the dissociation constant for the final complex is <4 p*M* (Komiyama *et al.*, 1994). Unlike most enzyme–serpin interactions, however, the ICE–CrmA complex readily dissociates when subjected to SDS–PAGE (Komiyama *et al.*, 1994). CrmA is a weak inhibitor of the ICE family member CPP32β, exhibiting a K_i>100 n*M*. It also appears to inhibit granzyme B (Thornberry *et al.*, 1996).

c. Baculovirus p35 Protein. Baculovirus encodes a 35-kDa protein (p35) that blocks apoptosis (Clem *et al.*, 1991). Like CrmA, p35 probably confers a significant evolutionary advantage on the virus. This protein is an inhibitor and substrate of ICE as well as the *Caenorhabditis elegans* cell death gene product Ced-3 (Bump *et al.*, 1995; Xue and Horvitz, 1995). Coexpression of p35 in cells inhibits autoactivation of the proenzyme and prevents cell death due to overexpression of ICE. Inhibition of ICE activity correlates with the cleavage of p35 and the formation of a p35–ICE complex. In addition to ICE and Ced-3, this protein irreversibly inhibits several ICE family members, including Ich-1, CPP32β, and ICE_{rel}-II (Bump *et al.*, 1995; Xue and Horvitz, 1995). Granzyme B is not inhibited by p35.

D. Molecular Biology

1. *Human ICE cDNA Sequences*

Extensive amino acid sequence information and high-resolution molecular mass determinations obtained through analysis of purified p22, p20, and p10 played a major role in cloning the human ICE cDNA and in determining the primary and secondary structures of the enzyme (Fig. 1) (Thornberry *et al.*, 1992). The cDNA for human ICE was amplified by polymerase chain reaction (PCR) and molecularly cloned from the mRNA of the human monocytic cell line THP.1. Degenerate oligonucleotide primers derived from the amino acid sequence of the enzyme were used for PCR amplification (Cerretti *et al.*, 1992; Thornberry *et al.*, 1992). Analysis of the cloned cDNAs revealed that sequences from both p20 and p10 were contained within a single cDNA. The full-length ICE cDNA (1490 nucleotides) encodes a 404-amino-acid proenzyme (M_r 45,158) that has been designated p45. The regions within p45 that corresponded to p22, p20, and p10 were established using NH_2-terminal

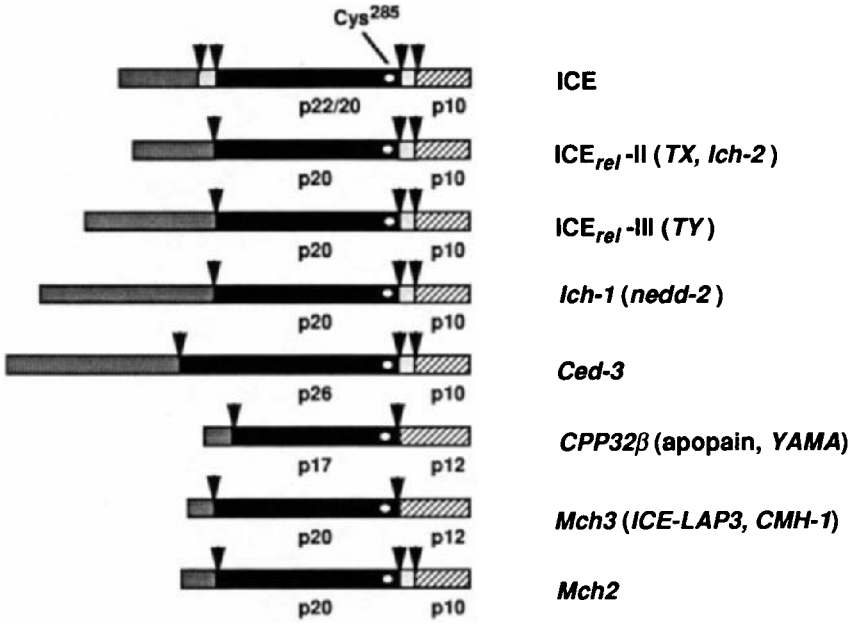

FIG. 1. Line drawings depicting the primary structures of the known ICE–Ced-3 family members. (Adapted from Miller *et al.*, 1996; Thornberry *et al.*, 1996.)

and internal peptide sequence information and the molecular mass of each subunit.

Taken together, the information gained from the purification, characterization, and cloning revealed that the enzyme was a heterodimer consisting of two subunits, p20 and p10, that are present in a 1:1 ratio in the active enzyme. Both subunits are required for catalytic activity and are derived from a 45-kDa proenzyme by autoproteolysis at sites similar to those used in the processing of pIL-1β. The sequences encoding p22/p20 and p10 are located in the central and COOH-terminal portions of p45, respectively. The catalytic Cys^{285}, identified by active site labeling, is located at the COOH terminus of p22/p20. An 11.5-kDa prodomain (p11) that precedes the p22/p20 subunit and a 2.0-kDa connecting peptide (p2) that lies between p22/p20 and p10 are removed proteolytically during maturation of the heterodimeric p20–p10 and p22–p10 complexes. The primary sequence of p45 revealed Asp-X sequences at Asp^{103}, Asp^{119}, Asp^{287}, and Asp^{316} that corresponded to the NH_2 and COOH termini of p22 and p20 and the NH_2 terminus of p10, strongly implying an autocatalytic mechanism is used to activate p45.

Cleavages at Asp^{103} and Asp^{119} generate the NH_2 termini of p22 and p20, respectively. Three of the four Asp-X sites are conserved in the primary sequences of mouse and rat ICE (Table III) (Nett *et al.*, 1992; Molineaux *et al.*, 1993; Keane *et al.*, 1995). Substitution of the catalytic Cys^{285} or of Aspartic acid at all four cleavage sites prevents proteolytic processing of the proenzyme, functionally confirming the autoactivation hypothesis (Rolando *et al.*, 1994; Wilson *et al.*, 1994; Howard *et al.*, 1995). ICE is not homologous to any of the previously known cysteine proteinases and thus became the first member of an entirely distinct class of cysteine proteinase.

2. *ICE mRNA Expression*

The mRNA for ICE is constitutively expressed at very low levels in human monocytes (<1 in 50,000 transcripts per cell). Three ICE transcripts corresponding to 2.3, 1.6, and 0.5 kb are detected by Northern blot hybridization of total or poly(A) RNA (Cerretti *et al.*, 1992; Nett *et al.*, 1992; Thornberry *et al.*, 1992; Molineaux *et al.*, 1993; Keane *et al.*, 1995). ICE transcripts are also found in a variety of other cells, including T and B lymphocytes and cells of monocytic origin, such as astrocytes (Cerretti *et al.*, 1992; Thornberry *et al.*, 1992; Munday *et al.*, 1995). Certain stimuli such as lipopolysaccharide (LPS) or interferon-γ (INF-γ) increase steady state ICE mRNA levels by approximately two-fold (Nett *et al.*, 1992; Keane *et al.*, 1995).

The primary RNA transcript for human ICE is alternatively spliced, giving rise to five different mRNA species and potentially five different isoforms of the enzyme, designated α–ϵ (Alnemri *et al.*, 1995). The mRNAs for ICE δ, γ, and ϵ are generated in part by an unusual splicing event that occurs within the coding sequence of exon 2 (Fig. 2). In each of these three mRNAs, an atypical splice donor site in the codon for Gly^{20} is used instead of the downstream splice site at the end of exon 2. Four of the isoforms—α, β, δ, and γ—contain the catalytic thiol residue Cys^{285}. However, neither ICE_δ nor ICE_γ appears to possess autocatalytic activity, even though both possess the catalytic cysteine residue. ICE_ϵ encodes a variant of p10 that is capable of complexing with the p20–p10 form of ICE (ICE_α). An alternatively spliced transcript corresponding to the β isoform of human ICE has also been identified for murine ICE (Molineaux *et al.*, 1993; Casano *et al.*, 1994). Other ICE gene family members appear to use this mechanism as well, perhaps as a way of regulating the activity of these molecules. An alternatively spliced transcript that lacks the COOH-terminal coding region of ICE–Ced-3 homolog-1 (Ich-1) has been identified that, when transfected into cells, is capable of modulating the apoptotic activity of

TABLE III

EVOLUTIONARY CONSERVATION OF ICE-LIKE CLEAVAGE SITE IN P45

Species	Cleavage Site 1 (p22N[a])	Cleavage Site 2 (p20N)	Cleavage Site 3 (p22/p20C[b])	Cleavage Site 4 (p10N)
Human	NYLNMQD SQGVL	QAVQD NPAMP	GVVWFKD SVGVS	EEFEDD AIKKA
Rat	TVFVTED SKGGH	NHS[c]	GVVLLKD SVGNS	AIFEDD GIKKA
Mouse	TFVATED SKGGH	NHS	GVVLLKD SVRDS	AIFEDD GIKKA

[a]N, NH_2 terminus.

[b]C, COOH terminus.

[c]NHS, no homologous site.

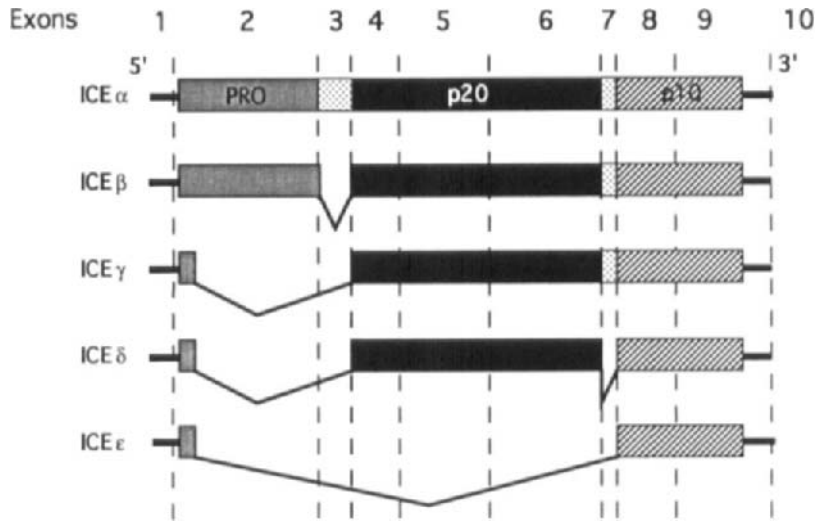

FIG. 2. Line drawings representing the structure of human ICE mRNA splice variants. (Adapted from Alnemri *et al.*, 1994.)

full-length Ich-1 (Wang *et al.*, 1994). Although the functional significance of each ICE isoform is not fully understood, it is now possible to ask if these splice variants are involved in regulating enzyme activity or altering its substrate specificity.

3. *ICE Sequences from Other Species*

ICE cDNAs have also been cloned from mouse and rat cells (Nett *et al.*, 1992; Molineaux *et al.*, 1993; Keane *et al.*, 1995). In contrast to human ICE, a single 1.6-kb transcript was detected by Northern blot hybridization in a panel of RNAs from murine cells, whereas 2.3- and 1.6-kb transcripts were found in various rat tissues. The rodent cDNAs encode proenzymes of 402 amino acids that display the same overall organization as human ICE: a prodomain, a large subunit, a short connecting peptide, and a small subunit. In both species, three of the four Asp-X cleavage sites are conserved. In the mouse and rat enzymes, the Asp-X processing site at the NH_2 terminus of p20 is missing due to the substitution of Asp^{119} by asparagine. The mouse enzyme, however, possesses an alternate processing site at Asp^{122}-Gly^{123}, while the rat does not. Thus the murine enzyme, like the human, can generate both p22–p10 and p20–p10 heterodimers, whereas the mature rat enzyme most likely consists solely of a p22–p10 complex (Keane *et al.*, 1995).

The rodent enzymes share over 90% amino acid sequence identity with one another and approximately 68% identity with human ICE (Nett *et al.*, 1992; Molineaux *et al.*, 1993; Keane *et al.*, 1995). The p10 subunit is the most highly conserved, possessing over 80% amino acid identity between all three species. The sequence identity shared between the p20 subunits is approximately 60%. One region of particular interest that is entirely conserved across species is a stretch of more than 19 residues in the p20 subunit that includes the active site Cys^{285} (Nett *et al.*, 1992; Molineaux *et al.*, 1993; Keane *et al.*, 1995).

4. *ICE Expression in Cells*

Meager amounts of inactive proenzyme are constitutively produced by monocytic cells (Ayala *et al.*, 1994). The vast majority of proenzyme resides in the cytoplasm (Singer *et al.*, 1995). A small pool of active enzyme is continuously drawn from the reservoir of cytosolic proenzyme. In virtually all cell types examined, the amount of active enzyme at any given time is so vanishingly small that it cannot be detected by immunoprecipitation or immunoblotting techniques (Ayala *et al.*, 1994). Consequently, the catalytically active form of the enzyme that is used by cells is not known. It is worth noting that the mature active p20–p10 complex isolated from cells is derived from autoactivation of p45 during incubation of cytosolic extracts (Miller *et al.*, 1993). Extremely sensitive immunoelectron microscopic staining techniques confirm the presence of the proenzyme in the cytoplasm of human monocytes and indicate that the active enzyme is localized on the external surface of their plasma membranes (Ayala *et al.*, 1994; Singer *et al.*, 1995). Epitopes to the p11 prodomain of human ICE were detected exclusively in the cytoplasm, whereas epitopes to the p20 subunit of ICE were detected both in the cytoplasm and on the plasma membrane. No differences in staining were observed between stimulated and resting cells. In addition, the staining patterns for an active site-directed biotinylated ICE inhibitor coincided with the staining for the p20 subunit (Singer *et al.*, 1995). These observations, taken together, strongly support the conclusion that the active enzyme is localized on the plasma membrane of cells and the proenzyme in the cytoplasm.

Certain stimuli such as LPS and INF-γ increase ICE mRNA levels approximately two-fold; however, there is no evidence as yet that this results in increased proenzyme production (Nett *et al.*, 1992; Keane *et al.*, 1995). A variety of cell types express p45; ICE activity, though, has only been detected in extracts of cells that also produce IL-1β, suggesting that activation of the enzyme is under strict control (Kostura *et al.*, 1989; Nett *et al.*, 1992; Molineaux *et al.*, 1993; Keane *et al.*, 1995; Mun-

day *et al.*, 1995). Notable exceptions include neutrophils and keratinocytes (Tocci and Schmidt, 1996). Keratinocytes do not appear to express ICE but do express small amounts of pIL-1β, which is not processed or secreted (Kupper *et al.*, 1986, 1988a,b; Blanton *et al.*, 1989; Tocci and Schmidt, 1996). In contrast, human neutrophils contain ICE mRNA but do not contain detectable levels of p45 (Tocci and Schmidt, 1996).

5. *Structure and Regulation of the Human ICE Gene*

ICE is encoded by a single gene in both the human and murine genomes (Casano *et al.*, 1994; Cerretti *et al.*, 1994). Both genes are approximately 10.5 kb in size and consist of nine introns and 10 exons (Fig. 3). The 10 exons are grouped into three clusters that are separated by the two largest introns. The first group of three exons (I–III) encodes the 5′UTR and the prodomain of the enzyme; the second group of three exons (IV–VI) encodes the majority of the p20 subunit; and the last group of four exons (VII–X) encodes the connecting peptide, the p10 subunit, and the 3′UTR. Individual exon boundaries generally parallel the functional domains in the enzyme (Table IV). The human gene, *IL1BC,* has been localized by fluorescence *in situ* hybridization on chromosome 11 to band q22.2–q22.3 (Cerretti *et al.*, 1994). This region of chromosome 11 contains the genes for the D2 dopamine receptor (*D2DR*) and the neuronal cell adhesion molecule (*NCAM*) and the ma-

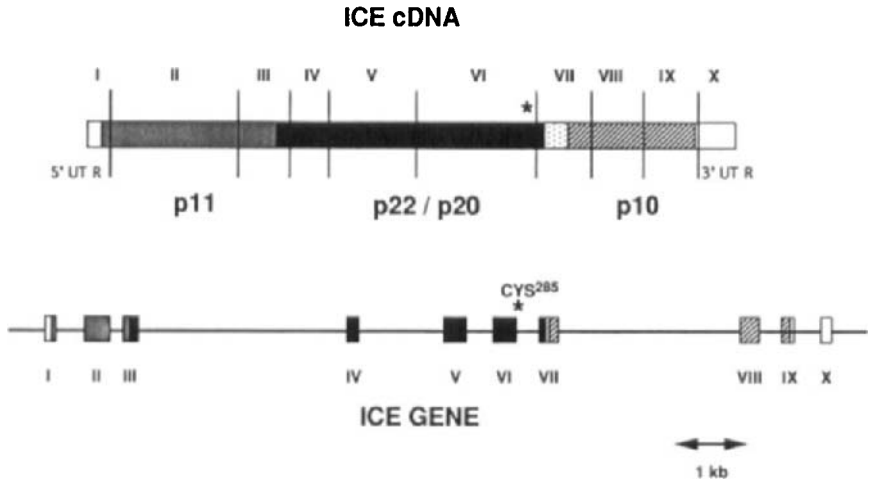

FIG. 3. Diagram showing the intron–exon structure of the human ICE gene (*IL1BC*). (Adapted from Cerretti *et al.*, 1993.)

TABLE IV
FUNCTIONAL ORGANIZATION OF EXONS IN THE HUMAN *IL1BC* GENE[a]

Exon	Size (bp)	Amino Acids	Coding Function
I	38	Met^{1}–Asp^{3}	5′UTR prodomain
II	267	Asp^{3}–Asp^{92}	Prodomain
III	63	Asp^{92}–Ala^{113}	Prodomain–p20 junction
IV	116	Ala^{113}–Glu^{151}	p20 subunit
V	174	Ile^{152}–Ser^{209}	p20 subunit
VI	235	Asp^{210}–Asp^{288}	p20 subunit
VII	144	Asp^{288}–Asp^{336}	p20–p10 junction
VIII	110	Asp^{336}–Lys^{372}	p10 subunit
IX	131	Val^{373}–His^{404}	p10 subunit
X	99	None	3′UTR

[a]Based on data from Thornberry *et al.* (1992) and Cerretti *et al.* (1992, 1994).

trix metalloproteinase gene cluster, which includes fibroblast collagenase (*CLG*), stromelysin 1 (*STMY1*), and stromelysin 2 (*STMY2*). The murine gene (*il1bc*) is located on chromosome 9 (Nett *et al.*, 1992).

The mRNA for ICE is expressed constitutively (Cerretti *et al.*, 1992; Nett *et al.*, 1992; Thornberry *et al.*, 1992; Molineaux *et al.*, 1993; Keane *et al.*, 1995) in a variety of human and murine cells. In monocytic cells, a single transcription start site has been identified at −33 bp upstream of the initiator Met codon for the human gene, while two start sites at −32 and −37 bp upstream of the ATG codon have been found for the murine gene (Casano *et al.*, 1994; Cerretti *et al.*, 1994). Neither the human nor the murine gene contains recognizable TATA, CAAT, or SP-1 consensus sequences. However, each contains a completely conserved 14-bp sequence (CTTTCAGTTTCAGT) that is located from −28 to −41 nucleotides upstream of the transcription start site. This sequence is identical to the consensus interferon regulatory factor-1 (IRF-1) element (Tamura *et al.*, 1995). The 14-bp sequence specifically binds IRF-1 and confers IRF-1 inducibility upon a transfected promoter element in mitogen-activated cells. The increased steady-state ICE mRNA levels observed upon stimulation of monocytes with LPS, INF-γ, or other mitogens are consistent with a role for IRF-1 in ICE gene expression (Nett *et al.*, 1992; Molineaux *et al.*, 1993; Casano *et al.*, 1994; Cerretti *et al.*, 1994; Keane *et al.*, 1995; Tamura *et al.*, 1995). Overexpression of IRF-1 in a pro-B cell line induced ICE mRNA, providing further support for this idea (Tamura *et al.*, 1995). The 5′ flanking region of the *IL1BC* gene also contains consensus binding sites for nuclear factor-κB,

activator protein-1, activator protein-3, and NFIL-6 within 600 bp of the transcription start site. The functional significance of these sites, though, has yet to be determined (Casano *et al.*, 1994; Cerretti *et al.*, 1994; Tamura *et al.*, 1995). Viable *il1bc*-deficient mice (ICE$^{-/-}$) have been generated through gene targeting techniques, demonstrating that ICE is not essential for growth and development (Kuida *et al.*, 1995; Li *et al.*, 1995).

6. *Recombinant Enzymes*

Recombinant forms of ICE (rICE) have been expressed in mammalian and insect cells and in *Escherichia coli* (Cerretti *et al.*, 1992; Thornberry *et al.*, 1992; Molineaux *et al.*, 1993; Walker *et al.*, 1994; Wang *et al.*, 1994b; Wilson *et al.*, 1994; Howard *et al.*, 1995; Ramage *et al.*, 1995). Both wild-type and mutant versions of the enzyme have been biochemically and functionally examined in several expression systems. These studies have provided interesting insights into the mechanism of proenzyme activation and the role of ICE in IL-1β release and provided large quantities of enzyme for structural determinations (Molineaux *et al.*, 1993; Walker *et al.*, 1994; Wilson *et al.*, 1994; Howard *et al.*, 1995; Ramage *et al.*, 1995).

a. Expression in COS7 Cells. Most of the mammalian cell expression studies on ICE have been performed in COS7 cells (Cerretti *et al.*, 1992; Thornberry *et al.*, 1992; Wilson *et al.*, 1994). When transfected with the full-length ICE cDNA, COS7 cells produce significant amounts of p45 but little or no detectable active enzyme. As with monocytes, ICE activity may be generated by incubation of transfected COS7 cell lysates, but even this approach is inefficient and produces variable amounts of activity. A number of cDNAs containing amino acid substitutions or truncations have been expressed in this system. Prodomain deletion mutants are constitutively active, suggesting that this sequence is important in enzyme activation. This finding provides further evidence that the activation of p45 is strictly regulated. Not surprisingly, the expression of cDNAs containing substitutions of the active site Cys^{285} or deletions of the p10 subunit yield inactive enzyme (Cerretti *et al.*, 1992; Wilson *et al.*, 1994).

b. Expression in E. coli. Milligram quantities of active enzyme have been obtained by expressing either the full-length proenzyme or the individual subunits in *E. coli* (Molineaux *et al.*, 1993; Walker *et al.*, 1994; Wilson *et al.*, 1994; Ramage *et al.*, 1995). The recombinant enzyme from *E. coli* is identical in its catalytic properties to the native enzyme isolated from THP.1 cells (Thornberry, 1994). Enzyme produced in *E. coli* was used to determine the three-dimensional crystal structure of ICE

by X-ray diffraction (Walker *et al.*, 1994; Wilson *et al.*, 1994). Proenzyme expressed in *E. coli* spontaneously converts to p20 and p10 during isolation and refolding (Ramage *et al.*, 1995). Incubation with a specific ICE inhibitor or refolding under nonreducing conditions prevents autoactivation of p45. Time-course studies indicate that the p10 subunit is released first during this activation process.

Active enzyme can also be produced by separately expressing the individual p20 and p10 subunits in *E. coli*. The purified subunits are refolded and combined in the presence of substrate to generate the active enzyme (Walker *et al.*, 1994). Thus the prodomain is not required for proper folding of the subunits, although in this case substrate could serve a role similar to that of the prodomain.

c. Recombinant Baculovirus Expression. Human ICE has been expressed in insect cells using recombinant baculovirus vectors (Wang *et al.*, 1994; Alnemri *et al.*, 1995; Howard *et al.*, 1995). This expression system has been used to produce large quantities of rICE, examine the catalytic activities of mutant enzymes, and reconstitute IL-1β processing and secretion in insect cells. The recombinant enzyme produced in insect *Sf9* cells is identical to native ICE with regard to its subunit composition and catalytic properties and is produced in amounts suitable for biochemical characterization (Howard *et al.*, 1995). The proenzyme is readily activated in baculovirus-infected *Sf9* cells. In virus-infected cells, a portion of the ICE prodomain is removed by an adventitious protease; the resulting 32-kDa protein (p32) spontaneously autoactivates, generating the mature p20–p10 heterodimeric enzyme (Wang *et al.*, 1994; Howard *et al.*, 1995). Mutation of the catalytic Cys^{285} prevents conversion to p20–p10, providing definitive proof for autocatalytic activation of the proenzyme (Wang *et al.*, 1994; Howard *et al.*, 1995). Similar results are obtained with a mutant proenzyme lacking all four Asp-X processing sites (Rolando *et al.*, 1994). Autocatalytic activation of p45 is also prevented by coexpression of *crmA* or the baculovirus p35 protein (Bump *et al.*, 1995; Howard *et al.*, 1995; Xue and Horvitz, 1995).

7. *Crystal Structure of ICE*

High-resolution structures of ICE (2.0 Å) complexed with tetrapeptide inhibitors have been determined by X-ray diffraction (Walker *et al.*, 1994; Wilson *et al.*, 1994). The tertiary structure of the enzyme bears no resemblance to the known class of cysteine proteinases and is unique. The core of the enzyme consists of six β-sheets that are protected from solvent exposure by six surrounding α-helices. Four of the β-sheets are contributed by p20 and the remaining two by p10. The structure reveals that both p20 and p10 contribute residues to the active site, explaining

the earlier finding that both subunits are required for catalytic activity and confirming their intimate association (Cerretti *et al.*, 1992; Thornberry *et al.*, 1992). Like all cysteine proteinases, the active site contains a catalytic dyad consisting of the sulfhydryl group of Cys^{285} and the imidazole group His^{237}. The crystal structure of human apopain (CPP32β), a member of the ICE gene family, is similar in many of these respects (Rotonda *et al.*, 1996).

In the crystal structure, ICE is a tetramer (Fig. 4), consisting of two p20–p10 heterodimers arranged in a head-to-tail fashion along a twofold axis of symmetry (Walker *et al.*, 1994; Wilson *et al.*, 1994). Crystals of apopain also form tetramers of this nature (Rotonda *et al.*, 1996). The two p20 subunits literally surround the two adjacent p10 subunits, which are buried in the center of the tetrameric complex. This finding may explain in part the difficulty in obtaining antibodies to the p10 subunit. The area of contact at this interface between the two p10 subunits covers nearly 5200 $Å^2$. Residues at the homodimer interface are highly conserved (81%), and mutation of one of these (His^{322}) inactivates the enzyme (Wilson *et al.*, 1994). This latter observation suggests that com-

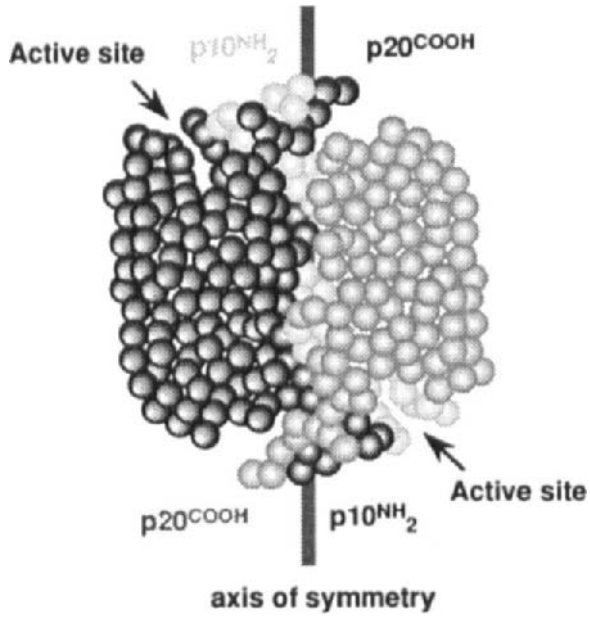

FIG. 4. Drawing of the tetrameric structure of ICE. (Adapted from Walker *et al.*, 1994; Wilson *et al.*, 1994.)

pounds that prevent dimer association may be useful as non-active-site-directed inhibitors. The distances between the subunits in the crystal structure further suggest that the p10 subunit from one p45 molecule interacts with the p20 subunit from the other p45 molecule in the homodimer (Walker *et al.*, 1994; Wilson *et al.*, 1994; Rotonda *et al.*, 1996). This arrangement of subunits in the $[p20–p10]_2$ homodimeric complex implies that activation of the proenzyme occurs via an intermolecular cleavage mechanism. Evidence supporting this mechanism has been obtained in cDNA transfection studies (Rolando *et al.*, 1994; Alnemri *et al.*, 1995; Gu *et al.*, 1995). The results of these studies show that one proenzyme molecule can process the other, and in at least one instance that different ICE family members can activate one another. Given the dimeric quaternary structure of the enzyme, it may be possible for two different ICE family members to form a mixed heterodimeric tetramer, consisting of the p20–p10 subunits from one family member and the p20–p10 subunits from another (Walker *et al.*, 1994; Wilson *et al.*, 1994; Gu *et al.*, 1995).

The active site of the enzyme resembles a shallow concave depression with a deep cavity at one end (Walker *et al.*, 1994; Wilson *et al.*, 1994). The deep cavity represents the S_1 subsite of the substrate binding groove where the P_1 aspartic acid residue of tetrapeptide-based inhibitors interacts. The S_1 pocket is formed by the catalytic dyad Cys^{285} and His^{237}, and side chains contributed by Arg^{179} and Gln^{283} from p20 and Arg^{341} and Ser^{347} from p10. The oxyanion of the tetrahedral transition state intermediate is stabilized by hydrogen bonding to the amide backbone protons of Cys^{285} and Gly^{238}. Residues from p10 make up the S_2–S_4 binding subsites. The P_2 and P_3 amino acid side chains of bound inhibitors are exposed to solvent, explaining the promiscuous nature of substitutions at these positions. Peptide backbone interactions between the inhibitor and the enzyme are largely responsible for binding at the S_2 and S_3 subsites. The P_4 tyrosine binds at the other end of the shallow substrate binding groove in a hydrophobic pocket (S_4 subsite) consisting of His^{342}, Pro^{343}, and Arg^{383}. Interestingly, the crystal structure shows that the bound conformation of the tetrapeptide aldehyde Ac-Tyr-Val-Ala-Asp-CHO does not mimic a transition state analog. Instead, the oxyanion of the inhibitor is stabilized by the active site His^{237} (Wilson *et al.*, 1994).

The crystal structure of apopain (CPP32β) has been determined by X-ray diffraction at 2.5-Å resolution (Rotonda *et al.*, 1996). The tertiary and quaternary structures of apopain and ICE are remarkably similar. The topology of the active site is highly conserved except for the S_4 subsite. Apopain prefers aspartic acid in the P_4 position of substrates and

TABLE V
POTENCY OF TETRAPEPTIDE ALDEHYDE INHIBITORS AGAINST ICE AND APOPAIN

	Inhibitor K_i (nM)[a]		
Enzyme	CrmA	Ac-Tyr-Val-Ala-Asp-CHO	Ac-Asp-Glu-Val-Asp-CHO
ICE	$<4.0 \times 10^{-3}$	7.6×10^{-1}	1.7×10^{1}
Apopain	$>1.0 \times 10^{2}$	1.0×10^{4}	3.5×10^{-1}

[a]Data from Thornberry *et al.* (1992, 1994, 1996) and Nicholson *et al.* (1995).

is less tolerant than ICE to substitutions in this position (Nicholson *et al.*, 1995; Thornberry *et al.*, 1996). The residues that interact with the P_4 aspartic acid are quite different in apopain and ICE. This observation is consistent with the known substrate specificities and inhibitor profiles of both enzymes (Table V).

IV. ICE GENE FAMILY MEMBERS

A number of sequences that are related to ICE have now been identified. The first sequence shown to have significant identity (28%) to ICE was the cell death gene *ced-3* from *C. elegans* (Yuan *et al.*, 1993). The *ced-3* gene was identified genetically as one of several genes that were required for programmed cell death in the nematode. This finding, along with studies showing that ectopic overexpression of an ICE–LacZ fusion protein in cells induced apoptosis and subsequent studies showing that CrmA could prevent this process, led to the hypothesis that ICE was the mammalian equivalent of Ced-3 (Miura *et al.*, 1993; Gagliardini *et al.*, 1994).

The once-unique sequence of ICE is now the prototype for defining new members of this rapidly expanding family of novel cysteinyl proteinases (Miller *et al.*, 1996; Thornberry *et al.*, 1996). At least seven family members have now been identified. Phylogenetically their sequences fall into two subfamilies, exemplified by ICE and Ced-3 (Fig. 5). All members of the family resemble ICE in their overall organization, possessing prodomains as well as a large and a small subunit of various lengths. The large and small subunits range in size from 17 to 26 kDa and from 10 to 12 kDa, respectively (Fig. 1). The greatest sequence variability occurs within the prodomains, suggesting that they are tailored for different purposes in each of the family members. As in

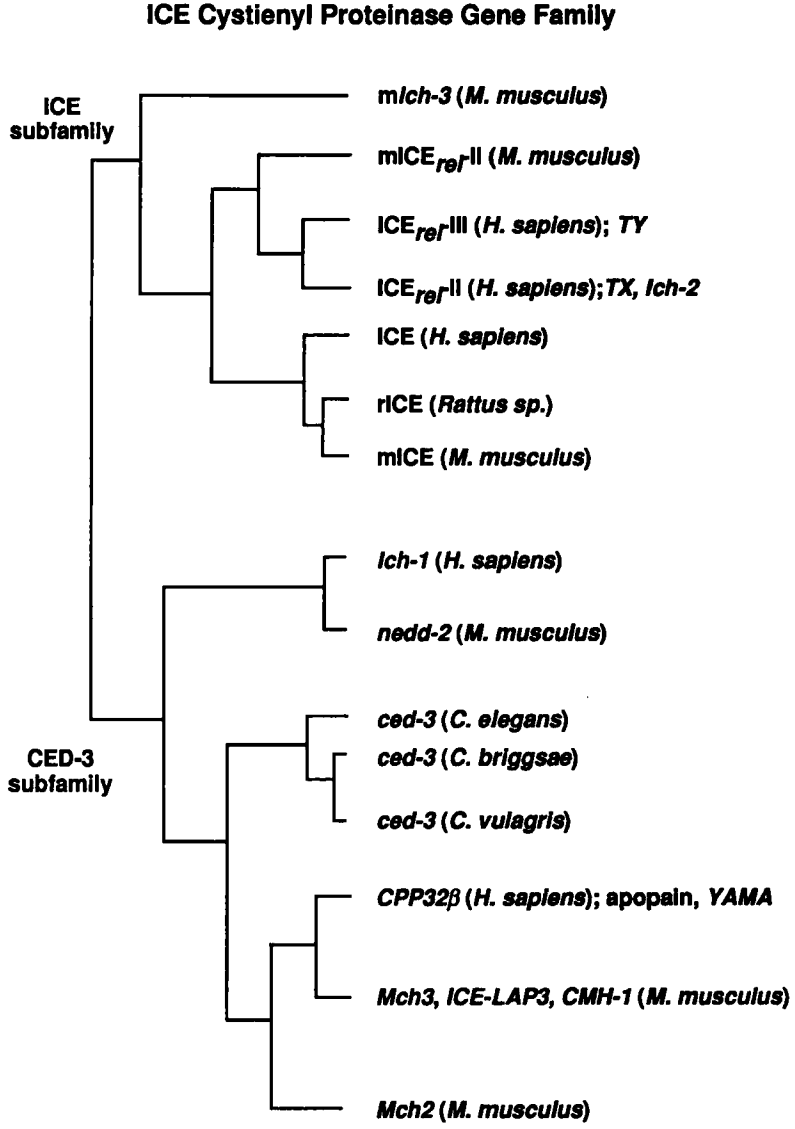

FIG. 5. Dendrogram depicting the phylogenic relationship of sequences comprising the ICE–Ced-3 gene family. (Adapted from Miller *et al.*, 1996; Thornberry *et al.*, 1996.)

ICE, the subunits are flanked by Asp-X cleavage sites; however, not all family members contain linker sequences between the subunits. This finding suggests that all members of this family are activated in an ICE-like fashion, by cleavages at the Asp-X sites. Family members possess significant sequence identities, particularly in residues associated with catalysis. The sequence Lys-Pro-Lys-X_4-Gln-Ala-Cys-Arg-Gly, encompassing the active site cysteine of ICE, is entirely conserved in virtually all of the homologs, further supporting the idea that these sequences also encode novel thiol proteinases. Many of the other key residues that are required for catalysis are also conserved (Miller *et al.*, 1996).

At this time the ICE subfamily consists of ICE itself and two other cDNAs encoding putative ICE-related thiol proteinases, ICE_{rel}-II and ICE_{rel}-III, also known as TX and TY, respectively (Munday *et al.*, 1995). Both of these sequences are expressed in monocytic cells along with ICE. Coexpression experiments indicate that ICE can cleave both ICE_{rel}-II and ICE_{rel}-III and that ICE_{rel}-II can cleave ICE (Gu *et al.*, 1995; Munday *et al.*, 1995).

The Ced-3 subfamily currently consists of Ich-1, its murine counterpart Nedd-2, Ich-2, Ich-3, Mch, and CPP32β (or YAMA) (Yuan *et al.*, 1993; Fernandes-Alnemri *et al.*, 1994; Kumar *et al.*, 1994; Wang *et al.*, 1994; Earnshaw *et al.*, 1995; Fearnhead *et al.*, 1995; Faucheau *et al.*, 1995; Nicholson *et al.*, 1995; Tewari *et al.*, 1995; Miller *et al.*, 1996; Thornberry *et al.*, 1996). All of these gene products are thought to be involved in apoptotic cell death due to their similarity to Ced-3 or their expression in apoptotic cells (Miller *et al.*, 1996; Thornberry *et al.*, 1996). Except for CPP32β, the relative potency and specificity of CrmA or peptide-based inhibitors for these ICE-related gene products are not yet known. The rate of association of CrmA for apopain ($k_{on} < 1.0 \times 10^4$ M^{-1} s^{-1}) is over 1000-fold slower than it is for ICE ($k_{on} = 1.7 \times 10^7$ M^{-1} s^{-1}) (Nicholson *et al.*, 1995; Thornberry *et al.*, 1996).

CPP32β cleaves the enzyme polyadenosine ribose polymerase (PARP), a marker of apoptosis at the sequence Asp-Glu-Val-Asp. The enzyme does not cleave pIL-1β, nor does ICE cleave PARP efficiently (Nicholson *et al.*, 1995). Moreover, CrmA or inhibitors based on the ICE cleavage site in pIL-1β (Ac-Tyr-Val-Ala-Asp-CHO) are significantly less effective against CPP32β (Table V), indicating that these enzymes may be inhibited selectively (Nicholson *et al.*, 1995; Tewari *et al.*, 1995). The active site of CPP32β, based on the X-ray structure of the enzyme, shows that the aspartic acid subsite (S_1) binding pocket is highly conserved. However, the residues that constitute the S_4 binding site and are critical in binding the P_4 residue are not conserved in CPP32β (Ro-

tonda *et al.,* 1996). Computer modeling studies based on the crystal structures of ICE and CPP23β suggest that differences in the S_4 subsites of the other ICE family members will allow even more diversity in substrate recognition.

V. Biological Role of ICE

A. Role of ICE in IL-1β Processing and Secretion

cDNA co-transfection experiments indicate that ICE not only processes pIL-1β but is also required for the export of IL-1β from cells (Wilson *et al.,* 1994; Howard *et al.,* 1995). These studies show that, as in human monocytes, insect *Sf9* cells processed pIL-1β and secreted mIL-1β only when ICE was coexpressed. Moreover, as in monocytes, mIL-1β is not detected within *Sf9* cells, suggesting that processing occurs at the plasma membrane and is concomitant with secretion. The coexpression of CrmA in *Sf9* cells blocks IL-1β processing and secretion and prevents ICE proenzyme activation. CrmA appears to act only as an intracellular inhibitor of ICE in this system and in human monocytes, further supporting the idea that processing by ICE does not occur on the outside of the cell (Howard *et al.,* 1995; Singer *et al.,* 1995). The localization of active ICE to the plasma membrane of human monocytes, coupled with the reconstitution of processing and secretion by coexpression of ICE in insect cells, suggests that ICE is part of a transmembrane pore complex that is required for IL-1β secretion (Fig. 6) (Howard *et al.,* 1995; Singer *et al.,* 1995). Evidence consistent with this idea has been obtained from ICE-deficient mice. Cells from *il1bc*-null mice do not process pIL-1β via an ICE-dependent mechanism and resemble fibroblasts transfected with the IL-1β cDNA in that they do not secrete IL-1β (Young *et al.,* 1988; Kuida *et al.,* 1995; Li *et al.,* 1995). Neither the primary sequence nor the quaternary structure of ICE provides any clues as to how the enzyme is targeted or tethered to the plasma membrane. Interactions with other cellular proteins will clearly play an important role in both of these processes.

B. ICE Knockout Mice

Gene targeting techniques have been used to generate mice that are deficient in *il1b* and *il1bc.* Phenotypic analyses of these mice have begun to generate interesting data on the biological functions of these genes and the redundancy in the IL-1 system, confirming previous stud-

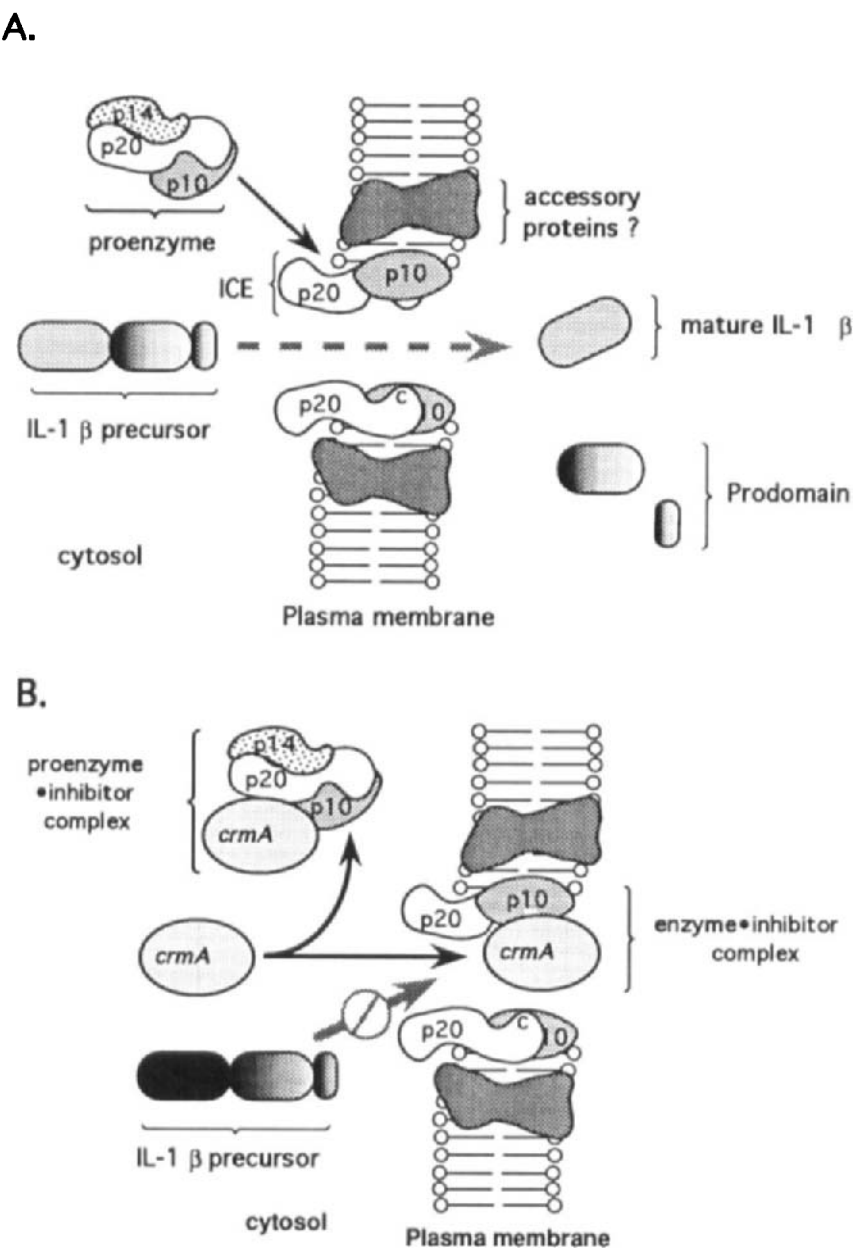

FIG. 6. (A) Drawing depicting the processing and secretion of IL-1β at the plasma membrane by ICE. (B) Model of ICE inhibition by the serpin product of the vaccinia virus *crmA* gene. (C) Model of ICE inhibition by a potent tetrapeptide aldehyde. (Adapted from Thornberry *et al.*, 1992; Howard *et al.*, 1995; Singer *et al.*, 1995.)

C.

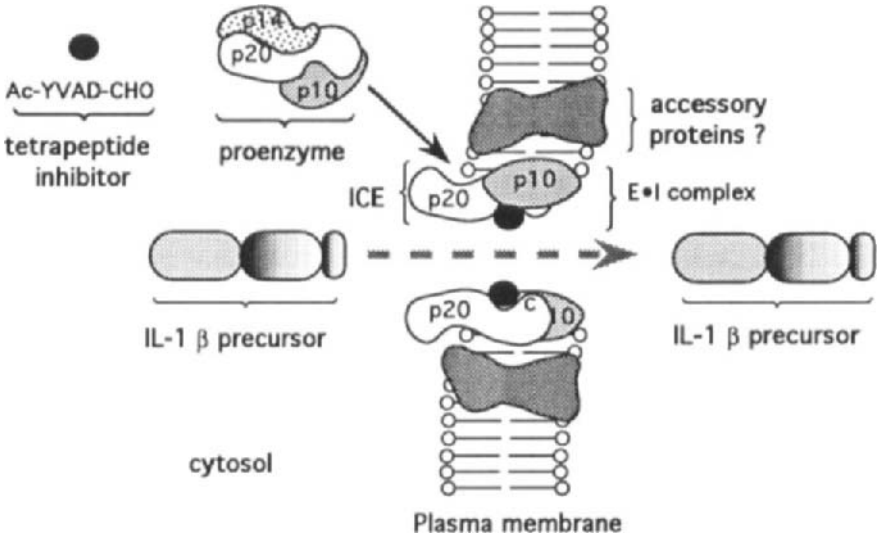

FIG. 6. (*continued*)

ies that used IL-1R antagonist or neutralizing antibodies to block the actions of IL-1.

The *il1b* gene was the first member of the *IL1* gene family to be targeted by homologous recombination in murine embryonic stem cells (Zheng *et al.*, 1995). As expected, *il1b*-null mice lack the ability to produce IL-1β, but produce normal amounts of IL-1α and tumor necrosis factor (TNF) when challenged with LPS or *Proprionibacterium acnes.* IL-1β-deficient mice are fertile and show no developmental abnormalities. These mice remain healthy under specific pathogen-free conditions. While they do not exhibit increased susceptibility to infection with the intracellular pathogen *Listeria monocytogenes,* they do exhibit a slight increase in susceptibility to and more rapid mortality from influenza virus infection (Kozak *et al.*, 1995; Zheng *et al.*, 1995). In contrast to findings from previous studies using IL-1β-neutralizing antibodies (Enk and Katz, 1991; Enk *et al.*, 1993), *il1b* knockout mice respond normally in models of contact or delayed-type hypersensitivity (Zheng *et al.*, 1995).

IL-1β-neutralizing antibodies have been shown to block interleukin-6 (IL-6) production and the febrile response in a mouse model of

turpentine-induced sterile abscess formation (Kluger, 1991). In this model, IL-1β-deficient mice do not to develop the fever or anorexia associated with the turpentine injection, nor do they produce IL-6 or acute-phase reactant proteins in response to this challenge (Zheng *et al.,* 1995). These studies confirm the central role of IL-1β in IL-6 induction and fever. In stark contrast to the turpentine challenge, IL-1β-deficient mice are not resistant to lethal LPS challenge. Unlike the turpentine challenge, the magnitude of the fever after LPS administration is reduced by only 50% in IL-1β-deficient mice, and there is no apparent difference in IL-6 or acute-phase protein synthesis compared to wild-type litter mates (Zheng *et al.,* 1995). B10.RIII mice are highly susceptible to collagen-induced arthritis (CIA). IL-1β-neutralizing antibodies block the onset as well as the progression of established CIA in B10.RIII mice (Geiger *et al.,* 1993; Van den Berg *et al.,* 1994). Studies show that IL-1β-deficient mice backcrossed (N3) onto the B10.RIII background fail to develop CIA, confirming the earlier neutralizing antibody studies (Christen *et al.,* 1996). The disease can be initiated and sustained in the deficient mice by injection of recombinant IL-1β.

The *il1bc*-null mice generated by gene targeting experiments have confirmed the role of ICE in IL-1β processing (Kuida *et al.,* 1995; Li *et al.,* 1995). These mice lack the 45-kDa ICE proenzyme. Cells from homozygous *il1bc* knockout mice contain normal intracellular levels of pIL-1β when activated, but the level of mature IL-1β is reduced by more than 95%. In heterozygous knockout mice, the level of IL-1β is 50% of that found in wild-type mice, indicating that mature IL-1β production is determined by the amount of ICE. However, a small amount of biologically active mature IL-1β is still generated in these animals. This material represents significantly less than 1% of the mature IL-1β produced by normal mice, and its production is not inhibited by a potent ICE inhibitor. Surprisingly, IL-1α levels in these mice are also reduced by >90% after LPS challenge. This observation suggests that IL-1α and IL-1β share a novel secretory pathway.

In stark contrast to IL-1β-deficient mice, ICE-deficient mice develop normal fevers in response to LPS or turpentine but are resistant to lethal LPS challenge (Kuida *et al.,* 1995; Li *et al.,* 1995). In addition, preliminary studies suggest that ICE-deficient mice also develop CIA. There are two possible explanations for these seemingly disparate results. The fever, anorexia, and arthritis responses can most likely be attributed to the small amounts of active IL-1β generated in these mice, while the resistance to LPS may result from a decrease in the levels of several major inflammatory cytokines, including IL-1α, IL-1β, and TNF (Kuida *et al.,* 1995; Li *et al.,* 1995; Zheng *et al.,* 1995). In the latter in-

TABLE VI
PHENOTYPES OF WILD-TYPE (wt), il1b-DEFICIENT (KO), AND il1bc-DEFICIENT (KO) MICE IN SEVERAL MODELS OF INFLAMMATION[a]

Model	Phenotype		
	wt	*il1b* KO	*il1bc* KO
Turpentine-induced fever	+	−	+
Turpentine-induced anorexia	+	−	+
LPS-induced shock	+	+	−
Collagen-induced arthritis	+	−	+

[a] Data from Christen *et al.* (1996), Kuida *et al.* (1995), Li *et al.* (1995), and Zheng *et al.* (1995).

stance, the overall cytokine burden is reduced and the animal is better able to survive the stress. Table VI summarizes the phenotypes of *il1b* and *il1bc* knockout mice.

The facts that ICE-deficient mice develop normally and that the majority of apoptotic process are unaffected in them indicate that ICE itself is not required for programmed cell death. Cytotoxic T-lymphocyte-mediated cell killing, as well as T-cell and macrophage apoptosis, are virtually unaffected in ICE-deficient mice. There is a modest effect on Fas-ligand-mediated apoptosis in these mice. In cells from normal mice, this process is inhibitable by CrmA and to some extent by a tetrapeptide ICE inhibitor (Enari *et al.*, 1995a,b; Fearnhead *et al.*, 1995; Los *et al.*, 1995; Tewari and Dixit, 1995). However, Fas-mediated apoptosis appears to be restricted to an extremely small subset of T lymphocytes; the significance of this is not yet understood.

VI. SUMMARY

An overwhelming body of evidence has shown that IL-1β is a major mediator of inflammatory disease (Tocci and Schmidt, 1996). The discovery of ICE, a unique processing enzyme involved in the production of active IL-1β, has provided a new approach to specifically block the production of this potent cytokine. Consequently, the discovery and development of inhibitors against the enzyme could hold great promise therapeutically. Potent inhibitors of the enzyme would be useful in the treatment of a number of important inflammatory diseases and potentially in the management of leukemia (Arend, 1993b; Estrov and Tal-

paz, 1996). A number of key questions must be answered before the therapeutic potential of such inhibitors can be realized. The development of a pharmaceutically acceptable cysteine proteinase inhibitor will almost certainly involve new chemical strategies gauged at safely inactivating the enzyme. For such inhibitors, it will be necessary to achieve selectivity for ICE from among the growing number of ICE family members while retaining potency. It will also be important to establish the level of inhibition of IL-1β required to achieve therapeutic efficacy. The studies comparing IL-1β- and ICE-deficient mice suggest that complete abrogation of IL-1β is required to achieve efficacy in models of inflammation. It is not known if this is the case in humans. Understanding the source of the residual IL-1β produced in ICE-deficient mice will be important in order to ascertain if a similar mechanism could generate active IL-1β in patients receiving an ICE inhibitor.

As for ICE itself, a number of formidable questions remain regarding its regulation and mechanism of activation. Answering these questions experimentally will present a major challenge due to the extremely low levels of enzyme present in cells. Studies on other family members may provide easier access to some of these questions and provide clues that can be applied to ICE. The components of the pathway involved in IL-1 trafficking and secretion are unknown, as are the mechanisms of ICE activation and regulation. Clearly other cellular proteins that have yet to be discovered will be involved in each of these processes, opening up new avenues of research in this field.

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The Role of the IGF-I Receptor in Apoptosis

RENATO BASERGA, MARIANA RESNICOFF, CONSUELO D'AMBROSIO, AND BARBARA VALENTINIS

Kimmel Cancer Center
Thomas Jefferson University
Philadelphia, Pennsylvania 19107

I. Introduction

The insulin-like growth factor-I receptor (IGF-IR) is a tyrosine kinase receptor (Ullrich and Schlessinger, 1990) with a 70% homology to the insulin receptor (IR) (Ullrich *et al.*, 1986). It is synthesized as a single precursor polypeptide of 1367 amino acids, including a 30-amino-acid signal peptide. The organization of the preproreceptor is NH_2–signal peptide–α subunit–β subunit–COOH. After removal of the signal peptide, the proreceptor is cleaved at the tetrapeptide RKRR after residue 706, to form the α and β subunits, linked by disulfide bonds (Werner *et al.*, 1991). The α subunit, entirely extracellular, is involved in ligand binding (Schumacher *et al.*, 1993); the transmembrane β subunit has a tyrosine kinase domain that is entirely intracellular (Fig. 1). The highest homology between insulin and insulin-like growth factor-I (IGF-I) receptors is in the tyrosine kinase domain (84%), with the lowest in the cysteine-rich pockets of the α subunit (48%). [Following Ullrich *et al.*

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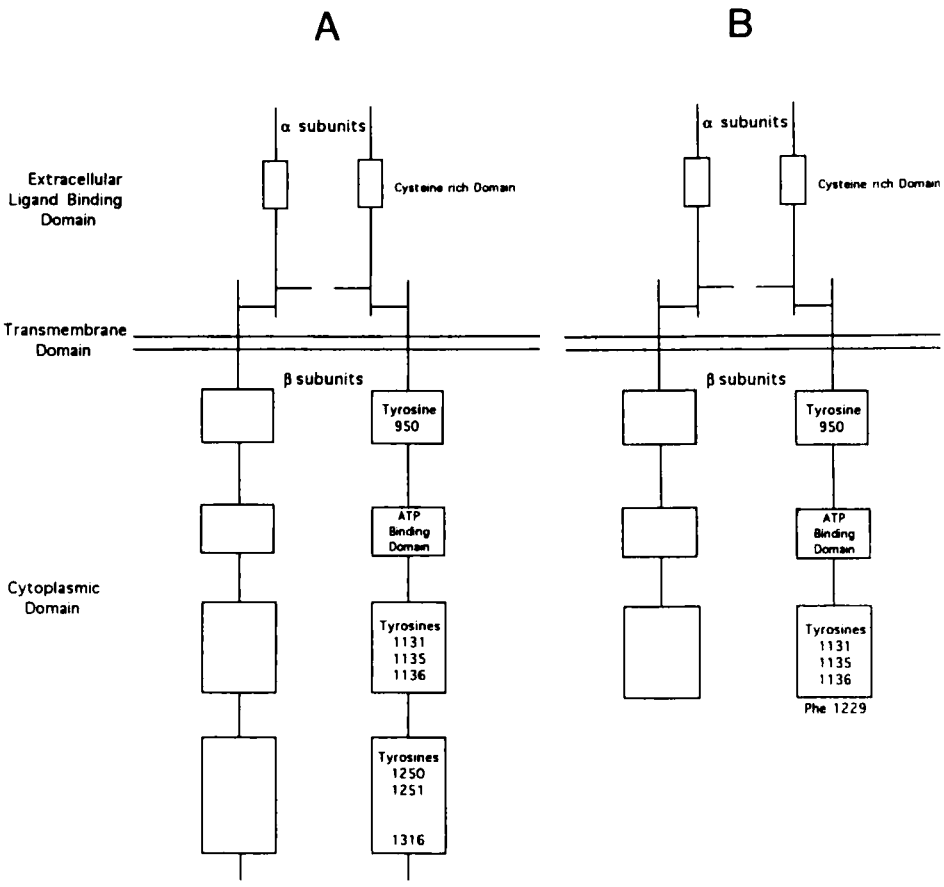

FIG. 1. Diagram of the IGF-I receptor. (A) The complete receptor, as a dimer, with the extracellular α subunits bound by disulfide bonds while the β subunits are largely cytoplasmic, although they do have extracellular and transmembrane domains. Some of the important residues in the β subunit (discussed in the text) are indicated. (B) A receptor with a C-terminal truncation. Receptors like this are nontransforming, although the tyrosine kinase domain and the binding site for IRS-1 and Shc are preserved.

(1986), in this review the amino acid residues are numbered from the first amino acid of the mature peptide up to 1337. Other authors include the signal peptide (30 extra amino acids) in their numbering system.] When activated by its ligands [IGF-I, insulin-like growth factor-II (IGF-II) or insulin], the IGF-IR transmits a signal to its major substrates, insulin receptor substrate-1 (IRS-1), insulin receptor substrate-2 (IRS-2), and Shc (White and Kahn, 1994; Myers *et al.*, 1993; Araki *et al.*, 1994),

a signal that is subsequently transduced via the common signal-transducing pathway, through Ras and Raf, all the way to the nucleus (Hill and Treisman, 1995). The IGF-IR, like the IR, has several functions, including anabolic functions, but it has three properties that are absent or markedly attenuated in the insulin receptor: it is mitogenic, it is required for the establishment and maintenance of the transformed phenotype, and it protects cells from apoptosis.

This review examines the role of the IGF-IR in apoptosis, but, since cellular proliferation and transformation are clearly related to apoptosis, we first examine the role of the IGF-IR in mitogenesis and in transformation, and then focus on its role in apoptosis.

II. The IGF-I Receptor in Mitogenesis

For many years, the IGF-IR, activated by its ligands, has been known to be mitogenic to cells in culture. However, in growth-regulated cells (like mouse 3T3 cells and human diploid fibroblasts), IGF-I, by itself, cannot sustain growth of cells in serum-free medium, but requires the cooperation of other growth factors—for instance, platelet-derived growth factor (PDGF) and/or epidermal growth factor (EGF)—that by themselves also fail to induce a mitogenic response (Scher *et al.*, 1979; Stiles *et al.*, 1979). The importance of the activated IGF-IR in cell growth has been rigorously demonstrated *in vivo* by Efstratiadis and co-workers, who showed that mouse embryos with a targeted disruption of the IGF-IR and IGF-II genes (*igfr*$^{-/-}$ mice) have a size at birth that is only 30% the size of wild-type litter mates (J. P. Liu *et al.*, 1993; Baker *et al.*, 1993). 3T3-like cells derived from these mouse embryos devoid of IGF-IRs (R^- cells) grow in 10% fetal bovine serum, albeit at a rate that is roughly 40% the rate of W cells, which are derived from mouse embryos of wild-type litter mates to the *igfr*$^{-/-}$ mice. In R^- cells, all phases of the cell cycle are equally elongated (Sell *et al.*, 1994), indicating that, at variance with established wisdom, the activation of the IGF-IR is important in maintaining the flow of cells through all phases of the cell cycle.

R^- cells do not grow at all in serum-free medium supplemented by a variety of growth factors that can sustain the growth of cells derived from wild-type litter mate embryos (W cells) and other 3T3 cells (Sell *et al.*, 1994). The important role of the IGF-IR and its ligands in cell proliferation seems to extend to a variety of cell types, which include fibroblasts, epithelial cells, mammary gland cells, hemopoietic stem cells and mature hemopoietic cells, osteoblasts, vascular smooth muscle

cells, and cells of the central nervous system. A list of cells requiring IGF-I for optimal growth can be found in Baserga and Rubin (1993). However, an important point to remember is that IGF-I is not essential for growth, as demonstrated by R^- cells that do grow in 10% serum (Sell *et al.*, 1994). What we should say is that the activated IGF-IR is required for *optimal* growth, *in vivo* and *in vitro,* in 10% serum but is not an absolute requirement. It becomes an absolute requirement only in serum-free medium supplemented with most of the known growth factors; the corollary is that there must be an unidentified growth factor in the serum (and in the animal), and/or an unidentified growth factor receptor, that can at least partially overcome the IGF-I requirement for cell growth.

One important observation made possible by R^- cells is that overexpression of the EGF (Coppola *et al.*, 1994), PDGF (DeAngelis *et al.*, 1995), and insulin (Miura *et al.*, 1995b) receptors is not sufficient for ligand-dependent growth, unless a functional IGF-IR is present. On the contrary, an overexpressed IGF-IR renders cell capable of growing in IGF-I only (Pietrzkowski *et al.*, 1992a), without any activation of the PDGF and EGF receptors. This would suggest a central role of the IGF-IR in the mitogenic action of other growth factors.

III. The Signal-Transducing Pathways of the IGF-I Receptor

Although it has several features in common with the IR (see review by Tavaré and Siddle, 1993), the β subunit of the IGF-IR is 10 times more mitogenic than the β subunit of the IR (Lammers *et al.*, 1989). Both the IR and the IGF-IR use the Ras pathway, discussed later, but through the intermediate of their docking protein, the IRS-1 protein (Sun *et al.*, 1992; Baltensperger *et al.*, 1993; Keller and Lienhard, 1994; O'Neill *et al.*, 1994). An excellent review on the insulin signaling system (including IRS-1), concise and clear and with important references, can be found in White and Kahn (1994). Myers *et al.* (1993) have also provided the evidence that the IGF-IR interacts with IRS-1, and Tobe *et al.* (1993) have shown that IRS-1 interacts with Grb2. The well-known Ras pathway (Fig. 4 in the review by White and Kahn, 1994) derives from IRS-1. Several reports have appeared clearly indicating that IRS-1 plays an essential role in the mitogenicity of both insulin and IGF-I in cells in culture (Waters *et al.*, 1993; Wang *et al.*, 1993; Rose *et al.*, 1994; Yamauchi and Pessin, 1994; D'Ambrosio *et al.*, 1995; and the experiments of White and co-workers mentioned earlier). The IR and the IGF-IR have a second direct substrate in the Shc proteins (see, e.g., Yamauchi and Pessin, 1994; Pronk *et al.*, 1993). There are three Shc

proteins, all apparently derived from a single gene, with molecular masses of 66, 52, and 46 kDa (Pelicci *et al.,* 1992). Both IRS-1 and Shc are tyrosyl phosphorylated when the IR or the IGF-IR are stimulated by their respective ligands; IRS-1 phosphorylation, however, seems more specific than Shc, since the latter is also tyrosyl phosphorylated by other growth factor receptors, such as the PDGF and EGF receptors (Basu *et al.,* 1994; Li *et al.,* 1994; Ohmichi *et al.,* 1994). Recently, a third substrate of the IGF-IR and the IR has been identified and designated as IRS-2 (Araki *et al.,* 1994), which has a weak homology to IRS-1 and can partially replace it, but, at the moment of writing, little information is available on its functions.

As mentioned earlier, the IGF-IR, through IRS-1, IRS-2, and Shc, connects with the pathway that, for convenience, we shall call the Ras pathway, which is reviewed extensively in several papers and involves, among others, phosphatidylinositol-3 kinase, Grb2, Sos, and other transducing molecules (Matuoka *et al.,* 1993; for reviews, see McCormick, 1993; Hill and Treisman, 1995). Blenis (1993) gives a list of the proteins involved in the signal transduction cascade, from Ras to c-Raf to MEK kinase, MEK, ERKK, p92, and finally transcription factors (see also Hill and Treisman, 1995). This pathway (schematized in Fig. 2) is used by the three growth factors, PDGF, EGF, and IGF-I, that together sustain the growth of wild-type mouse embryo cells. Although the evidence is overwhelming that Ras activation is required for optimal cell proliferation and transformation (Lu *et al.,* 1989; Cai *et al.,* 1992; Medema and Bos, 1993), our findings indicate that it is not sufficient, since a constitutively activated Ras fails to induce growth of R^- cells in serum-free medium supplemented with individual growth factors. Ras-independent pathways for growth (and transformation; see later) have also been suggested independently by others (Falco *et al.,* 1988; Aaronson, 1991; Silvennoinen *et al.,* 1993; Sakaue *et al.,* 1995; Buscher *et al.,* 1995). Barone and Courtneidge (1995) have reported that the oncogene v-*src* may bypass the Ras pathway in PDGF-induced activation of c-Myc expression. This pathway is a good candidate for the Ras-independent pathway originating from the IGF-IR, especially in view of the fact that v-Src can tyrosyl phosphorylate both the IGF-IR and IRS-1 (Peterson *et al.,* 1994).

IV. The IGF-I Receptor in Transformation

Overexpression and/or constitutive activation of IGF-IR in a variety of cell types leads to ligand-dependent growth in serum-free medium and to the establishment of a transformed phenotype—that is, one ca-

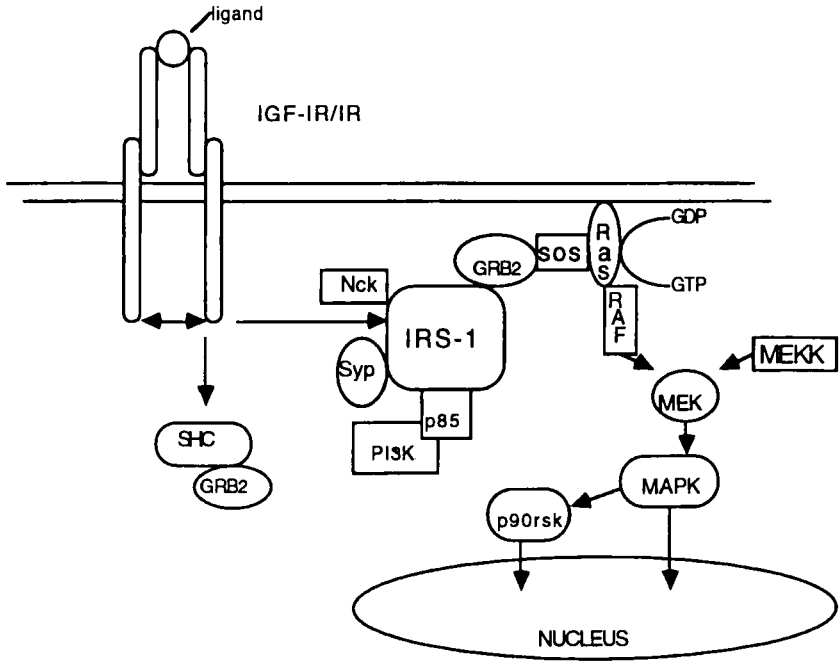

FIG. 2. A simplified view of the signal-transduction pathway of the IGF-I and insulin receptors. This is essentially the Ras pathway common to other growth factor receptors, except that these two receptors use a docking protein, IRS-1 (and probably another one, IRS-2), to make a connection with the Ras pathway. As explained in the text, other unidentified substrates and pathways are necessary to explain all the functions of the IGF-I receptor.

pable of forming colonies in soft agar or producing tumors in nude mice (Kaleko *et al.*, 1990; McCubrey *et al.*, 1991; Pietrzkowski *et al.*, 1992; D. Liu *et al.*, 1993; Sell *et al.*, 1994; Coppola *et al.*, 1994; Rogler *et al.*, 1994). This by itself is not a unique property, since a great variety of overexpressed gene products can transform cells, including a number of growth factor receptors (for a list of these receptors, see Coppola *et al.*, 1994, and later).

The crucial finding that clearly pointed out the major role played by the IGF-IR in transformation was the demonstration that R^- cells are refractory to transformation by simian virus 40 (SV40) large T antigen, an activated Ras, or a combination of both (Sell *et al.*, 1993, 1994); by bovine papillomavirus E5 protein (Morrione *et al.*, 1995); or by overexpressed growth factor receptors, such as the EGF receptor (Coppola *et al.*, 1994), the PDGF β receptor (DeAngelis *et al.*, 1995), and the IR

(Miura *et al.,* 1995b)—all conditions that readily transform cells from wild-type litter mate embryos or other 3T3-like cells with a physiological number of IGF-IRs. It would be exaggerated to claim that R^- cells are untransformable; we have been growing these cells in our laboratory for over 4 years, and we have observed spontaneous transformation, at a rate roughly three logarithms lower than mouse cells, with a physiological number of IGF-IRs. 3T3-like cells have such a propensity to chromosomal rearrangements and spontaneous transformation that it is easy to visualize how a gain-of-function mutation in a signal-transducing molecule downstream of the IGF-IR may bypass the IGF-IR requirement for transformation.

If deletion of the IGF-IR genes renders cells refractory to transformation, it is not surprising that a reversal of the transformed phenotype can be obtained in a variety of tumor cells by decreasing the number of IGF-IRs or by interfering with their function. For instance, antisense expression plasmids or antisense oligodeoxynucleotides (ODN) against either IGF-II (Christophori *et al.,* 1994), IGF-I (Trojan *et al.,* 1993), or the IGF-IR (Sell *et al.,* 1993; Resnicoff *et al.,* 1994a,b; Shapiro *et al.,* 1994; Neuenschwander *et al.,* 1995), antibodies to the IGF-IR (Arteaga, 1992; Kalebic *et al.,* 1994), and dominant negative mutants of the IGF-IR (Prager *et al.,* 1994; Li *et al.,* 1994; Burgaud *et al.,* 1995) can all reverse the transformed phenotype, inhibit tumorigenesis, and induce loss of the metastatic phenotype (Long *et al.,* 1995).

In theory, and possibly also in practice, targeting the IGF-IR may be more efficient than targeting its ligands. The adult rat has negligible plasma levels of IGF-II, but other animals, including humans, have substantial circulating concentrations of both IGFs (Conlon *et al.,* 1995). Interference with one of the ligands could leave the other one free to activate the IGF-IR.

V. Mutational Analysis of the IGF-I Receptor

Mutations in the various domains of the IGF-IR can affect its various functions; for instance, a single point mutation at K1003 (the ATP binding site) results in an IGF-IR that has essentially lost all its functions (Kato *et al.,* 1993; Coppola *et al.,* 1994). A triple mutation of the three tyrosines (1131, 1135, 1136) in the tyrosine kinase domain results in a markedly reduced level of autophosphorylation and in the abolition of both mitogenicity and transforming activity (Gronborg *et al.,* 1993; Li *et al.,* 1994). Similarly, R^- cells stably transfected with a human IGF-IR mutated at Y950 cannot grow in serum-free medium supplemented

solely with IGF-I and do not form colonies in soft agar, although the medium in this assay is 10% serum (Miura *et al.*, 1995a). These mutations affect both the mitogenic and transforming activities of the IGF-IR, but mitogenicity here must be defined as ability of cells in monolayers to respond to IGF-I only or to a combination of IGF-I, PDGF, and EGF (see earlier). Mitogenicity of a wild-type or mutant IGF-IR cannot be tested in serum, since R^- cells have shown that the IGF-IR is not an absolute requirement for growth in 5–10% serum (see earlier). The fact that the soft agar assay is done in 10% serum already suggests that the growth factor(s) in serum that can bypass the IGF-IR for growth in monolayers cannot replace it for colony formation in soft agar. A corollary of these findings is that inhibitors of IGF-IR function will have very little effect on the growth of cells in monolayers in serum-supplemented medium, while having much more dramatic effects on growth in soft agar of *in vivo*.

More interesting are those mutant receptors in which only selective functions are deleted. Using a constitutively activated IGF-IR, with a glycosaminoglycan protein in lieu of the α domain, D. Liu *et al.* (1993) found that truncations in the C-terminal domain of the receptor affected its ability to induce transformation. These authors, however, did not test for mitogenic activity. It was Surmacz *et al.* (1995) who clearly dissociated the mitogenic and transforming activities of the IGF-IR by showing that the deletion of the C-terminal 108 amino acids from the human IGF-IR resulted in a receptor that had conserved its ability to transmit an IGF-I-mediated mitogenic signal but was no longer capable of transforming activity. R^- cells stably transfected with this receptor, truncated at residue 1229, grow in serum-free medium supplemented solely with IGF-I but cannot form colonies in soft agar, although the soft agar assay is done in 10% serum. Subsequently Miura *et al.* (1995b) showed that a single point mutation at Y1251 (to phenylalanine) also greatly reduced the transforming activity of the IGF-IR, without affecting its mitogenicity. Interestingly, a Y1250F mutation had no effect on the receptor, which behaved essentially as a wild-type IGF-IR (Miura *et al.*, 1995b). In these experiments, we transfected mutant receptors in R^- cells to generate stable cell lines with no other IGF-IR but the transfected one. The absence of endogenous receptors is a distinct advantage in a mutational analysis of this type because the intermolecular transactivation that often occurs among growth factor receptors and the possible formation of hybrid receptors (Ballotti *et al.*, 1989; Lammers *et al.*, 1990; Taouis *et al.*, 1994; Takata and Kobayashi, 1994; Chantry, 1995; Burgaud and Baserga, 1996) can transactivate receptors that, per se, are inactive.

We have extended these investigations to other mutants of the IGF-IR (Hongo *et al.*, 1996; Li *et al.*, 1996), and the results are summarized in Table I.

The conclusions from these experiments can be summarized as follows: (1) mitogenicity and transforming activity of the IGF-IR can be separated (Surmacz *et al.*, 1995; Miura *et al.*, 1995b; Table I), clearly indicating that there is at least one pathway for transformation that is additional to and distinct from the mitogenic pathways, and is very likely an unidentified Ras-independent pathway (Sell *et al.*, 1994); (2) the transforming domain of the IGF-IR can be localized between residues 1245 and 1310 (Hongo *et al.*, 1996; see Table I); and (3) Y1251 and the serine quartet at 1280–1283 are very important for transformation (Miura *et al.*, 1995b; Table I), with the region around 1293 playing some

TABLE I

MUTATIONAL ANALYSIS OF THE IGF-I RECEPTOR IN GROWTH AND TRANSFORMATION

Receptor[a]	Reference	Mitogenicity[b]	Transformation[c]
Wild-type	Ullrich *et al.* (1986)	+++	+++
ATP-binding mutant	Kato *et al.* (1993)	–	–
Y950*	Miura *et al.* (1995a)	–	–
3Y*	Li *et al.* (1994)	–	–
	Gronborg *et al.* (1993)		
Single Ys*	Li *et al.* (1994)	++	–
Y1250*	Miura *et al.* (1995b)	+++	+++
Y1251*	Miura *et al.* (1995b)	+++	–
Serine mutant*	Li *et al.* (1996)	+++	–
1293–1294*	Hongo *et al.* (1996)	++	+
Y1316F*	Hongo *et al.* (1996)	+++	+++
del. 1229	Surmacz *et al.* (1995)	+++	–
del. 1245*	Hongo *et al.* (1996)	+++	–
del. 1293*	Hongo *et al.* (1996)	++	+
del. 1310*	Hongo *et al.* (1996)	+++	+++

[a] 3Y has Y-to-F mutations at 1131, 1135, and 1136 (kinase domain). We also made mutants at each single tyrosine. The serine mutant is a mutant in which the four serines at 1280–1283 have been mutated to alanine. The 1293–1294 mutant has the two basic amino acids at those residues replaced by alanine. del. indicates receptors that have been truncated at the indicated residue (the amino acid number is the one proposed by Ullrich *et al.*, 1986).

[b] Mitogenicity is defined as ability to grow in serum-free medium supplemented solely with IGF-I.

[c] Transformation is the ability to form colonies in soft agar.

*Indicates mutants developed in our laboratory.

role in it. When these residues are mutated, the transforming activity of the IGF-IR is severely impaired, whereas its mitogenic activity in response to IGF-I is perfectly normal. *It should not be surprising that the transforming pathway is additional to and distinct from the mitogenic pathways, since transformed cells are anchorage independent, and this characteristic is not required by cells growing in monolayers. What is novel in these findings is that the two pathways originate from different domains of a single growth factor receptor.*

An overexpressed IRS-1 can transform 3T3-like cells with a physiological number of IGF-IRs but cannot transform R^- cells (D'Ambrosio *et al.*, 1995). In our hands, Shc, which was reported to be a transforming protein (Pelicci *et al.*, 1992; Salcini *et al.*, 1994), is nontransforming, whether in R^- cells or in BALB/c 3T3 cells (Table II). We mentioned before that the SV40 large T antigen, singly transfected, cannot transform R^- cells; however, a combination of T antigen and IRS-1 is transforming. Interestingly, T antigen binds to IRS-1, and its binding is necessary for the co-transformation of R^- cells (Zhou-Li *et al.*, 1995).

Table II summarizes the transforming potential of the IGF-IR and its major substrates. It is significant that the wild-type IGF-IR, by itself, is fully transforming (R^+ and p6 cells), as already reported by several laboratories (see earlier), while its two major substrates, IRS-1 and Shc,

TABLE II
TRANSFORMING POTENTIAL OF THE IGF-I RECEPTOR AND ITS MAJOR SUBSTRATES

Cell Line and Receptor Number[a]	Colony Formation in Soft Agar
R^- cells (0)	0
W cells (30×10^3)	0
BALB/c 3T3 (25×10^3)	0
R^+ (10^6)	140
p6 cells (5×10^5)	159
W/IRS-1	35
R^-/IRS-1	0
W/Shc (9 clones)	0
BALB/c 3T3/Shc (5 clones)	0–5
R^-/IRS-1/Shc (4 clones)	0

[a] R^- and W cells were established from litter mate embryos, with or without a targeted disruption of the IGF-IR genes (Sell *et al.*, 1993). R^+ are R^- cells stably transfected with a wild-type human IGF-IR cDNA. p6 cells are like R^+ but with a BALB/c background. All the IRS-1 and Shc clones markedly overexpressed the respective protein (D'Ambrosio *et al.*, 1995, unpublished data).

singly or in combination, are not (W/IRS-1, W/Shc, Balb/Shc, and R^-/IRS-1/Shc cells). The latter observation is intriguing, especially in view of the fact that both Surmacz *et al.* (1995) and Miura *et al.* (1995b) reported an increased tyrosyl phosphorylation of IRS-1 and Shc in cells expressing the mutant IGF-IRs that are mitogenic but nontransforming. It indicates essentially two alternatives: Either (1) the transforming pathway of the IGF-IR C terminus is totally independent of IRS-1 and Shc (something also suggested by the fact that it is a Ras-independent pathway), or (2) the transforming domain of the IGF-IR causes in IRS-1 and Shc more subtle changes than can be detected by conventional techniques.

VI. The IGF-I Receptor and Its Ligands in Apoptosis

A historical review of apoptosis and its morphological aspects is provided by Majno and Joris (1995), who also compare it to other forms of cell death. Steller (1995) has given a lucid discussion of the genetic basis of cellular suicide, and Fisher (1994) and Thompson (1995) have pointed out the possible therapeutic implications. Since apoptosis is the subject of this entire volume, we refer the reader to other articles for details and limit ourselves to the role of the IGF-IR in the process.

There have been sporadic reports that IGF-I may protect cells, *in vivo* or *in vitro,* from cell death in general and apoptosis in particular. For instance, Gluckman *et al.* (1992) reported a protective effect of IGF-I in ischemic injuries or the central nervous system, and D'Mello *et al.* (1993) found that IGF-I inhibited low-potassium-induced apoptosis of cerebellar granule neurons. There is also an intriguing report by Rodriguez-Tarduchy *et al.* (1992) that IGF-I inhibits apoptosis in interleukin (IL)-3-dependent hemopoietic cells. But the interest in the role of the IGF-IR in apoptosis received its impetus with the finding by Harrington *et al.* (1994) that IGF-I (and, to a lesser extent, PDGF, but not EGF or fibroblast growth factor) exerts a protective effect on c-Myc-induced apoptosis (Evan *et al.,* 1992; Askew *et al.,* 1992). Subsequently, our laboratory showed that an overexpressed IGF-IR protected cells *in vitro* from etoposide-induced apoptosis (Sell *et al.,* 1995), and, even more dramatically, that a decrease in IGF-IR levels below wild-type levels caused massive apoptosis of tumor cells *in vivo* (Resnicoff *et al.,* 1995a,b). These *in vivo* experiments are detailed in the next section. The role of the IGF-IR in apoptosis, and the possibility that it may discriminate between normal and tumor cells, have been discussed in two reviews by Baserga (1994, 1995).

VII. The *in Vivo* Method for Determining the Extent of Apoptosis

The reports mentioned earlier (see Section IV) on the role of the IGF-IR in tumorigenesis strongly suggested that tumor cells with an impaired IGF-IR function must have died when injected into rodents. The complete absence of tumor development in the first place suggests that the effect of interference with the action of the IGF-IR is not simply to slow down growth, but actually to kill cells, either by apoptosis or some other mechanism of cell death. The experiments in nude mice with human melanoma cells (Resnicoff *et al.*, 1994a) further confirmed the role of cell death in IGF-IR-mediated inhibition of tumor growth. But it is difficult to demonstrate cell death in general (Fernandez *et al.*, 1994), or apoptosis in particular, *in vivo* in cells injected into the subcutaneous tissue of rodents. If the cells die, one finds very little at the site of injection, certainly not enough to make a quantitative assessment of apoptosis. We therefore had to devise a method for demonstrating cell death in general, and apoptosis in particular, of tumor cells *in vivo* (Resnicoff *et al.*, 1995a). For this purpose, we used a diffusion chamber implanted into the subcutaneous tissue of rats or mice. This diffusion chamber (Abraham *et al.*, 1993) is a Lucite ring closed at both ends by a Millipore filter with pores 0.1 μm in diameter. The pore size allows the passage of proteins, nutrients, antibodies, and the like but not of cells (Lange *et al.*, 1994), and it is essentially the same type of diffusion chamber that was used many years ago by tumor immunologists to prove rigorously that tumor cells in the immune animal could not be destroyed unless the cells of the host came in direct contact with the tumor cells used as antigen. It is still used by immunologists (Lange *et al.*, 1994), although it is not as popular as it was many years ago. The cells to be studied are placed in the previously sterilized diffusion chamber, which is then inserted, under anesthesia, into the subcutaneous tissue of rats or mice. The diffusion chambers can then be removed at the desired intervals after insertion. Cells from several transplantable tumors of human or rodent origin, when placed in diffusion chambers, double in number in 24 hr during *in vivo* incubation, indicating that the conditions are optimal for their survival and proliferation. Because of the limited size of the chamber, there is crowding when the cells reach a certain number. For instance, 5×10^5 tumor cells double in number in 24 hr *in vivo* (Resnicoff *et al.*, 1995a,b), but, if 10^6 cells are loaded into the chamber, then the number of cells increases only by 50%. Conversely, if lower numbers of cells are used, they will grow in the chamber for several days until they reach a total of $1.5–1.6\times10^6$ cells per

chamber. Not all cells grow or even survive in the chamber; for instance, 3T3 cells die in the chamber, just as they die when injected into the subcutaneous tissue of syngeneic or nude animals. However, all the transplantable rodent tumor cells we have tested so far do very well in the diffusion chamber. In short, cells in the diffusion chamber behave *in vivo* as cells injected into the subcutaneous tissue of rodents.

VIII. The IGF-I Receptor Protects Tumor Cells from Apoptosis *in Vivo*

While several rodent and human cell lines from transplantable tumors grow very well in diffusion chambers, a totally different result is obtained when the same cells are stably transfected with a plasmid expressing an antisense RNA to the IGF-IR RNA or are treated with antisense ODN to the IGF-IR RNA. These cells have a decreased number of IGF-IRs, and they undergo massive apoptosis when loaded into diffusion chambers that are then inserted into the subcutaneous tissue of either rats or mice (Resnicoff *et al.*, 1995a,b). Apoptosis is almost complete within 4 hr after incubation *in vivo*. An illustration of the rapidity with which cells expressing an antisense plasmid to the IGF-IR RNA die is given in Fig. 3. The tumors tested included the C6 rat glioblastoma, a rat rhabdomyosarcoma, a human melanoma, and a mouse melanoma. In each instance, control cells (wild-type or "sense" cells) doubled in number in 24 hr, whereas cells expressing an antisense plasmid or antisense-treated wild-type cells died, with recovery ranging from 0 to 1–2% (Resnicoff *et al.*, 1995a). Therefore, one can say that targeting the IGF-IR to induce apoptosis in tumor cells is not limited to a single cell type. Another advantage of this assay is that apoptosis is so rapid that one can also study nonsyngeneic cells, since everything is over before any immune response may set in. For the same reason, it makes no difference whether the diffusion chambers are inserted into the subcutaneous tissue of rats or of mice.

There is actually a correlation between concentration of antisense ODN, the IGF-IR numbers, apoptosis, growth in nude mice, and growth in syngeneic animals (Resnicoff *et al.*, 1995b). This correlation is summarized in Table III. In control cells (untreated or treated with a random ODN at very high concentrations), the number of receptors remain normal; the cells double in number in 24 hr in the diffusion chambers, produce tumors palpable after only 4 days in nude mice, and produce tumors in syngeneic rats. These tumors continuously increase in size and eventually kill the animals. Treatment with antisense ODN

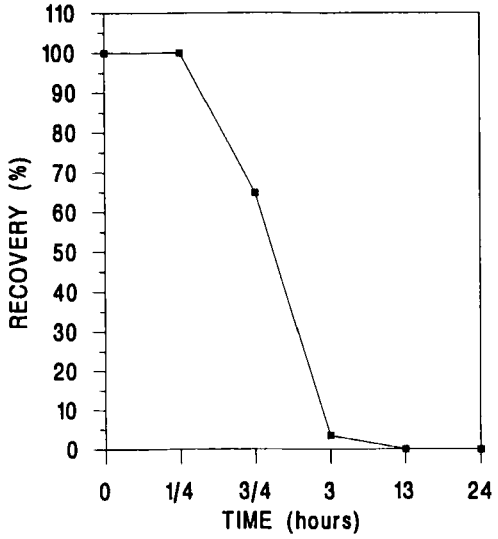

FIG. 3. Induction of apoptosis *in vivo* by a decrease in the number of IGF-I receptors. The cells shown are C6 rat glioblastoma cells, stably transfected with a plasmid expressing an antisense RNA to the IGF-IR RNA. After preincubation *in vitro,* the cells were inoculated into a diffusion chamber (permeable to nutrients but not to cells), which was then inserted into the subcutaneous tissue of rats. Chambers were removed at the intervals indicated on the abscissa and the surviving cells counted. The percentage of surviving cells (recovery) is given on the ordinate. The controls are given in Resnicoff *et al.* (1995a); suffice it to say that wild-type cells or C6 cells expressing a sense RNA double in number in 24 hr (200% recovery).

against the IGF-IR RNA causes a concentration-dependent decrease in receptor number, which in turn causes a concentration-dependent extent of apoptosis *in vivo.* In nude mice, delay in tumor growth is proportional to the extent of apoptosis, indicating a close relationship between the ability to induce apoptosis *in vivo* and the retardation of tumor growth. In syngeneic rats, tumors develop at the lowest concentrations of antisense ODN, but the tumors eventually regress and never recur. At higher ODN concentrations, the tumors do not even appear. The difference between nude mice and syngeneic animals clearly indicates that there is also a host response, which is discussed later.

Another striking observation is that a decrease in the number of IGF-IRs is much more effective in inducing apoptosis *in vivo* than in monolayer cell cultures. Conditions that *in vitro,* in monolayer cultures, result only in inhibition of IGF-I-mediated growth, an inhibition often very modest, will cause massive apoptosis *in vivo* (Resnicoff *et al.*,

TABLE III
EFFECT OF A DECREASE IN THE NUMBER OF IGF-I RECEPTORS ON APOPTOSIS, TUMORIGENESIS IN NUDE MICE, AND TUMORIGENESIS IN SYNGENEIC RATS[a]

Concentration (μM)	Receptor Number	Recovery (%)	Tumorigenesis	
			Nude Mice (days)	Syngeneic rats
Control	100	>200	4	6/6
0.15	77	54	6	0/6
0.80	85	48	ND	0/3
1.50	80	35	11	0/3
2.50	82	35	ND	0/3
3.0	56	1.3	17	0/3
6.5	56	0.4	17	0/3
13.0	42	0.010	17	0/3
16.0	43	0.008	17	0/3
19.0	36	0.001	24	0/3

[a] C6 rat glioblastoma cells were treated for 24 hr at the indicated concentrations of antisense ODN to the IGF-IR RNA; controls were treated with 19 μM random ODN or left untreated. The cells were then tested for receptor number (by Scatchard analysis), inoculated into diffusion chambers, or injected into nude mice (10^5 cells) or into syngeneic BD IX rats (10^7 cells subcutaneously). Receptor number is expressed as percentage of untreated (or random ODN-treated) cells. Apoptosis is expressed as percentage of cells recovered (from 5×10^5 cells) after 24 hr in the diffusion chambers inserted into the subcutaneous tissue of rats. Growth in nude mice is expressed as time required in days for the appearance of palpable tumors. In the case of syngeneic rats, at the lowest concentrations of antisense ODN, tumors actually appeared after a few days but they completely regressed. (Modified from Resnicoff *et al.*, 1995b.)

1995a,b). Colony formation in soft agar is somewhat intermediate; reduction in the number of colonies in soft agar is much more pronounced than growth inhibition in monolayer cultures but not as dramatic as the extent of apoptosis *in vivo*. These findings have also been repeated on several cell types, and our two preferred explanations for this difference are (1) the fact that high concentrations of serum can also produce apoptosis *in vitro* (Kurita and Namiki, 1994), or (2) more likely, the fact that soft agar growth or growth in the subcutaneous tissue of animals requires anchorage independence, a property that is not tested in monolayers. The importance of the IGF-IR in protecting cells from high concentrations of serum is demonstrated in Fig. 4. Wild-type C6 cells, C6 cells expressing a sense, and C6 cells expressing an antisense RNA to the IGF-IR RNA were incubated in increasing concentrations of serum. Figure 4 shows that, whereas all three cell lines grow well in 10%

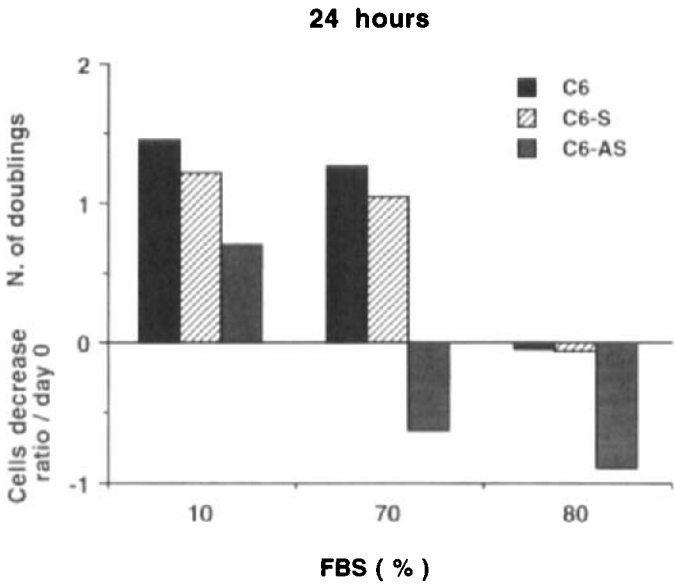

FIG. 4. Effect of high concentrations of serum on apoptosis of C6 cells. Wild-type C6 cells and C6 cells expression either a sense (C6-S) or an antisense (C6-AS) RNA to the IGF-IR RNA were plated in the concentrations of fetal bovine serum indicated on the abscissa. The number of cells in the plates were counted after 24 hr; the results are expressed as increase or decrease over numbers of cells plated. Note that all cells grow in 10% serum and all die in 80% serum. In 70% serum, wild-type and sense cells still grow while cells expressing the antisense plasmid die.

serum, at 80% serum the antisense-expressing cells dramatically decrease in number while the other two cell lines survive. This is reasonable because R cells do grow in 10% serum, which means that, under the usual culture conditions, the IGF-IR is not required for protection from apoptosis. It is in the absence of growth factors (Rodriguez-Tarduchy *et al.*, 1992; Harrington *et al.*, 1994) or in the presence of high concentrations of serum or plasma (as in the living animals and in Fig. 4), that the protective effect of the IGF-IR is strikingly evident. This raises some legitimate questions about the wisdom of using conventional cell culture techniques to test drugs and agents for their ability to kill cancer cells. The use of diffusion chambers would probably save a lot of time and money in the identification of drugs that are active *in vivo*.

Since the IGF-IR can induce the transformed phenotype and its functional ablation can induce apoptosis, it is legitimate to ask whether the two processes are the mirror images of the same function. If this were

true, nontransforming IGF-IRs (see Table I) should not protect from apoptosis. Although the results are preliminary, this seems not to be the case. Certain IGF-IRs that are nontransforming are very good at protecting cells from apoptosis, and vice versa. The problem is therefore somewhat complex, and the two processes, while obviously overlapping, may be distinct, just as mitogenicity and transforming activity are distinct (see Table I).

IX. The IGF-I Receptor and Apoptosis in Normal Cells

We have previously hypothesized (Baserga, 1994, 1995) that a decrease or a loss of function of the IGF-IR will cause massive apoptosis in tumor cells, while being much less effective on normal cells that can take refuge in the G_0 state. There are findings in the literature that support the hypothesis that normal cells may be more resistant to apoptosis due to loss of function of the IGF-IR than tumor cells. In mouse embryos with a targeted disruption of the IGF-IR genes (Baker *et al.*, 1993; J. P. Liu *et al.*, 1993), there is no evidence of increased apoptosis. Similarly, the cells derived from these mouse embryos, (R^- cells; (see earlier) grow more slowly but do not seem more prone to apoptosis by serum deprivation than the cells generated from wild-type litter mates. Indeed, even the observation by Harrington *et al.* (1994), already mentioned, indicates that, to induce apoptosis in the absence of IGF-I, one needs an overexpressed c-Myc (i.e., an oncogene) (Evan *et al.*, 1992).

Another experiment that supports this hypothesis is summarized in Table IV. WI-38 human diploid fibroblasts, untreated or treated with random or antisense ODN to the IGF-IR, were inoculated into diffusion chambers that were then inserted into the subcutaneous tissue of rats. The number of cells recovered after 24 hr was essentially the same in the three different conditions, suggesting that treatment with antisense ODN to the IGF-IR RNA (at a concentration of 19μM) has little effect on normal fibroblasts. Uptake of ODN in WI-38 cells is somewhat less than in C6 cells, but one should remember that, with C6 cells, we already had extensive apoptosis with as little as 0.15 μM. There is, though, a caveat with this experiment: we cannot obtain a full recovery even from untreated cells. We do not know the reason why recovery of WI-38 cells from the diffusion chamber is low, but we can say, at least, that antisense strategies do not affect the extent of cell death in these cells. These experiments, in fact, are in agreement with the report by Wang (1995) that human diploid fibroblasts are more resistant to apoptosis induced by serum deprivation than 3T3 cells. The latter, although

TABLE IV
RESISTANCE OF HUMAN DIPLOID FIBROBLASTS TO *IN VIVO* APOPTOSIS INDUCED BY ANTISENSE ODN TO THE IGF-I RECEPTOR RNA[a]

Treatment	Percent Recovery
Untreated	50
Random ODN (120 μg/ml)	49
Antisense ODN (120 μg/ml)	46

[a] WI-38 human diploid fibroblasts were treated as indicated above; 5×10^4 cells were then inoculated in diffusion chambers and the percent recovery determined after 24 hr incubation *in vivo*. The ODN used are the same as in Table III, and the recovery figures given are the mean of six determinations.

often considered normal cells, are in fact immortalized, aneuploid, and very prone to spontaneous transformation; human diploid fibroblasts, on the contrary, do not transform spontaneously and have a finite life span. If normal cells are indeed more resistant than cancer cells to apoptosis induced by a functional ablation of the IGF-IR, then targeting of the IGF-IR could truly have a unique therapeutic value in the treatment of tumors and metastases.

X. DOMINANT NEGATIVES

There are many direct ways in which one can interfere with the function of a gene product. In the case of a growth factor receptor such as the IGF-IR, several approaches can be used. These are summarized in Table V. We discuss here the dominant negatives, first because dominant negatives have been used extensively with many other gene products, and second, because, in the case of growth factor receptors, they constitute a special case, as discussed later.

First of all, we can say that there are dominant negatives of the IGF-IR. Prager *et al.* (1994) have described one, an IGF-IR that was truncated at residue 952. Transfected into Rat-1 cells, this mutant receptor acted as a dominant negative, completely inhibiting tumorigenesis, when the stably transfected cells were injected into nude mice.

We have tested several other mutants of the IGF-IR for their ability to act as dominant negatives. Some of these mutants, listed in Table I,

TABLE V
USE OF DIFFERENT METHODOLOGIES TO CAUSE LOSS OF FUNCTION OF THE IGF-I RECEPTOR

Method	Reference
Deletion of gene	J.-P. Liu *et al.* (1993)
Antibodies to receptor	Arteaga (1992)
Antisense to receptor	Pietrzkowski *et al.* (1992)
Dominant negatives	Prager *et al.* (1994)
Antisense to ligand(s)	Trojan *et al.* (1993)
Peptide analogs to ligand	Pietrzkowski *et al.* (1992c)

were transfected into C6 rat glioblastoma cells, which grow in serum-free medium in monolayers and form many colonies in soft agar. When the transfectants were tested in soft agar, several mutant receptors acted as dominant negatives, including the triple tyrosine mutant at 1131, 1135, and 1136 (Li *et al.*, 1994), the K1003 mutant (Coppola *et al.*, 1994), the Y950F mutant, and the Y1251F mutant (Burgaud *et al.*, 1995). The Y1250F mutant, which is both mitogenic and transforming, did not act as a dominant negative in these assays.

However, when these same transfectants were tested for their ability to induce apoptosis *in vivo* in diffusion chambers, none of the mutants mentioned induced apoptosis (Burgaud *et al.*, 1995). With some of them, growth in the diffusion chambers were decreased but no apoptosis was detectable. The only exception was observed in our laboratory when C6 cells were stably transfected with a plasmid that makes a soluble IGF-IR, that is, a receptor that has only the first 516 amino acids of the α domain, including the 30 amino acids of the signal peptide (D'Ambrosio *et al.*, 1996). This receptor, which we call 486/STOP, engineered by a point mutation of the human IGF-IR cDNA that causes a frameshift, has 486 amino acids after cleavage of the signal peptide, but the correct sequence of the IGF-IR terminates at residue 481. This sequence comprises the binding domain for IGF-I, which has been localized between residues 135 and 315 (Schumacher *et al.*, 1993). The 486/STOP receptor is secreted in the medium and inhibits IGF-IR function. C6 cells transfected with the 486/STOP receptor undergo massive apoptosis in the diffusion chamber *in vivo*. Accordingly, these cells are no longer tumorigenic in syngeneic rats.

Thus, one can say that there are different definitions of a dominant negative: there are those that inhibit growth in soft agar (the most numerous) and those that cause apoptosis (rare). We propose the follow-

ing hypothesis to explain the different levels of dominant negative behavior. Growth factor receptors are known to be rather promiscuous, easily forming hybrid receptors and transactivating each other intracellularly (Ballotti *et al.*, 1989; Lammers *et al.*, 1990; Taouis *et al.*, 1994; Chantry, 1995). In fact, Burgaud and Baserga (1996) have shown that crippled IGF-IRs, such as the triple tyrosine mutant and the Y950F mutant, which have lost both mitogenicity and transforming activity, can be transactivated by an overexpressed EGF receptor, their properties being fully restored. They can now tyrosyl phosphorylate IRS-1, which is a specific substrate of IR and the IGF-IR, after stimulation with EGF, something they cannot do when stimulated with IGF-I. They can even become transforming, while the truncated receptor (at residue 1229) still is nontransforming, regardless of the use of EGF or IGF-I, once more confirming that the C-terminus domain of the IGF-IR is required for transformation. Thus, only the Prager *et al.* mutant (1994) and our 486/STOP receptor, which is secreted in the medium, cannot be transactivated (or transactivate endogenous receptors) and therefore can act as dominant negatives in the apoptosis test. In growth assays, the mutant receptors simply act as competitors for the ligands, even in soft agar, but in apoptosis something more is required, perhaps more inhibition or perhaps something else that has yet to be identified.

XI. Critique of Antisense Strategies

The use of antisense strategies in biology has been severely criticized, sometimes with very good reasons. In some instances, antisense ODN have caused inhibition of growth without reducing the amount of the target protein; in other instances, the effect on growth was aspecific and largely due to the reactivity of the ODN with cellular proteins (for the pitfalls of antisense strategies, see, e.g., Sainio *et al.*, 1994; Chiasson *et al.*, 1994; Giles *et al.*, 1995; Guieysse *et al.*, 1995). While recognizing these problems, the case for the IGF-IR in transformation and in protection from apoptosis remains strong for several reasons:

1. In the first place, there is evidence that is not based on antisense strategies. Thus, R^- cells, devoid of IGF-Is, are refractory to transformation by several agents that readily transform cells with a physiological number of IGF-IRs (see earlier), clearly indicating the importance of the receptor in the establishment and maintenance of the transformed phenotype.
2. Inhibition of growth and/or tumorigenesis has also been obtained by other methods, as discussed previously (i.e., use of dominant nega-

tives or antibodies to the IGF-I). Thus, induction of apoptosis by interference with the function of the IGF-IR can also be obtained by methods that have no connection with antisense strategies.

3. The inhibition of IGF-I-mediated growth and tumorigenesis by antisense strategies has been obtained in two different ways: (1) with an expression plasmid coding for an antisense fragment to the IGF-IR RNA (Resnicoff *et al.,* 1994a,b; Shapiro *et al.,* 1994; Neuenschwander *et al.,* 1995) and (2) with antisense ODN (Resnicoff *et al.,* 1994a, 1995a,b). It would be difficult to believe that an antisense RNA and an antisense ODN would exert the same aspecific affects.

4. We have repeatedly shown that both the expression plasmid and the ODN cause a decrease in the target protein, the IGF-IR (Resnicoff *et al.,* 1994b, 1995a,b). The decrease is proportional to the concentration of antisense ODN used (see earlier). We have never been able to bring the receptor level down to zero, an observation confirmed by Neuenschwander *et al.* (1995), but the levels of IGF-IR protein are tightly regulated (Rubini *et al.,* 1994) and a decrease in receptor number promptly stimulates the synthesis of receptor RNA.

5. Different antisense ODN have different effects. We tested several antisense ODN both *in vitro* and *in vivo. In vitro,* we determined the effect of the antisense ODN on IGF-I-mediated growth, while *in vivo* we measured their effect on induction of apoptosis. The first sequence in Table VI is the sequence used in previous papers (Resnicoff *et al.,* 1994b, 1995a,b), which begins three nucleotides downstream from the methionine codon; the third sequence is an antisense beginning at the methionine codon, while the second sequence begins nine nucleotides downstream. They are all active in inhibiting cell growth *in vitro* or in inducing apoptosis *in vivo,* but the antisense of the second sequence is somewhat better, especially in inducing apoptosis at low concentrations. The sequence from the middle of the IGF-IR cDNA, under the same conditions, has no effect, confirming once more that sequences at the 5′ end of an mRNA are more effective (Sainio *et al.,* 1994).

6. The differences between antisense ODN and random ODN (control) are dramatic. The antisense induces partial apoptosis of C6 cells at concentrations as low as 0.15 μM (see earlier), while the random ODN is totally ineffective even at concentrations as large as 32 μM. A 200-fold difference in effective concentration does not support the possibility of totally aspecific effects.

In short, apart from the many controls that have been used with antisense strategies, the role of the IGF-IR in transformation and apoptosis is sustained by the use of other methodologies unrelated to antisense ODN.

TABLE VI
EFFECTS OF DIFFERENT ANTISENSE ODN TO THE IGF-IR RNA[a]

Sequence	% Growth Inhibition	% Apoptosis
From the 5′ region:		
TCCTCCGGAGCCAGACTT	48	46
same, ends capped	43	46
GGACCCTCCTCCGGAGCC	58	67
same, ends capped	26	58
CCGGAGCCAGACTTCAT	52	52
same, ends capped	54	54
From the middle region:		
CTGCTCCTCCTCTAGGATGA	0	0
same, ends capped	0	0

[a]For each sequence, the first is of fully thioate ODN and the second is the same sequence but with capped 3′ and 5′ ends. Percent growth inhibition was determined as described by Resnicoff *et al.* (1995a) and represents inhibition of IGF-I-mediated growth. Percent apoptosis was determined in a diffusion chamber (Resnicof *et al.*, 1995a). Both assays were carried out on C6 rat glioblastoma cells. *In vitro,* the concentration of ODN used was 120 μg/ml, whereas for *in vivo* studies (apoptosis in the diffusion chamber) we used a concentration of 1 μg/ml.

XII. MECHANISMS

While there is convincing evidence that the activated IGF-IR protects cells from apoptosis, very little is known about its mechanism. However, an important step in our understanding has been accomplished by Haim Werner (National Institutes of Health), who reported that the p53 tumor suppressor protein is a very efficient repressor of transcription from the IGF-IR promoter, causing a reduction in receptor number (Werner *et al.*, 1996). This finding could have remarkable repercussions in the field of apoptosis, bringing together two major players, p53 and the IGF-IR. It seems indeed that p53 may be extensively involved with the IGF system, since Buckbinder *et al.* (1995) reported that p53 induces the growth-inhibitory IGF binding protein-3. Apart from this clue, very little is known about the mechanism(s) by which the IGF-IR protects tumor cells from apoptosis. It has been reported that tumor necrosis factor (TNF)-α inhibits the phosphorylation of the insulin receptor and its major substrate IRS-1 (Feinstein *et al.*, 1993; Hotamisligil *et al.*, 1994); presumably the IGF-IR will also be inhibited, but at

this point it is still a tenuous connection. Yet the IGF-IR signaling must connect somewhere with the world of Bcl-2, Fas, integrins, TNF, and related proteins (Ruoslahti and Reed, 1994; Smith *et al.,* 1994; Cleveland and Ihle, 1995; Nicholson *et al.,* 1995; Whyte and Evan, 1995; Reed, this volume). At present, we can only suggest hypotheses.

One possibility is that the IGF-IR sends a signal that simply neutralizes the death signals originating through other receptors and other pathways that induce apoptosis. For instance, the TNF receptor may send a signal that induces apoptosis in tumor cells in the absence of the IGF-IR (see earlier). A second possibility is that loss of function of the IGF-IR results in the production of a peptide or peptides capable of inducing apoptosis, in a manner that is independent of the other apoptotic pathways.

A third possibility, suggested by the findings of Feinstein *et al.* (1993) and Hotamisligil *et al.* (1994), is that proteins with the death domain interact with the IGF-IR and/or its immediate substrates, causing the equivalent of a loss of function. For instance, Hotamisligil *et al.* (1996) have reported that TNF-α phosphorylates IRS-1 on serines, and that the serine-phosphorylated IRS-1 inhibits the IR activity. If this turns out to be true also for the IGF-IR, a mechanism for apoptosis by TNF would readily become apparent. Interestingly, IRS-1 also interacts with integrins (Vuori and Ruoslahti, 1994), and integrins are known to be involved in apoptosis (Brooks *et al.,* 1994; Montgomery *et al.,* 1994). It should also be noted that IRS-1 by itself is nontransforming, but it binds to SV40 T antigen (Zhou Li *et al.,* 1995), and together they can transform cells in the absence of an IGF-IR (D'Ambrosio *et al.,* 1995). These three possibilities envisage completely different roles for the IGF-IR in apoptosis. In the first two instances, it would act independently of other pathways, as an antidote in the first case and an active inducer of apoptosis in the second case. In the last possibility, it would cooperate closely with the other mechanism of apoptosis in causing it. A similar cooperation may be true also in the case of p53, if the results of Haim Werner (see earlier) can be extended.

Another clue is given by the fact that the activated IGF-IR protects cells from apoptosis induced by withdrawal of IL-3 (McCubrey *et al.,* 1991; Rodriguez-Tarduchy *et al.,* 1992). It suggests that the IGF-IR may activate the pathway originally activated by the IL-3 receptor. The elucidation of the mechanism involved in IGF-I-modulated apoptosis is, of course, crucial to a rational development of therapeutic approaches to either induction or prevention of apoptosis. For the moment, we can say that the IGF-IR is obviously central to the various pathways that lead to cell death in general and apoptosis in particular.

XIII. Host Response

For simplicity, we limit ourselves to the host response elicited in BD IX rats, host response that confers protection to a challenge with 10^7 wild-type C6 rat glioblastoma cells. In all instances the rats were pretreated with different types of cells, either injected subcutaneously or incubated in a diffusion chamber that was subsequently removed. The cells used were wild type, expressed sense or antisense plasmids against the IGF-IR RNA, or were treated with ODN or other agents. Details on these cells and the induction of apoptosis are given in previous papers (Resnicoff *et al.*, 1994a,b, 1995a). In each case, after appropriate pretreatment, all rats were challenged with 10^7 wild-type C6 cells injected subcutaneously. The challenge was carried out usually 1 week after the pretreatment, but we have gone for periods as short as 1 day or as long as 90 days; there were no significant differences in the host response within this range.

The results are summarized in Table VII, where the cells and the modalities used for the pretreatment are given in the first two columns. The last column, Tumor Development, notes the appearance (+) or lack of appearance (−) of tumors following a challenge with 10^7 wild-type C6 cells. (The challenge was always done with wild-type C6 cells, regardless of the type and species of cells used for the pretreatment.) When there was no protection (Table VII), the tumors appeared in 4–5 days; when there was protection, tumors never appeared. In some cases we kept the rats for as long as 1 year, and we never observed a tumor in animals with a positive host response. It is clear from Table VII that

1. When cells with a decreased number, or an impaired function, of the IGF-IR are injected into the subcutaneous tissue of rats or mice, the animals invariably develop a host response that makes them completely resistant to subsequent challenge with wild-type C6 cells.
2. The same results can be obtained if the cells are placed into a diffusion chamber that is subsequently removed. We could not find any difference in host response whether the cells used for pretreatment were injected subcutaneously or placed in a diffusion chamber.
3. There is cross-reaction between tumors and even species. Several types of cells, even WI-38 human diploid fibroblasts, when treated with antisense strategies, elicit a complete host response (i.e., resistance to subsequent challenge with wild-type C6 cells).
4. R^- cells, with no IGF-IRs (Sell *et al.*, 1993, 1994), elicit a host response but 3T3 cells do not.

TABLE VII
HOST RESPONSE ELICITED IN RATS BY TREATMENT OF CELLS WITH AGENTS THAT INDUCE APOPTOSIS BY TARGETING THE IGF-I RECEPTOR

Cell Type[a]	Strategy[b]	Tumor Development[c]
C6 wild-type	SC, CH	+
C6, sense plasmid or random ODN	SC, CH	+
C6, antisense plasmid		
45 min	CH	+
3 hr	CH	−
24 hr	CH	−
C6, antisense ODN	SC, CH	−
Human melanoma cells	CH	+
do, with antisense	CH	−
Mouse melanoma, wt	CH	+
do. antisense	CH	−
Rat rhabdomyosarcoma (AS)	CH	−
BALB/c 3T3	CH	+
R	CH	−
R'	CH	+
C6 plus etoposide	CH	−
C6 with soluble receptor	CH	−
WI-38 human diploid fibroblasts		
+ random ODN	CH	+
+ antisense ODN	CH	−

[a] In each instance, the rats were treated with the type of cells indicated; information is also included on the use of antisense, random, or sense ODN. Plasmid means that the cells were stably transfected with a plasmid expressing a sense or an antisense RNA to the IGF-IR RNA (Resnicoff *et al.*, 1995a).

[b] The strategy used to induce apoptosis: the cells either were injected subcutaneously (SC) or placed in the diffusion chamber (CH). In every case, the pretreated rats were subsequently challenged with 10^7 wild-type C6 cells injected subcutaneously from 1 to 30 days after the first treatment.

[c] +, Appearance of tumors; −, protection (no tumor).

5. A soluble IGF-IR (D'Ambrosio *et al.*, 1996) induces apoptosis and a protective host response, indicating that this host response is independent from antisense strategies.
6. Induction of apoptosis with etoposide also induces a host response.
7. In no case did untreated wild-type cells or cells treated with a sense plasmid or a random ODN ever elicited a host response.

We are perfectly aware that these results are somewhat unorthodox and difficult to explain by current immunological rules. But these are the results we have obtained, repeatedly and repeatably through at

least 2 years of experimentation. When cells (many kinds of cells) undergo apoptosis, either in subcutaneous tissue or in a diffusion chamber, they elicit a host response that renders the rat completely immune to subsequent challenge with wild-type C6 cells. This host response is especially vigorous when there is interference with the IGF-IR. The mechanism by which this host response is elicited is obviously one of the priorities in our future studies.

XIV. Significance

There are some interesting conclusions that can be drawn from the literature discussed in the preceding pages, and also some intriguing implications. The conclusions can be summarized as follows:

1. The IGF-IR, activated by its ligands, plays a major role in cell proliferation in at least three ways: it participates in mitogenesis, it is required for the establishment and maintenance of transformation (at least in several cell types), and it regulates the extent of apoptosis.
2. The transforming domain of the IGF-IR can be separated from its mitogenic signaling.
3. The IGF-IR protects tumor cells from apoptosis, and its protective role is much more evident *in vivo* than *in vitro*. In fact, cells in monolayers in serum-supplemented medium are very little affected by impaired function or even the absence of an IGF-IR.
4. Tumor cells and oncogene (e.g., c-*myc*)-driven cells are more sensitive to IGF-IR-mediated apoptosis than normal, growth-regulated cells.
5. When tumor cells with a loss of function of the IGF-IR undergo apoptosis, they elicit a host response that seems to be able to eliminate the surviving cells.
6. In some cases—for instance, with peptide analogs of IGF-I (but also with some dominant negatives; see earlier)—inhibition of IGF-IR function does not produce apoptosis but only inhibition of growth. This effect could be used to advantage in certain situations that do not require apoptosis but only temporary inhibition of growth (e.g., in re-stenosis of the coronary arteries [Hayry *et al.*, 1995]).
7. The literature indicates that the IGF-IR may be involved in the genesis and growth of certain human tumors (see reviews by Macaulay, 1992; Baserga, 1995; see also Bergmann *et al.*, 1995).

The implications are obvious. We are perfectly cognizant of the fact that results obtained in animals are very rarely reproduced in humans, especially when the animal results have been provided by transplantable tumors. We are also painfully aware of the difficulties encountered in delivering plasmids, peptides, or ODN to tumors *in vivo*. But, having made these disclaimers, we can say that, at least in animals, the targeting of the IGF-IR has some aspects that are unique. In the first place, interference with the IGF-IR causes extensive apoptosis of tumor cells while being much less effective on normal cells; second, the apoptotic cells induce a host response that, in syngeneic animals, eliminates the surviving tumor cells. The fact that cells overexpressing the IGF-IR do not need the activation of other growth factor receptors for growth, whereas an activated IGF-IR is still required for the mitogenic action of other growth factors (see earlier), also suggests that the IGF-IR is, so to speak, downstream from other growth factors, making it a more vulnerable target. If the animal results were to be reproducible in humans, the IGF-IR would indeed become a target for an intelligent bullet, since it is a two-edge sword (it induces apoptosis *and* a host response) and can apparently discriminate between normal and tumor cells.

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Bcl-2 Family Proteins and the Hormonal Control of Cell Life and Death in Normalcy and Neoplasia

JOHN C. REED

The Burnham Institute
Cancer Research Center
La Jolla, California 92037

I. Introduction

A continuous cycle of cell birth and death occurs in essentially all tissues with self-renewal capacity, with new cells being produced through cell division and old cells dying principally through programmed cell death and its morphological counterpart, apoptosis. So massive is this flux of cells through our bodies that, in the course of a typical year, it has been estimated each of us will produce and simultaneously eradicate a mass of cells equivalent to nearly our entire body weight. This delicate balance between cell production and cell death ensures that overall numbers of cells are maintained within physiologically appropriate ranges. Consequently, disturbances in the homeostatic mechanisms that normally balance cell production with cell death can contribute to the origins of cancer, imparting neoplastic cells with a

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selective growth advantage because of accelerated rates of cell proliferation, decreased rates of cell demise, or both. Moreover, while defects in cell cycle checkpoint mechanisms and other disturbances in cell proliferation often render tumor cells relatively more susceptible to the cytotoxic effects of chemotherapeutic drugs and radiation compared to normal cells, defects in the cell death pathway can promote chemo- and radioresistance by allowing tumor cells to survive despite having been damaged by drugs or radiation. Thus, the same genetic and biochemical disturbances in the physiological cell death pathway that contribute to the pathogenesis of tumors in the first place can also endow tumors with resistance to cytotoxic chemotherapeutic drugs, antihormonal agents, and X-irradiation, possibly explaining the "intrinsic" resistance of many types of solid tumors to currently available treatment.

Among the more prominent regulators of apoptosis are members of the Bcl-2 family of proteins. These proteins appear to regulate a distal step in an evolutionarily conserved pathway for physiological cell death and apoptosis, with some members functioning as suppressors of apoptosis and others as promoters of cell death. The expression of Bcl-2 can be regulated by steroid hormones under some circumstances, providing a potential mechanistic link between these factors and the control of cell life and death. Aberrant expression of Bcl-2 and some of its homologs has been observed in several types of human cancer, and may contribute to hormone-independent growth or impart resistance to retinoid- and glucocorticoid-induced cell death in certain subgroups of solid tumors, lymphomas, and leukemias. Though the biochemical mechanisms of action of Bcl-2 family proteins remain enigmatic, studies of the physical interactions of these apoptosis-regulating proteins with each other as well as with other nonhomologous partner proteins are beginning to provide insights into the molecular biology of apoptosis regulation and may reveal novel approaches to the treatment of cancer.

II. The Bcl-2 Protein Family

The *Bcl-2* gene was discovered by virtue of its involvement in the t(14;18) chromosomal translocations commonly found in non-Hodgkin's B-cell lymphomas, thus the name *B-cell lymphoma/leukemia-2 (BCL-2)* (Tsujimoto *et al.*, 1985). The protein encoded by the *BCL-2* gene is a potent blocker of programmed cell death (Vaux *et al.*, 1988; Hockenbery *et al.*, 1990; Reed *et al.*, 1990). Deregulation of the production of this protein as a result of t(14;18) translocations contributes to neoplastic

B-cell expansion by preventing cell turnover rather than by accelerating rates of cell division (McDonnell *et al.*, 1989, 1990; Katsumata *et al.*, 1992), making *BCL-2* the first example of a human proto-oncogene that functions through effects on cell death rather than cell cycle.

Since the discovery of Bcl-2 over a decade ago, several proteins with significant amino acid sequence homology have subsequently been identified that comprise the Bcl-2 protein family (Table I). Interestingly, some of these proteins block apoptosis when overexpressed by gene transfer methods, whereas others promote cell death. To date, at least nine mammalian homologs of Bcl-2 have been described, including the antiapoptotic proteins Bcl-X_L, Mcl-1, and A1/Blf-1; the proapoptotic proteins Bax, Bcl-X_S, Bad, Bak, and Bik; and an additional homolog BRAG-1 that is overexpressed in glioblastomas and presumably functions as a cell death blocker (Oltvai *et al.*, 1993; Boise *et al.*, Kozopas *et al.*, 1993; E. Y. Lin *et al.*, 1993, 1996; Das *et al.*, 1996; Choi *et al.*, 1995; Reynolds *et al.*, 1994; Kiefer *et al.*, 1995; E. Yang *et al.*, 1995; Chittenden *et al.*, 1995b; Farrow *et al.*, 1995; Boyd *et al.*, 1995). An additional homolog of Bcl-2, called Nr-13, has also been identified in avian cells transformed with v-*src* and has been speculated to function as a blocker of cell death (Gillet *et al.*, 1995). Moreover, several of the genes

TABLE I
BCL-2 FAMILY PROTEINS

Protein	Source	Primary Function
Bcl-2	Mammalian	Antiapoptotic
Bcl-X_L	Mammalian	Antiapoptotic
Bcl-X_S	Mammalian	Proapoptotic
Bax	Mammalian	Proapoptotic
Mcl-1	Mammalian	Antiapoptotic
A1/Bfl-1	Mammalian	Antiapoptotic
Brag-1	Mammalian	Antiapoptotic[a]
Bad	Mammalian	Proapoptotic
Bak	Mammalian	Proapoptotic
Bik	Mammalian	Proapoptotic
Nr13	Avian	Antiapoptotic[a]
Ced-9	Nematode	Antiapoptotic
E1b-19 kDa	Adenovirus	Antiapoptotic
BHRF1	Epstein–Barr virus	Antiapoptotic
LMW5-HL	African swine fever virus	Not tested
ORF-16	Herpes virus Saimiri	Not tested

[a] Function not formally tested but overexpression in tumors and presence of BH4 domains suggest antiapoptotic.

encoding these apoptosis-regulating proteins can also generate less abundant additional protein isoforms through alternative splicing mechanisms. Among the most striking examples are the Bcl-X_L and Bcl-X_S proteins, which arise through alternative mRNA splicing mechanisms from a common gene and which function in diametrically opposed fashions as cell death blockers and promoters, respectively (Boise *et al.,* 1993). In addition, four homologs of Bcl-2 have been discovered in viruses: 19-kDa E1b (adenovirus), BHRF-1 (Epstein–Barr virus), LMH-5W (African swine fever virus), and ORF-16 (herpesvirus Saimiri) (Rao *et al.,* 1992; Golemis *et al.,* 1994; Neilan *et al.,* 1993; Smith, 1995). An antiapoptotic homolog has also been identified in the nematode *Caenorhabditis elegans* that has been termed Ced-9 (Hengartner and Horvitz, 1994).

Several of these homologous proteins can interact with each other, thus constituting a network of homo- and heterodimers that regulate programmed cell death (Oltvai *et al.,* 1993; Sato *et al.,* 1994a; Yin *et al.,* 1994; Hanada *et al.,* 1995; Bodrug *et al.,* 1995; Sedlak *et al.,* 1995). The relative ratios of these various pro- and antiapoptotic members of the Bcl-2 family have been shown to determine the ultimate sensitivity or resistance of cells to diverse apoptotic stimuli, including chemotherapeutic drugs and radiation, growth factor deprivation, loss of cell attachment to extracellular matrix proteins (an issue of potential relevance to mechanisms of tumor metastasis), hypoxia (a common occurrence in the centers of large tumors), and lysis by cytolytic T- cells (reviewed in Reed, 1994, 1995). The cell death pathway regulated by Bcl-2 family proteins appears to be evolutionarily conserved, based on the observations that (a) the human Bcl-2 protein can partially substitute for the endogenous homolog Ced-9 in preventing developmental cell death in the nematode *C. elegans* (Vaux *et al.,* 1992; Hengartner and Horvitz, 1994); (b) Bcl-2 can rescue superoxide dismutase (*sod*)-deficient strains of budding yeast from the growth-inhibitory effects of aerobic conditions (Kane *et al.,* 1993); (c) the proapoptotic protein Bax confers a lethal phenotype on yeast, which can be reversed by coexpression of the antiapoptotic proteins Bcl-2, Bcl-X_L, or Mcl-1 (Sato *et al.,* 1994a, 1995; Bodrug *et al.,* 1995); and (d) Bcl-2 can protect insect cells from baculovirus-induced apoptosis (Alnemri *et al.,* 1992b).

III. Mechanisms of Bcl-2 Protein Action

The mechanism by which Bcl-2 and its homologs control the programmed cell death pathway remains enigmatic to date, due mostly to

the lack of any significant homology between Bcl-2 family proteins and other proteins whose biochemical mechanism of action is know. The human Bcl-2 protein has a molecular mass of ~26 kDa and contains a stretch of hydrophobic amino acids at its C terminus that allows it to posttranslationally insert into intracellular membranes, primarily the outer mitochondrial membrane, nuclear envelope, and endoplasmic reticulum (Krajewski *et al.*, 1993; Jacobson *et al.*, 1993; Monaghan *et al.*, 1992; Silvestrini *et al.*, 1994). Most other members of the Bcl-2 family also contain a transmembrane domain near the C termini and, where examined to date, appear to reside within approximately in the same intracellular membrane compartments (T. Yang *et al.*, 1995; González-Garcia *et al.*, 1994).

Several theories have been advanced as to how Bcl-2 and its homologs control cell life and death. Data have been presented, for example, that argue both in favor and against the possibility of an effect of Bcl-2 on an antioxidant pathway in cells (Kane *et al.*, 1993; Hockenbery *et al.*, 1993; Jacobson and Raff, 1995; Shimizu *et al.*, 1995). It has also been suggested that Bcl-2 may regulate the homeostasis of Ca^{2+} in cells, based on experiments that have shown an ability of Bcl-2 overexpression to influence the sequestration of Ca^{2+} within the endoplasmic reticulum and (in some cases) to prevent the accumulation of Ca^{2+} in mitochondria of cells subjected to an apoptotic stimulus (Baffy *et al.*, 1993; Lam *et al.*, 1994). Evidence has also been presented suggesting that Bcl-2 can control the transport of proteins across biological membranes, particularly the nuclear envelope (Ryan *et al.*, 1994; Meikrantz *et al.*, 1994). In this regard, electron microscopic studies have demonstrated the presence of Bcl-2 protein in association with what appear to be nuclear pore complexes (Krajewski *et al.*, 1993). It has been hypothesized that Bcl-2 may control the activity of a family of cysteine proteases with homology to the interleukin-1β converting enzyme (ICE), based on genetic arguments from studies of cell death genes in the nematode *C. elegans* (Yuan *et al.*, 1993). Indeed, biochemical evidence supporting this idea has been obtained through experiments that have demonstrated that overexpression of Bcl-2 can prevent the proteolytic processing and activation of the ICE homolog CPP32β/YAMA in mammalian cells (Chinnaiyan *et al.*, 1996; Boulakia *et al.*, 1996). Moreover, Bcl-2 may influence the activities of certain transcription factors under some circumstances, including p53 and nuclear factor-κB (Upadhyay *et al.*, 1995; K.-I. Lin *et al.*, 1995; Albrecht *et al.*, 1994; Shen and Shenk, 1994). Finally, the association of Bcl-2 with the kinase Raf-1 and reports of interactions with the GTPases R-Ras and H-Ras have raised the possibility that Bcl-2 may control a signal transduction pathway that is fo-

cused on the intracellular membrane compartments where Bcl-2 resides, rather than the plasma membrane, where such enzymes are associated with growth factor receptor function (Wang *et al.,* 1994, 1995; Fernandez-Sarbia and Bischoff, 1993; Chen and Faller, 1996). In no case, however, has a direct cause-and-effect relation been demonstrated between Bcl-2 and these processes, and at this point it is unknown whether the effects of Bcl-2 on redox state, Ca^{2+} compartmentalization, protein transport, transcription factors, and protease activation represented direct effects of Bcl-2 or downstream events that are hundreds of steps removed from Bcl-2.

A. Identification of Functionally Important Domains in Bcl-2 Family Proteins: Correlations with Homo–Heterodimerization and Function

In the absence of a clear function for the Bcl-2 protein, several groups have attempted to map functionally important domains in the Bcl-2 protein, asking what the roles of these domains are both for antiapoptotic function and for interactions with other proteins. At present, at least four well-conserved domains can be recognized in the Bcl-2 protein based on sequence comparisons with the Bcl-2 proteins derived from various species (human, rat, mouse, chicken, worm) and from other Bcl-2 family proteins, as well as from functional analysis of the repercussions of deleting or mutating these domains. Though various names for these domains can be found in the literature, we have recently proposed the terms BH1, BH2, BH3, and BH4, where BH stands for Bcl-2 *h*omology *d*omain as originally suggested by Oltvai *et al.* (1993; Zha *et al.,* 1996a). For historical reasons, these fours domains are unfortunately not ordered sequentially along the protein from the N to the C terminus.

Figure 1 depicts the structures of Bcl-2 and its cellular homologs. In the human Bcl-2 protein, the BH1, BH2, BH3, and BH4 domains reside at amino acid positions 136–155 (BH1), 187–202 (BH2), 93–107 (BH3), and 10–30 (BH4). The transmembrane domain (TM) of Bcl-2 resides at positions 219–237. Of interest, the BH4 domain (also known as the A-box) (Sato *et al.,* 1994a,b; Hanada *et al.,* 1995) is not found in most proapoptotic Bcl-2 family proteins, including Bax, Bak, Bik, and Bad, suggesting that this domain may play a unique role in the function of the antiapoptotic proteins such as Bcl-2, Bcl-X_L, Mcl-1, A1/Bfl-1, and Ced-9. The BH4 domain, however, is found in the cell death-promoting protein Bcl-X_S, which suggests either that the BH4 domain is not directly involved in the antiapoptotic function of Bcl-2 family proteins or,

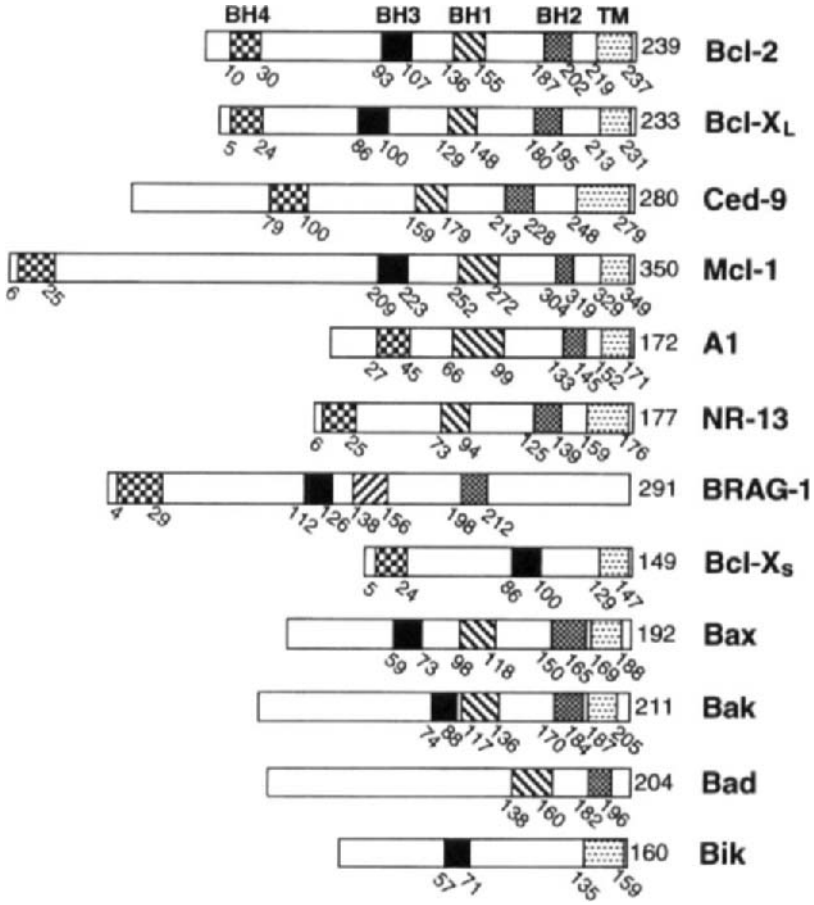

FIG. 1. Structures of Bcl-2 family proteins.

more likely, that the Bcl-X_S protein can function at least in part as a dominant inhibitor of Bcl-2 and its antiapoptotic homologs by competing for whatever proteins or protein domains might normally interact with BH4. Also of note in Fig. 1 is the observation that one of the proapoptotic proteins, Bik, contains only the BH3 domain, implying that this particular domain may be uniquely important in the promotion of apoptosis (Boyd *et al.,* 1995). Indeed, deletional analysis of the Bax and Bak proteins suggests that this is the case, as is discussed later in more detail (Zha *et al.,* 1996a; Chittenden *et al.,* 1995a). Though some antiapoptotic proteins do contain regions with homology to BH3,

there exist clear sequence differences that distinguish the BH3 domains of the proapoptotic proteins Bax, Bak, and Bik from the antiapoptotic proteins Bcl-2, Bcl-X_L, and Mcl-1 (Zha *et al.*, 1996a). Moreover, it has been demonstrated that substituting the BH3 domain of Bax for the corresponding domain in Bcl-2 converts the Bcl-2 protein from a cell death blocker to a cell death promoter (Hunter and Parslow, 1996).

Deletion of the N-terminal BH4 domain of Bcl-2 as well as the downstream BH1 and BH2 domains prevents the resulting mutant Bcl-2 proteins from forming homodimers with themselves, though they can still bind the wild-type Bcl-2 protein, forming mutant–wild-type heterodimers (Hanada *et al.*, 1995). These mutants also are deficient in antiapoptotic function in mammalian cells (Borner *et al.*, 1994), suggesting the possibility that Bcl-2–Bcl-2 homodimerization is important for function. In this regard, data suggest that deletion of the BH4 domain of Bcl-2 converts the protein to a dominant inhibitor of the wild-type Bcl-2 protein, suggesting that mutant–wild-type heterodimers of Bcl-2 may be dysfunctional dimers (Hunter *et al.*, 1996). This observation also provides insights into the potential mechanism by which the Bad protein may promote cell death, since it contains the BH1 and BH2 domains but lacks the BH4 domain (Fig. 1).

The capacity of deletion mutants of Bcl-2 to retain their ability to bind the wild-type Bcl-2 protein can be explained by data that suggest that Bcl-2–Bcl-2 homodimerization involves a head-to-tail association in which sequences located in the first ~80 amino acids of the protein, where the BH4 domain resides, form an interaction with sequences located in the more distal portions of the protein (from approximately amino acid 85 to the TM), where the BH1, BH2, and BH3 domains are located (Sato *et al.*, 1994b). Thus, a Bcl-2 mutant protein in which the BH1 and/or BH2 domains have been deleted, for example, can still bind via its BH4 domain to the wild-type Bcl-2 protein or to Bcl-2 protein fragments that retain an intact distal region (from approximately amino acid 85 to the TM) where BH1, BH2, and BH3 reside. This observation may provide some insights into the mechanism by which the Bcl-X_S protein promotes apoptosis, given that it contains the BH4 domain but is lacking the BH1 and BH2 domains and can therefore bind to Bcl-2, presumably sequestering the protein in dysfunctional Bcl-2–Bcl-X_S heterodimer (Sato *et al.*, 1994b).

It should be noted, however, that it has been suggested that the head-to-tail interaction between the N-terminal region where BH4 lies and the more distal region where BH1 and BH2 reside may occur intramolecularly versus intermolecularly in the case of at least some Bcl-

2 family proteins (Minn *et al.*, 1996). These proteins may have little capacity for homodimerization but are still able to heterodimerize with certain other members of the Bcl-2 protein family.

In contrast to Bcl-2–Bcl-2 homodimerization, the BH4 domain of the Bcl-2 protein is completely expendible for dimerization with Bax. Indeed, the first ~80 amino acids of Bcl-2 can be removed without impairing heterodimerization with Bax (Hanada *et al.*, 1995). Given that the Bax protein lacks a BH4 domain, this observation suggests that the structural features by which Bcl-2 interacts with Bax versus with itself are considerably different. Though BH4 is not required for heterodimerization with Bax, the BH1 and BH2 domains are needed (BH3 has not been tested to date) (Yin *et al.*, 1994; Hanada *et al.*, 1995). Indeed, single amino acid substitutions in the BH1 and BH2 domains of Bcl-2 have been shown to impair binding to Bax and abrogate antiapoptotic function in lymphoid cells (Yin *et al.*, 1994). These data argue that, for Bcl-2 to suppress cell death, it must be capable of heterodimerizing with Bax. (It is important to note, however, that while we describe the interactions of Bcl-2 with itself and Bax as dimers, in fact the stoichiometry of these protein–protein interactions is unknown at present.) Notwithstanding these data implying an essential role of heterodimerization of Bcl-2 with Bax, the finding that the N-terminal region of Bcl-2, where BH4 resides, is needed for function but not for dimerization with Bax argues that Bcl-2–Bax heterodimer formation is insufficient by itself for blocking apoptosis and that the Bcl-2 protein must fulfill other functions as well. In this regard, not only are deletion mutants of Bcl-2 lacking the BH4 domain incapable of suppressing apoptosis in mammalian cells, but they also fail to rescue yeast from Bax-induced lethality despite binding to Bax (Sato *et al.*, 1994a; Hanada *et al.*, 1995). Thus, whatever the role of the N-terminal domain in suppressing Bax-mediated cell death, it appears to be evolutionarily conserved.

One idea is that BH4 domain of Bcl-2 may be required to sterically interfere with the binding of Bax to some other death effector protein or to somehow modulate posttranslational modifications of the Bax protein. It is also possible, however, that this domain is required for Bcl-2–Bcl-2 homodimerization or for interactions of Bcl-2 with other proteins that require the BH4 domain for their association with Bcl-2–Bax complexes. In this regard, data indicate that the association of BAG-1 and Raf-1, two proteins that have been demonstrated to associate with protein complexes containing Bcl-2 and that cooperate with Bcl-2 in cotransfection assays to suppress apoptosis (Takayama *et al.*, 1995; Wang *et al.*, 1994), is dependent on the BH4 domain. Thus, to some extent, the N-terminal region of Bcl-2, where BH4 resides, can be thought of as

an effector domain that may link Bcl-2 to other proteins such as BAG-1 and Raf-1, whereas the distal portion of Bcl-2, where the BH1 and BH2 domains reside, may represent a dimerization domain that serves to target Bcl-2 and its associated proteins to Bax. Alternatively, it is possible that the dependence on the BH4 domain for associations with BAG-1 and Raf-1 is an indirect consequence of the need for this domain for formation of functional Bcl-2–Bcl-2 homodimers.

In an effort to understand some of the structural features of the Bax protein that allow it to homodimerize with itself and heterodimerize with Bcl-2, we have tested the ability of deletion mutants of Bax lacking the BH1, BH2, or BH3 domains to interact with the wild-type Bax and Bcl-2 proteins using yeast two-hybrid assays (Zha *et al.*, 1996b). These studies showed that the BH1 and BH2 domains of Bax are expendible for both homodimerization with Bax and heterodimerization with Bcl-2. In addition, Bax deletion mutants lacking BH1 or BH2 are also able to bind to themselves (i.e., mutant to mutant) as well as to the wild-type Bax protein. In contrast, the BH3 domain of Bax is absolutely required for binding to both wild-type Bax and Bcl-2.

The structural features of Bax that permit it to homodimerize with itself and to heterodimerize with Bcl-2 therefore are considerably different from those required for Bcl-2 protein function in terms of dimerization events. These data suggest that, despite their amino acid sequence homology, the Bax and Bcl-2 proteins are probably extensively different in their three-dimensional structures, but X-ray crystallographic or nuclear magnetic resonance studies are required to confirm this idea. Consistent with this idea, however, we have also explored the effect of deleting the N-terminal domains of Bcl-2 and Bax on their dimerization with each other. As mentioned earlier, removal of the N-terminal first ~80 amino acids of Bcl-2 does not impair its ability to bind to wild-type Bax. Moreover, a Bcl-2 (ΔN) mutant of this type can also form heterodimers with an N-terminal truncation mutant of Bax that lacks the first 58 amino acids (i.e., everything upstream of BH3). Thus, unlike Bcl-2–Bcl-2 homodimerization, which involves a head-to-tail interaction, Bax–Bcl-2 heterodimerization appears to occur through a tail-to-tail interaction. N-terminal truncation mutants of Bax can also homodimerize with themselves, indicating that Bax–Bax homodimerization also occurs via a tail-to-tail interaction that is independent of the proximal region of the Bax protein upstream of BH3 (Zha *et al.*, 1996b).

Studies indicate that mutants of Bax that have altered BH3 domains and that fail to homodimerize are incapable of promoting cell death in both mammalian cells and yeast (Zha *et al.*, 1996b). Interestingly, how-

ever, the BH3 domain of Bak was shown to be not only necessary but also sufficient for Bak-induced apoptosis and for dimerization with Bcl-X_L (Chittenden *et al.*, 1995a). (Bak homodimerization was not explored.) Moreover, the proapoptotic protein Bik contains a BH3 domain but lacks the BH1, BH2, or BH4 domains, suggesting that BH3 in Bik is also functionally important for this protein's interactions with Bcl-2 and other antiapoptotic Bcl-2 family proteins (Boyd *et al.*, 1995). Taken together, these observations concerning the BH3 domains of proapoptotic proteins such as Bax, Bak, and Bik can be interpreted in at least three ways. First, BH3-mediated homodimerization of Bax with some unknown cell death effector protein may be required for promotion of apoptosis by Bax. Second, it could be that the BH3 domain of proapoptotic proteins such as Bak, Bax, and Bik engages directly some unknown effector protein that promotes cell death, and that the dependence on the BH3 domain for homodimerization is merely a misleading coincidence. Third, expression of this domain may act as a "decoy," binding to Bcl-X_L, Bcl-2, or other antiapoptotic proteins and thereby preventing them from interacting with the endogenous wild-type Bak, Bax, or Bik proteins and thus leaving these proapoptotic proteins unopposed.

Taken together, the data currently available about Bcl-2, Bax, and other Bcl-2 family proteins fail to provide conclusive information about the functional significance of Bcl-2–Bcl-2, Bcl-2–Bax, and Bax–Bax dimers. However, several models can be imagined, most of which are not mutually exclusive (Fig. 2). For example, Bax–Bax homodimers can be envisioned as the active moiety that promotes cell death, with Bcl-2 functioning essentially as a dominant inhibitor of Bax by sequestering the protein in a heteromeric Bax–Bcl-2 complex. Alternatively, Bcl-

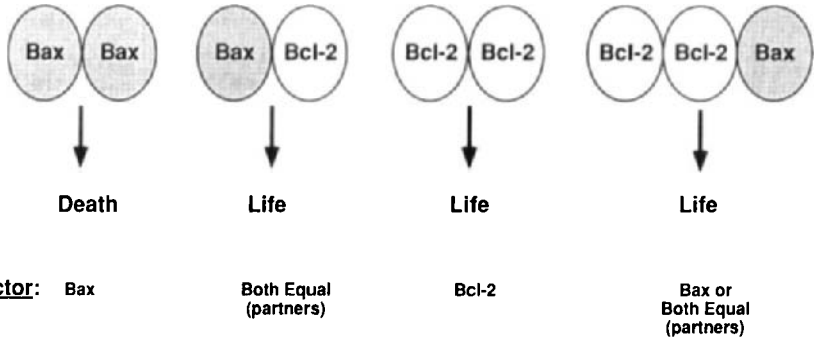

FIG. 2. Models of Bcl-2 family protein dimerization.

2–Bcl-2 homodimers may actively provide "signals" for cell survival, with Bax functioning as a dominant inhibitor that prevents Bcl-2–Bcl-2 dimerization. It is also formally possible that the Bcl-2–Bax heterodimers represent the most critical structure, with the proteins functioning as equal partners in suppressing cell death by analogy to the α- and β-chains of many growth factors or dimeric transcription factors such as Myc–Max. Though *bax* knockout experiments argue against this co-partner model, it cannot be formally excluded because of the possibility of redundancy among known and perhaps currently undiscovered proapoptotic members of the Bcl-2 family. Indeed, the paradoxical observation that loss of *bax* can result in excessive cell death in the developing spermatocytes of the testes raises the possibility of a need for Bax for suppression of apoptosis in some contexts (Knudson *et al.*, 1995). A similar phenomenon has been observed for the proapoptotic protein Bak, in that overexpression of Bak paradoxically enhances cell survival in a particular Epstein–Barr virus-immortalized lymphoblastoid cell line (Kiefer *et al.*, 1995). Finally, because Bcl-2 can dimerize with itself through a head-to-tail interaction and yet bind to Bax via a tail-to-tail interaction, there exists the potential for Bcl-2 to simultaneously bind to Bax via is distal domain, where BH1 and BH2 reside, and bind another molecule of Bcl-2 via its BH4 domain-containing proximal domain, thus complexing with Bax in a 2:1 ratio (Fig. 2). This last model would provide the advantage that both Bcl-2–Bax heterodimerization and Bcl-2–Bcl-2 homodimerization are simultaneously required for antiapoptotic function, which fits well with the currently available data suggesting that both Bcl-2–Bcl-2 homodimerization and Bcl-2–Bax heterodimerization are necessary but insufficient for Bcl-2 protein function.

One caveat about the models presented in Fig. 2, however, is that a report involving an analysis of various single amino acid substitution mutants in the BH1 and BH2 domains of the Bcl-X_L protein has suggested that this protein can suppress apoptosis without necessity for binding to Bax or Bak (Cheng *et al.*, 1996). Thus, it is possible that our current concepts about the significance of interactions of antiapoptotic proteins such as Bcl-X_L and Bcl-2 with proapoptotic proteins such as Bax and Bak are erroneous. It should be borne in mind, however, that while some substitution mutants of Bcl-X_L that retain cell death-blocking activity are apparently defective in binding to Bax or Bak *in vitro* and could not be detectably coimmunoprecipitated with Bax or Bak from cells, it cannot be excluded that such mutants do retain at least weak affinity for Bax and Bak and that this reduced affinity is nevertheless sufficient for function. Clearly, some direct comparisons of the

affinities (K_d), as well as kinetics (k_{on},k_{off} rates), of the interactions of wild-type and mutant Bcl-X_L proteins with Bax and Bak would shed some light on this controversial issue. The effects of these substitution mutants on Bcl-X_L–Bcl-X_L homodimerization were not explored, leaving open the possibility that homodimerization is critical for the anti-apoptotic function of this protein.

B. Interactions of Bcl-2 with Nonhomologous Proteins

To date, at least nine proteins have been described that can interact with Bcl-2, either directly or indirectly, as determined by yeast two-hybrid assays or coimmunoprecipitation studies. Among these potential interacting proteins are (1) Raf-1, a serine/threonine kinase (Wang *et al.,* 1994); (2) p21H-Ras and p23R-Ras, members of the Ras family of small-molecular-weight GTPases (Fernandez-Sarbia and Bischoff, 1993; Chen and Faller, 1995, 1996); (3) Nip-1, -2, and -3, a group of proteins that bind to both Bcl-2 and the 19-kDa E1b protein of adenovirus and that encode proteins with domains that share homology with the catalytic domain of phosphodiesterase, Ca^{2+}-binding proteins, and calbindin-D, respectively (Boyd *et al.,* 1993); (4) PrP, a cellular prion protein that has been implicated in neurodegenerative diseases (Kurschner and Morgan, 1995); (5) p53BP2, a protein that binds to wild-type but not mutant p53 (Naumovski and Cleary, 1996); and (6) BAG-1, a novel protein that contains a ubiquitin-like domain (Takayama *et al.,* 1995). It should be noted, however, that no data exist to date that demonstrate a dependence on any of these interacting proteins for Bcl-2 function.

Of these Bcl-2-interacting proteins, functional data that suggest a regulatory effect on apoptosis are available thus far only for Raf-1 and BAG-1. In co-transfection studies, both BAG-1 and activated versions of Raf-1 that have constitutively high kinase activity can cooperate with Bcl-2, providing synergistic protection from apoptotic stimuli (Takayama *et al.,* 1995; Wang *et al.,* 1994). Both BAG-1 and Raf-1 depend on the BH4 domain for their interactions with Bcl-2. Moreover, BAG-1 can also bind Raf-1, suggesting that these proteins may interact with Bcl-2 as a complex. In addition, we have shown that BAG-1 can activate this kinase, presumably through a protein–protein interaction that involves binding of BAG-1 to the kinase domain of Raf-1 (Razin and Gromova, 1995). Since Bcl-2 is primarily found integrated via its C-terminal TM into the outer membrane of mitochondria, nuclear envelope, and parts of the endoplasmic reticulum, with the protein exposed to the cytosol (Krajewski *et al.,* 1993; Lithgow *et al.,* 1994), presumably interactions

with Bcl-2 help to target BAG-1 and Raf-1 to these intracellular membranes. Thus, at least one function of Bcl-2 may be to serve as an adaptor protein that helps to target Raf-1 to unique substrates involved in the control of programmed cell death. These substrates presumably are different from MEK or other substrates normally associated with Raf-1's role as a transducer of signals from growth factor receptors in the plasma membrane. It is hoped that discovery of those substrates will provide new insights into the function of Bcl-2 and its homologs. At present, we have been unable to find evidence that Raf-1 phosphorylates Bcl-2, Bax, or BAG-1 under normal circumstances (Wang *et al.*, 1994; Razin and Gromova, 1995), but evidence that the antimicrotubule drug taxol can induce phosphorylation of Bcl-2 through what may be a Raf-1-dependent mechanism suggests that perhaps Raf-1 can phosphorylate Bcl-2 in some contexts (Blagosklonny *et al.*, 1995a,b; Haldar *et al.*, 1996). Interestingly, though conclusive evidence has yet to be obtained, it has been suggested that the phosphorylated form of Bcl-2 may be inactive, in part due to a reduced ability to homodimerize with Bax (Haldar *et al.*, 1995, 1996).

IV. Bcl-2 Family Proteins as Determinants of Tumor Resistance to Chemotherapeutic Drugs, Glucocorticoids, Retinoids, Antiandrogens, and Antiestrogens

Gene transfer-mediated elevations in Bcl-2 protein levels were first shown to protect a murine lymphoma and a leukemia cell line from cell death induced by a wide variety of anticancer drugs, including dexamethasone, etoposide (VP-16), methotrexate, cisplatin, cyclophosphamide, vincristine, and Ara-C (Miyashita and Reed, 1992). Since then, these observations have been extended to a wide variety of cancer cell lines and anticancer drugs (reviewed in Reed, 1995). The protection afforded by Bcl-2 against chemotherapeutic agents defines a novel type of drug resistance, in that high levels of Bcl-2 do not prevent drugs from entering cells, finding their biochemical targets, or inducing damage to DNA and other molecules. Nor does Bcl-2 alter the rates of repair of drug-induced damage. Rather, Bcl-2 prevents the translation of drug-induced damage into effective signals for cell death, thus allowing neoplastic cells to survive for longer periods of time after becoming injured. This prolonged survival could then provide additional time for development of biochemical or genetic alterations that then allow cancer cells to acquire classical resistance to pharmacophores, or simply allow drug-damaged tumor cells to live until chemotherapeutic

drugs are discontinued (such as between cycles of chemo- or radiotherapy), when they can repair the damage and resume normal functions. Because drug- or radiation-induced damage often causes malignant cells to arrest in cell cycle, Bcl-2 essentially converts anticancer drugs from cytotoxic to cytostatic, thus leaving a reservoir of viable tumor cells from which relapses may arise.

Probably some of the best examples of the selectivity with which Bcl-2 affects cell death pathways, as opposed to cell proliferation and differentiation, come from investigations of the effects of steroid hormones on leukemia and lymphoma cells. For example, when Bcl-2 is overexpressed in lymphoid leukemia and lymphoma cell lines by gene transfer techniques, apoptosis induced by glucocorticoids is completely prevented but G_0–G_1 cell cycle arrest occurs unimpeded (Miyashita and Reed, 1992, 1993). Indeed, in the pre-B-cell acute lymphocytic leukemia cell line 697, we have observed that gene transfer-mediated increases in Bcl-2 can allow these cells to sit in a quiescent G_0–G_1 state with ~99% cell viability for over 1 month in culture when exposed to dexamethasone. Upon washing out the synthetic glucocorticoid, these Bcl-2-overexpressing leukemia cells then resume proliferation and give rise to clonigenic cells at normal frequencies (unpublished data). Thus, Bcl-2 does not prevent glucocorticoids from entering lymphoid cells, binding glucocorticoid receptors, and altering gene expression, but rather acts downstream of these effects of glucocorticoids and selectively prevents the apoptotic response that normally would ensue. Interestingly, glucocorticoids have been reported to cause a depletion of Ca^{2+} from the endoplasmic reticulum of lymphoid leukemia cells; overexpression of Bcl-2 prevents this alteration in Ca^{2+} storage (Lam *et al.*, 1993, 1994). Since enhancing Ca^{2+} sequestration in the endoplasmic reticulum by overexpressing the Ca^{2+}-binding protein calbindin-D can delay glucocorticoid-induced apoptosis (Dowd *et al.*, 1992), these findings suggest the possibility that Bcl-2 may prevent glucocorticoid-induced apoptosis by helping to maintain the endoplasmic reticulum pools of Ca^{2+}. In this regard, Bcl-2 has been reported to decrease the rate of spontaneous Ca^{2+} efflux from the endoplasmic reticulum (Lam *et al.*, 1994), but whether this is a direct effect of Bcl-2 or reflects an event hundreds of steps downstream remains to be determined.

Similarly, the induction of apoptosis by certain retinoids, such as all-*trans*-retinoic acid and 4-hydroxyphenyl-retinamide, in the acute myelogenous leukemia cell line HL-60 can be prevented by gene transfer-mediated overexpression of Bcl-2 (Naumovski and Cleary, 1994; Benito *et al.*, 1995; Delia *et al.*, 1995). At the same time, however, the effects of these retinoids on cell cycle arrest and differentiation are not prevent-

ed by Bcl-2. Indeed, elevated Bcl-2 can give the appearance of enhanced differentiation, probably because it helps to maintain the survival of terminally differentiated cells that would otherwise die.

Further evidence that Bcl-2 family proteins play an important role in controlling the relative resistance of neoplastic cells to apoptosis induction by steroid hormone agonists and antagonists, as well as other types of cytotoxic anticancer drugs, has come from antisense experiments and studies where proapoptotic Bcl-2 homologs have been overexpressed in tumor cell lines. For example, experimental reductions in Bcl-2 protein levels achieved through use of antisense oligonucleotides or antisense expression plasmids can render leukemia and lymphoma cell lines more sensitive to the cytotoxic effects of anticancer drugs as well as glucocorticoids (Kitada *et al.,* 1994; Campos *et al.,* 1994). In addition, gene transfer-mediated elevations in the proapoptotic protein Bcl-X_S were reported to increase the sensitivity of MCF-7 breast cancer cells to chemotherapeutic drugs (Sumantran *et al.,* 1995). Interestingly, estrogens upregulate *BCL-2* gene expression in MCF-7 cells, whereas antiestrogens reduce Bcl-2 protein levels and render these breast cancer cells more sensitive to Adriamycin (Teixeira *et al.,* 1995). Similarly, androgens increase *BCL-2* expression in the LNCaP prostate cancer line, whereas *BCL-2* antisense oligonucleotides prevent the androgen-induced increase in Bcl-2 protein levels and thereby increase sensitivity to etoposide (Berchem *et al.,* 1995). These observations provide further evidence that Bcl-2 family proteins are important regulators of chemosensitivity and resistance, including resistance to steroid hormone analogs. They also provide proof-of-concept evidence that agents that manipulate the levels or function of Bcl-2 and its homologs could potentially offer new approaches to the treatment of at least some types of cancer.

High levels of *BCL-2* expression have been correlated with low response rates, faster time to relapse, shorter survival, or other indicators of poor clinical outcome in patients with several types of cancer. The implication, therefore, is that this antiapoptotic gene is clinically relevant for at least some subgroups of cancer patients, including those with some types of non-Hodgkin's lymphoma, acute myelogenous leukemia, and prostate cancer (Yunis *et al.,* 1989; Piris *et al.,* 1994; Offit *et al.,* 1989; Hermine *et al.,* 1996; Campos *et al.,* 1993; McDonnell *et al.,* 1992; Maung *et al.,* 1994; Bubendorf *et al.,* 1996). Though some clinical-correlative studies have paradoxically suggested an association between Bcl-2 and favorable prognosis, the treatment of these patients was primarily or even exclusively surgical in most of these studies, and thus the findings are not relevant to the issue of regulation of chemore-

sponses by Bcl-2. In other instances (e.g., breast cancer), the association of Bcl-2 with good prognosis may be misleading because of lack of information about other Bcl-2 family proteins such as Bax (Krajewski *et al.,* 1995a) (discussed later). At this juncture, additional studies involving large groups of patients who received uniform therapy are needed to unequivocally establish the prognostic significance of Bcl-2, Bax, and other members of the Bcl-2 protein family for various types of cancer.

V. *In Vivo* Patterns of *Bcl-2* Family Gene Expression in Hormone-Dependent Tissues and Tumors Derived From Them

The expression of Bcl-2 and several of its mammalian homologs has been examined by immunohistochemical and other techniques in hormone-dependent tissues and the tumors that arise from these organs. Some of the currently available information is summarized here.

A. Breast

In the adult breast, the numbers of mammary epithelial cells expand and contract in response to estrogens and other hormones. In fact, one of the classical examples of programmed cell death is the involution of the postlactating mammary gland, where the loss of lactogenic hormones results in collapse of the gland in large part because of elimination of differentiated mammary epithelial cells by apoptosis (Daniel and Silberstein, 1987; Li *et al.,* 1996a). Several Bcl-2 family proteins are expressed in normal mammary epithelium, including the antiapoptotic proteins Bcl-2, Bcl-X, and Mcl-1 and the proapoptotic protein Bax (Krajewski *et al.,* 1994a,b, 1995b). The proapoptotic protein Bak is also expressed in normal breast but primarily is found in the myoepithelial cells that line the basement membranes of the tubuloalveolar glands, rather than in the differentiated luminal epithelial cells (Krajewski *et al.,* 1996). These long-lived stem cells, however, also express Bcl-X, which presumably offsets the death-promoting effects of Bak (Krajewski *et al.,* 1994b). Bcl-2, in contrast, is not expressed in the myoepithelial cells (Krajewski *et al.,* 1994a,b; Nathan *et al.,* 1993).

Expression of Bcl-2 in breast cancer cell lines has been shown to be dependent on estrogen, whereas Bax and Bcl-X are not (Teixeira *et al.,* 1995; Wang and Phang, 1995). In the breast carcinoma cell line MCF-7, estradiol upregulates the relative levels of Bcl-2 mRNA and protein, whereas the pure antiestrogen ICI-164,384 reduces Bcl-2 expression at

both the mRNA and protein levels (Teixeira *et al.*, 1995). The levels of Bcl-2 protein in MCF-7 cells, as determined by immunoblotting, have been reported to be comparable to those found in the B-cell lymphoma cell line Karpas 231, which contains a t(14;18) chromosomal translocation (Nathan *et al.*, 1994), implying that functionally significant levels of this protein can be found in breast cancers. In this regard, several studies have documented very strong correlations between estrogen receptor (ER) positivity and higher percentages of Bcl-2-immunopositive tumor cells in primary adenocarcinomas of the breast (Silvestrini *et al.*, 1994; Nathan *et al.*, 1994; Leek *et al.*, 1994; Hellemans *et al.*, 1995; Gasparini *et al.*, 1995; Joensuu *et al.*, 1994; Doglioni *et al.*, 1994; Binder *et al.*, 1995). Furthermore, consistent with the generally more aggressive nature of tumors that have progressed to an ER^-, hormone-independent state, tumors with an ER^+ and Bcl-2-immunopositive phenotype have been associated with longer disease-free and overall survival in several clinical-correlative studies. Some of these clinical-correlative studies involved surgery with or without local radiation as the primary therapy, thus rendering the data potentially irrelevant to the issue of Bcl-2 and its role as a chemoresistance protein (Silvestrini *et al.*, 1994; Joensuu *et al.*, 1994). However, in two other studies where higher percentages of Bcl-2-immunopositive tumors cells were associated with better outcome, patients with node-positive disease were treated with either combination chemotherapy (cyclophosphamide–methotrexate–5-fluorouracil) or tamoxifen (Gasparini *et al.*, 1995; Hellemans *et al.*, 1995).

Though at first glance these data may seem paradoxical, given the overwhelming evidence that Bcl-2 can promote cell survival and provide protection from apoptosis induced by cytotoxic drugs and estrogen deprivation, it should be remembered that the immunohistochemical analysis of Bcl-2 provides only a single snapshot in time of what surely must be a dynamic process of *in vivo* fluctuations in Bcl-2 expression in response to endogenous estrogens and other stimuli. Thus, these correlations may represent little more than a reflection of the estrogen-dependent status of the tumors, with the most estrogen-responsive tumors having the highest Bcl-2 expression. Evidence supporting the idea that estrogen analogs can influence Bcl-2 expression *in vivo* has come from a longitudinal study of breast cancer patients whose tumors were biopsied before and then again after treatment with tamoxifen for ~1–5 weeks (Johnston *et al.*, 1994). Among the 12 tumors that were ER^+, 3 were deemed to be Bcl-2 immunopositive (i.e., >10% invasive cancer cells immunostained) prior to tamoxifen, compared to 8 after treatment (Johnston *et al.*, 1994). In contrast, tamoxifen had no impact on Bcl-2

expression in 6 of 6 ER^- tumors. It was speculated that these results may have reflected an agonistic effect of tamoxifen, resulting in increased rather than decreased Bcl-2 expression (Johnston *et al.,* 1994), and suggested a need for investigations of compounds with purer antiestrogenic activity as clinical tools for downregulating Bcl-2 protein levels in ER^+ breast cancers. Alternatively, the *in vivo* influence of estrogens and antiestrogens on Bcl-2 expression in breast tumors may be more complex than suggested by *in vitro* studies of cell lines, as evidenced by a report that the patterns of Bcl-2 immunostaining in normal breast do not vary with the menstrual cycle (Nathan *et al.,* 1993).

Another potential contributing factor to the association of Bcl-2 with favorable prognosis in breast cancer has been revealed by studies of Bax expression in human mammary carcinomas. Using immunohistocemical techniques to evaluate Bax protein levels in primary tumors derived from 119 women with metastatic breast cancer, Krajewski *et al.* (1995a) demonstrated heterogeneous percentages of Bax-immunopositive invasive malignant cells among tumor specimens, with 34% of cases having $<10\%$ Bax-positive cells. The subgroup of patients whose tumors contained $<10\%$ Bax-positive invasive cells also generally did not respond to therapy (21% vs. 43%; $P < 0.02$) and typically experienced shorter time to tumor progression (median 3.7 vs. 9.0 months) and shorter overall survival (10.7 vs. 17.1 months), which were statistically significant in both univariate and multivariate analyses (Krajewski *et al.,* 1995a). Interestingly, a strong positive correlation was found ($P = 0.005$) between the percentages of Bcl-2- and Bax-immunopositive tumor cells, implying that the same tumor cells that had lost Bcl-2 expression were also the ones with reduced Bax levels. Similarly, Bargou *et al.* (1995a) reported that Bax mRNA levels were reduced compared to normal breast tissue in 10 of 10 breast cancer specimens, whereas levels of mRNA for the antiapoptotic proteins Bcl-2 and Bcl-X_L were similar in tumor and normal breast tissue. Moreover, a correlation was observed *in vitro* in human breast cancer cell lines between lower Bax mRNA levels and resistance to apoptosis induction by serum growth factor deprivation and anti-Fas antibody treatment. In a subsequent study, these investigators extend this analysis, showing reductions in Bax mRNA levels in 35 of 36 malignant breast cancer specimens and demonstrating that gene transfer-mediated restoration of Bax protein levels in two breast cancer lines promoted apoptosis induced by growth factor deprivation and slowed the growth of these lines as tumors in immunocompromised (severe combined immunodeficiency) mice (Bargou *et al.,* 1995b).

The hypothesis that can be raised, therefore, is that, while Bcl-2 lev-

els tend to decline during progression of breast cancers, so too do the levels of Bax. Furthermore, since it is the ratio of Bcl-2 to Bax that presumably determines sensitivity to apoptosis, rather than the absolute levels of either protein, these findings suggest that advanced breast cancers with reduced Bcl-2 are probably no more sensitive to apoptosis than their Bcl-2-immunopositive, less aggressive counterparts, and indeed may be more resistant to apoptosis when one considers that other antiapoptotic Bcl-2 family proteins, including Bcl-X_L and Mcl-1, are usually present in these breast cancers (unpublished observations).

In addition to the pathogenesis and progression of breast cancers, a potential role for changes in the expression of Bcl-2 family proteins has also been suggested in the involution of the mammary gland following cessation of nursing, when lactogenic hormone levels plummet (Daniel and Silberstein, 1987). For example, increases in the relative levels of Bax and Bcl-X_S mRNA have been reported during postlactation-associated apoptosis in the involuting mammary glands of mice (Li *et al.*, 1996b). Interestingly, these increases in Bax mRNA accumulation occurred equally well in p53 knockout and normal mice, indicating that p53 is not required for induction of Bax in this circumstance. Involution of the mammay glands at weaning was also p53 independent. Conversely, in transgenic mice that carried a simian virus 40 large-T antigen (TAg) under the control of the hormone-responsive whey acidic protein gene promoter, where a >100-fold increase in the percentage of apoptotic mammary epithelial cells was detected during late pregnancy compared to normal litter mate control mice, the levels of Bax mRNA and protein were elevated, as well as the ratio of Bcl-X_S to Bcl-X_L mRNA (Li *et al.*, 1996b). Thus, simultaneous loss of both p53 and Rb function in these TAg-expressing mammary cells appears to create conditions that promote expression of Bax and Bcl-X_S. In the future, it will undoubtedly be informative to evaluate mammary gland function during pregnancy and after lactation in the Bcl-2 and Bax knockout mice.

B. Endometrium

The expression of several Bcl-2 family proteins has been reported in endometrial tissues, based on immunohistochemical analysis. Based on relative immunointensity, Bcl-2 and Bak appear to be expressed at high levels in the endometrium, Bcl-X at moderate levels, Mcl-1 at low levels, and Bax at low to undetectable levels (Krajewski *et al.*, 1994a,b, 1995a, 1996). The smooth muscle cells of the myometrium contain relatively high levels of immunostaining for Mcl-1, Bax, and Bak but more modest levels of Bcl-2 and Bcl-X.

The effects of hormonal fluctations on expression of Bcl-2 family proteins during the menstrual cycle has been examined to date only for Bcl-2. High levels of Bcl-2 immunostaining are seen in the endometrial epithelium during the proliferative phase of the cycle, when estrogen levels are on the rise, and levels then decline during the secretory phase, when progesterone is produced (Gompel *et al.,* 1994; Tabibzadeh *et al.,* 1995). In contrast, Bcl-2 immunoreactivity reportedly increases in the endometrial stromal cells during the latter half of the menstrual cycle, as the predecidualization changes occur. Taken together, these findings suggest that Bcl-2 expression is under hormonal control in the endometrium. The expression of Bcl-2 and its homologs in uterine cancers has not been examined to date.

C. Ovary

Though a detailed time course of changes with the ovulatory cycle has not been developed, expression of several Bcl-2 family proteins has been immunolocalized in the granulosa cells of the developing ovarian follicles and in the granulosa–lutein and thecalutein cells of the corpus luteum in the ovaries of mice and humans (Krajewski *et al.,* 1994a,b, 1995b, 1996). Gonadotrophin is known to be important for supporting the survival and follicular development of ovarian germ cells (reviewed in Tilly, 1993). In rats, administration of exogenous gonadotrophin reportedly had no effect on the constitutive expression of Bcl-2 and Bcl-X but produced marked reductions in Bax mRNA levels, to approximately one-third of control, based on Northern blot analysis of RNA isolated from whole ovaries (Tilly *et al.,* 1995). Moreover, antral follicles cultured *in vitro* without hormonal support displayed an approximate doubling of their levels of Bax mRNA, while Bcl-X_L mRNA levels became reduced slightly to ~70% of control. Supplying gonadotrophin to these cultures resulted in reduced levels of Bax mRNA while the pretreatment expression of Bcl-2 and Bcl-X_L was sustained, thus altering the ratio of Bax relative to Bcl-2 and Bcl-X_L and rescuing the cells from apoptosis.

Bcl-2 expression was reported in 60% of ovarian carcinomas in a study where immunohistochemical methods were used to detect Bcl-2 protein in primary tumor specimens (Eliopoulos *et al.,* 1995). The expression, however, was heterogeneous among the tumor cells, suggesting either that local factors control expression of Bcl-2 or that activation of the *BCL-2* gene represents a relatively late event in the progression of these tumors. In another report where Bcl-2 immunostaining was assessed in ovarian adenocarcinomas derived from 70 pa-

tients, Bcl-2 immunopositivity was found in 57% of cases. Higher percentages of Bcl-2-immunopositive tumors cells had a slight tendency to be associated with improved survival (Herod *et al.*, 1996). Since other Bcl-2 family proteins have yet to be analyzed in primary ovarian cancers, it is difficult to interpret the biological significance of this observation, particularly since it is the ratios of anti- and proapoptotic Bcl-2 family proteins that ultimately determine relative resistance to apoptosis.

Gene transfer-mediated elevations of Bcl-2 protein levels in the ovarian cancer cell line A2780 resulted in enhanced resistance to the cytotoxic effects of cisplatin (Eliopoulos *et al.*, 1995). Interestingly, increased Bcl-2 caused a delay and a reduction in the total amount of endogenous p53 protein accumulation after exposure of A2780 cells to cisplatin, suggesting that Bcl-2 affects a step in the DNA damage response pathway upstream of p53. Cisplatin-induced increases in Bax protein levels also were impaired in the Bcl-2 transfectants compared to control transfectants, with Bax protein levels increasing only ~3-fold in the Bcl-2 transfectants versus >10-fold in the control cells. These findings are consistent with evidence that Bax is a p53 response gene in at least some types of tumors and normal tissues (Miyashita *et al.*, 1994b; Selvakumaran *et al.*, 1994; Miyashita and Reed, 1995; Kitada *et al.*, 1996). DNA-damaging drugs, radiation, and other inducers of genotoxic stress are known to stimulate increases in the levels of p53, a DNA binding protein that stimulates transcription of target genes through specific interactions with a 10-bp consensus DNA sequence (Vogelstein and Kinzler, 1992). DNA sequence analysis of the *BAX* gene promoter has revealed the presence of at least four sites with homology or identity to the 10-bp consensus sequence for p53 binding. Furthermore, functional studies of the *BAX* promoter have demonstrated that p53 directly binds to and transactivates this gene (Miyashita and Reed, 1995). However, the ability of p53 to stimulate increases in Bax expression appears to be highly tissue and tumor type specific (Kitada *et al.*, 1996; Zhan *et al.*, 1994), suggesting that the response is under genetic control and modulated by other as-yet unidentified factors that may operate at either the transcriptional or posttranscriptional levels.

Of relevance to the potential role of p53 in controlling Bax expression, analysis of another ovarian cancer cell line, IGROV-1, and two cisplatin-resistant sublines derived from it after continuous culture in the presence of drug revealed striking reductions in Bax mRNA and protein levels in both of the drug-resistant sublines compared to the parental drug-sensitive cells (Perego *et al.*, 1996). These drug-resistant sublines also contained mutations in both alleles of p53, whereas p53

was determined by single-strand conformation polymorphism analysis to be wild type in the parental cells. Experiments involving use of *BAX* promoter–reporter gene plasmids confirmed reduced transactivation of the *BAX* promoter in the drug-resistant lines. Though Bax expression was markedly reduced in the cisplatin-resistant sublines, the relative levels of mRNA and protein for Bcl-2, Bcl-X, and Mcl-1 were not altered. Taken together, these data suggest that mutations in p53 resulted in reduced expression of Bax, thereby contributing to the emergence of cisplatin resistance in this *in vitro* model system. It remains to be determined, however, whether similar events occur in ovarian cancers *in vivo* in patients treated with cisplatin.

D. Prostate

The patterns of Bcl-2 expression in the normal prostate gland correlate with the androgen-dependent and androgen-independent phenotypes of the secretory epithelial and basal cells, respectively. By immunostaining, high levels of Bcl-2 protein appear to be present selectively in the basal cells that line the basement membrane, whereas no Bcl-2 immunoreactivity is seen in the differentiated epithelial cells that face the lumina of the gland (Hockenbery *et al.,* 1991; Krajewski *et al.,* 1994a,b; McDonnell *et al.,* 1992). In castration models, it is the Bcl-2-negative epithelial cells that undergo rapid programmed cell death, thus demonstrating the androgen dependence of these cells (Kyprianou and Isaacs, 1988). In contrast, the Bcl-2-positive basal cells, which differentiate to give rise to the luminal secretory epithelial cells, represent a long-lived population of stem cells that lack androgen receptors and that display no dependence on testosterone for survival.

Like Bcl-2, expression of the proapoptotic protein Bak is found primarily in the basal cells. Thus, a delicate balance between the antiapoptotic effects of Bcl-2 and the proapoptotic actions of Bak may help to regulate the overall numbers of basal cells. It will be of interest in the future, therefore, to contrast the expression of Bcl-2 and Bak in basal cell hyperplasia. Intense immunostaining for Bcl-2 has been reported in basal cell hyperplasia, but Bak has yet to be evaluated (Shabaik *et al.,* 1994). In contrast to Bcl-2 and Bak, expression of the proapoptotic protein Bax is higher in the secretory cells than in the basal cells, consistent with the differential apoptotic tendencies of these two types of cells (Krajewski *et al.,* 1994a). However, the antiapoptotic protein Mcl-1 is also expressed at higher levels in the secretory than the basal cells, whereas Bcl-X immunostaining is fairly evenly distributed between these two cell populations (Krajewski *et al.,* 1994b,

1995b). The normal prostate therefore expresses several members of the *BCL-2* gene family, thus affording opportunities for several interactions among these homologous proteins and thereby presumably allowing for fine tuning of the regulation of apoptosis by hormones and other agents in this gland. Interestingly, in p53 knockout mice, the relative intensity of immunostaining was reported to be increased for Bcl-2 and decreased for Bax, suggesting that p53 may regulate the expression of these genes in prostate tissue (Miyashita *et al.*, 1994a). These findings are consistent with reports that p53 can function as a transcriptional repressor of Bcl-2 and an activator of Bax (Miyashita *et al.*, 1994b; Miyashita and Reed, 1995). However, p53 gene ablation in transgenic mice only delays and does not prevent programmed cell death in the prostate due to castration (Berges *et al.*, 1993). Thus, programmed cell death induced by androgen deprivation is p53 independent, despite the apparent changes in the basal steady-state levels of Bcl-2 and Bax seen in p53 knockout mice.

McDonnell *et al.* (1992) reported that, after castration, Bcl-2 mRNA levels slowly rise in the rat prostate and showed that this increase in Bcl-2 expression can be suppressed by administration of exogenous testosterone. The implications of this observation, however, remain unclear and could reflect either a suppressive effect of androgens on Bcl-2 expression or a compensatory expansion of the population of Bcl-2-expressing basal cells after castration. Similarly, in the G-subline of the Dunning rat prostate carcinoma tumor R-3327, castration resulted in upregulated expression of Bcl-2 (Furuya *et al.*, 1996). It was speculated that this ability of the G-subline to upregulate Bcl-2 might account for its androgen-sensitive but not androgen-dependent phenotype where *in vivo* tumor growth is concerned.

Immunohistochemical studies of Bcl-2 expression in adenocarcinomas of the prostate have suggested that Bcl-2 expression is generally, but not always, associated with androgen independence and progression to metastatic disease. For example, McDonnell, *et al.* (1992) reported Bcl-2 immunostaining in only 6 of 19 primary tumors that displayed androgen dependence based on clinical response after administration of androgen-ablative therapy, compared to Bcl-2 immunopositivity in 10 of 13 androgen-independent primary tumors. Similarly, Colombel *et al.* (1993) reported Bcl-2 immunopositivity in 62% of 37 primary adenocarcinomas of the prostate, compared to 100% of 9 primary tumors and metastatic lesions derived from men with hormone-refractory tumors. Shabaik *et al.* (1994) found Bcl-2 immunopositivity in only 3 of 41 cases of primary adenocarcinoma of the prostate. All three of these Bcl-2-immunopositive cases were poorly differentiat-

ed tumors (Gleason histological grade 9 or 10), suggesting that Bcl-2 gene expression generally becomes upregulated in prostate cancers as a relatively late event. Consistent with this idea, none of 20 cases of high-grade prostate intraepithelial neoplasia (PIN) were found to be Bcl-2 immunopositive in the same study (Shabaik *et al.,* 1994). Furuya *et al.* (1996) studied Bcl-2 expression in lymph node and bone metastases from men with prostate cancer who either had been previously untreated ($n = 45$) or had failed androgen-ablative therapy ($n = 12$). Homogeneous Bcl-2 immunostaining was detected in 5 of 30 (17%) nodal metastases and 14 of 27 (52%) bone metastases. No significant difference, however, was noted between the frequency of Bcl-2-immunopositive specimens when comparing bone metastases derived from previously untreated men ($n = 15$) with those of patients who had failed androgen-ablative therapy ($n = 12$) (53% vs. 42% Bcl-2-immunopositive cases) (Furuya *et al.,* 1996). In a report by Krajewska *et al.,* (1996a) that involved an immunohistochemical analysis of Bcl-2 expression in 64 adenocarcinomas and 24 cases of PIN, Bcl-2 immunopositivity (i.e., >1% immunopositive cells) was observed more frequently in high-grade primary tumors (Gleason grade 8–10) and lymph node metastases compared to low-grade (Gleason grade 2–7) primary tumors ($p < 0.05$). Higher levels of Bcl-2 expression were also confirmed for high-grade primary tumors and nodal metastases compared to low-grade primary tumors and PIN lesions by use of a method for scoring immunostaining that incorporated both percentage immunopositive tumor cells and immunointensity ($P = 0.05$). However, when the analysis excluded metastatic disease, neither this study nor any of the others cited previously found significant correlations of Bcl-2 immunostaining with histology (Gleason grade) among primary prostate cancers.

The absence of an association between Bcl-2 and histological grade raises the possibility that Bcl-2 may provide a novel independent prognostic marker for men with prostate cancer. To date, only one study has attempted to correlate Bcl-2 expression in prostate cancer with clinical outcome (Bubendorf *et al.,* 1996). Based on an analysis of 115 men with prostate cancer for whom follow-up data were available (median 5.2 years), Bcl-2 immunopositivity (i.e., >10% immunopositive tumor cells) was more frequently associated with advanced clinical stage ($P = 0.001$) and shorter survival ($P = 0.03$). Bcl-2 lost its prognostic significance, however, in multivariate analysis, which identified Ki67 immunopositivity and advanced stage (pT3) as independent indicators of poor prognosis. Interestingly, tumors that were both Bcl-2 and Ki67 positive defined a subgroup of patients with particularly short survival. The results of this study, however, may have little relevance to the issue of

androgen dependence, since only 6 of the patients underwent antiandrogen therapy. However, they may be of importance for predicting progression of prostate cancers to metastatic disease, inasmuch as *BCL-2* gene transfer can protect epithelial cells from apoptosis that results from loss of integrin attachments to extracellular matrix proteins (Frisch and Francis, 1994; Ruoslahti and Reed, 1994; Zhang *et al.*, 1995).

Support for the hypothesis that Bcl-2 can contribute to androgen resistance in at least some circumstances, however, has come from immunohistochemical analysis of Bcl-2 expression in Dunning rat prostate cancer sublines that had progressed to an androgen-independent state. High levels of Bcl-2 immunostaining were detected in four of six such sublines, whereas the androgen-dependent R-3327 parental line was evidently Bcl-2 negative (Furuya *et al.*, 1996). Moreover, gene transfer-mediated elevations in Bcl-2 protein levels in the human prostate cancer line LNCaP abrogate the dependence of these tumor cells on androgens for tumor formation in mice (Raffo *et al.*, 1995). In addition, transfection of Bcl-2 expression plasmids into the androgen-independent rat prostate cancer line AT-3 increases the resistance of these cells to the cytotoxic effects of the chemotherapeutic drugs 5-fluorouracil and 4-hydroxycyclophosphamide, as well as the Ca^{2+} ionophore ionomycin and the Ca^{2+} ATPase inhibitor thapsigargin (Furuya *et al.*, 1996). Bcl-2, however, did not prevent DNA-damaging drugs from inducing increases in the relative levels of GRP-78 and tissue transglutaminase (TTG) and clusterin mRNAs. Elevated Bcl-2 also did not prevent the Ca^{2+}-altering agents from inducing increases in cytosolic free Ca^{2+} concentrations, or increases in the expression of GRP-78, TTG, calmodulin, or α-prothymosin (Furuya *et al.*, 1996). Thus, as in other studies, Bcl-2 appears to block a downstream step in the cell death pathways through which disturbances in Ca^{2+} homeostasis and DNA-damaging drugs trigger apoptosis in these prostate cancer cells.

To date, relatively little is known about the expression of other members of the *BCL-2* gene family in prostate cancers and the influence that androgens may have on them. Krajewska *et al.* (1996b) reported that most primary tumors express Bcl-X (100%), Mcl-1 (79%), and Bax (100%), as defined by >1% immunopositive tumor cells. In general, progression to more advanced histology (Gleason grade 8–10) and metastatic disease were associated with increases in Bcl-X and Mcl-1 immunostaining, with relatively little change in Bax. For example, comparisons of high-grade (Gleason grade 8–10; $n = 17$) primary tumors with low-grade (grade 2–7; $n = 31$) cancers revealed significantly higher intensity of Bcl-X immunoreactivity ($P < 0.001$), higher propor-

tions of cases with >50% Bcl-X-immunopositive tumor cells ($P < 0.03$), and higher overall immunostaining scores (intensity × percentage) ($P = 0.002$) in the more histologically advanced neoplasms. Immunostaining for Bcl-X was also significantly higher in lymph node and bone metastases than in low-grade primary tumors. Immunoblot analysis of normal prostate, human prostate cancer cell lines, and rat Dunning tumors suggests that most, if not all, of the Bcl-X immunoreactivity seen in prostate cancers can probably be ascribed to the antiapoptotic Bcl-X_L protein, as is typically the case for all other normal tissues and tumors (Krajewski *et al.*, 1994b; Krajewska *et al.*, 1996a; González-Garcia *et al.*, 1994). Similarly, the proportion of specimens with Mcl-1 immunopositivity (i.e., >1% immunopositive tumor cells) was greater among primary tumors than PIN lesions (70% of 10 vs. 38% of 24; $P < 0.001$), and the percentages of Mcl-1 immunopositive tumor cells ($P < 0.05$) as well as the overall Mcl-1 immunostaining scores ($P = 0.03$) were significantly higher among high-grade tumors compared to low-grade primary cancers. Mcl-1 immunostaining was also higher for lymph node and bone metastases than PIN lesions and low-grade primary tumors (Krajewska *et al.*, 1996a).

Taken together, the data available to date suggest that an upregulation of the expression of some antiapoptotic members of the Bcl-2 family, including Bcl-2, Bcl-X_L, and Mcl-1, occurs during the pathogenesis and/or progression of adenocarcinomas of the prostate. The increased levels of these proteins relative to proapoptotic Bcl-2 family proteins such as Bax may contribute to the development of hormone-refractory metastatic disease. However, conclusive proof of this idea and evidence that Bcl-2, Bcl-X_L, Mcl-1, or other Bcl-2 family proteins provide prognostically useful information for patients with prostate cancer await further experimental analysis.

E. Thyroid

The normal thyroid gland expresses Bcl-2 at high levels, whereas Bax immunostaining is only weak or entirely absent from most follicular thyroid cells (Pilotti *et al.*, 1994; Krajewski *et al.*, 1994a). Bcl-X and Mcl-1 immunoreactivity have also been reported to be rather weak or absent from thyroid epithelium (Krajewski *et al.*, 1994b, 1995b). It has yet to be determined what the effects of thyroid-stimulating hormone are on the expression of Bcl-2 and other *BCL-2* family genes in the normal thyroid.

Bcl-2 expression has been evaluated by immunohistochemical methods in 135 carcinomas of the thyroid. Expression of Bcl-2 was detected

in ~79% of 94 well-differentiated (90% of 20 follicular; 76% of 74 papillary) and 84% of 19 poorly differentiated thyroid cancers but in only ~14% of 22 undifferentiated tumors (Pilotti *et al.*, 1994). Moreover, Bcl-2 immunopositivity was significantly correlated with thryoglobulin immunostaining. Thus, Bcl-2 tends to be expressed at high levels in differentiated thyroid cancers.

In another study by Branet *et al.* (1996), expression of Bcl-2, Bcl-X, Bax, and Mcl-1 was evaluted by immunohistochemical methods in 23 papillary and 10 follicular thyroid cancers. Among well-differentiated thyroid cancers, the intensity of Bcl-2 immunostaining was typically higher in follicular than in papillary carcinomas. Bax was expressed in all papillary carcinomas examined (n = 23) and most (80%) follicular tumors (n = 10). Interestingly, the intensity of both Bcl-2 and Bax immunostaining was generally lower in undifferentiated than differentiated tumors. In contrast, Bcl-X was expressed in 6 of 6 (100%) undifferentiated tumors and in 22 of 23 (96%) papillary carcinomas, compared to only 5 of 10 (50%) cases of follicular tumors. Mcl-1 was also frequently expressed in undifferentiated tumors. During progression of thyroid cancers to undifferentiated tumors, therefore, it appears that, in general, expression of Bcl-2 and Bax appears to decline while Mcl-1 and particularly Bcl-X expression increases. Immunoblotting suggested that Bcl-X_L was the major isoform of Bcl-X expressed in these tumors.

F. Thymus

The T-cell precursors of the thymus are exquisitely sensitive to the induction of apoptosis by glucocorticoids, thus making this gland another example of a hormonally responsive tissue. In the thymus, the normal patterns of Bcl-2 expression correlate with the relative sensitivity and resistance of thymocytes to glucocorticoids, with high levels of Bcl-2 immunostaining residing in the more mature, glucocorticoid-resistant thymocytes of the medulla and essentially no Bcl-2 expression appearing in the immature, highly sensitive thymocytes of the cortex (Pezzella *et al.*, 1990; Hockenbery *et al.*, 1991). The levels of endogenous Bcl-2 protein also correlate with relative sensitivity to glucocorticoids in leukemia cell lines, with higher levels of Bcl-2 being associated with resistance (Alnemri *et al.*, 1992a). Transgenic animal experiments have confirmed an important role for Bcl-2 in the control of resistance to glucocorticoid-induced apoptosis in the thymus. For example, when Bcl-2 is overexpressed in the cortical thymocytes of transgenic mice, striking resistance to glucocorticoid-induced apoptosis is noted both *in vivo* and

in thymocytes derived from these animals in culture (Strasser *et al.*, 1991; Sentman *et al.*, 1991; Siegel *et al.*, 1992). Conversely, thymocytes derived from Bcl-2 knockout mice display enhanced sensitivity to glucocorticoid-induced apoptosis (Veis *et al.*, 1993b; Kamada *et al.*, 1995). Since Bcl-2 is not normally expressed in the immature cortical thymocytes ($CD4^+CD8^+$), with the exception of a small population of $CD4^-CD8^-$ very immature T-cell precursors (Veis *et al.*, 1993a), the enhanced sensitivity presumably reflects loss of resistance to glucocorticoids in the mature $CD4^+CD8^-$ and $CD4^-CD8^+$ medullary T cells. Interestingly, Bcl-2 knockout mice initially develop normal immune systems but at ~4 weeks of age suffer massive apoptosis of their lymphocytes and subsequently die. Since endogenous glucocorticoid levels rise at about this time because of the elevations in adrenocorticotropic hormone that occur with puberty, it is intriguing to speculate that this apoptotic collapse of the immune systems of Bcl-2 knockout mice is due to production of these steroid hormones.

The antiapoptotic protein Bcl-X is also expressed in the normal thymus, but in a reciprocal pattern compared to Bcl-2, with strong Bcl-X immunostaining residing in the immature cortical thymocytes and little if any Bcl-X expression occurring in the mature medullary thymocytes. Western blotting experiments suggest that most of the Bcl-X protein in the thymus is the antiapoptotic Bcl-X_L isoform, despite the exquisite sensitivity of cortical thymocytes to glucocorticoids. Overexpression of Bcl-X_L in the cortical thymocytes in transgenic mice confers resistance to glucocorticoids (Chao *et al.*, 1995), suggesting that having higher levels of Bcl-X_L can overcome the apoptotic effects of glucocorticoids on these immature T cells and raising the question of whether glucocorticoids might possibly downregulate the endogenous levels of Bcl-X_L protein as part of the mechanism by which these steroid hormones induce apoptosis of cortical thymocytes. Gene transfer-mediated elevations in Bcl-X_L in the pro-B-cell line FL5.12 also produces resistance to glucocorticoids, as well as a variety of cytotoxic anticancer drugs (Minn *et al.*, 1995). Interestingly, studies of chimeric Bcl-X knockout mice suggest that Bcl-X is required for maintenance of the survival of immature thymocytes but not mature peripheral T cells, suggesting that the expression of Bcl-X in cortical thymocytes is essential for their survival (Motoyama *et al.*, 1995). The sensitivity to glucocorticoids of mature T cells lacking Bcl-X was not explored.

The proapoptotic protein Bax is expressed in the thymus with a distribution similar to Bcl-2, in that higher levels of Bax are generally found in the mature medullary thymocytes than in the immature cortical cells (Krajewski *et al.*, 1994a). In Bax knockout mice, increased

numbers of thymocytes are found compared to litter mate controls, but no alteration in sensitivity to dexamethasone *in vitro* was observed (Knudson *et al.*, 1995). Bax, therefore, appears to be relatively unimportant for the induction of apoptosis by glucocorticoids. Hence, glucocorticoid-induced apoptosis of thymocytes can proceed normally in the absence of Bax as well as p53 (Berges *et al.*, 1993) but is suppressible by overexpression of Bcl-2 and Bcl-X_L. The simplest interpretation of these results is that glucocorticoids kill via a pathway that can be regulated by Bcl-2 family proteins, but presumably some other proapoptotic member(s) of the family can supplant the role that Bax might play or Bax itself is not particularly important for glucocorticoid-mediated cell death.

VI. Conclusions

Though the mechanisms of action of Bcl-2 and its homologs remain unclear, this family of proteins seems to play a critical role in regulating a distal step in what may represent a final common pathway for programmed cell death and apoptosis. The finding that the expression of Bcl-2 and some of its homologs is regulated in concert with cellular expansion and eradication in hormone-sensitive tissues suggests that these apoptosis regulators serve an important function as intracellular mediators of the biological effects of several steroid hormones. Moreover, aberrations in the regulation of the expression of Bcl-2 family genes may underlie some of the abnormalities in hormone dependence and hormone sensitivity that occur during the pathogenesis and progression of cancers. Improved knowledge about the mechanisms that control the expression of these genes and the biochemical mechanisms of action of their encoded proteins offers the promise of improved strategies for the treatment of human malignancy and other diseases that arise in whole or in part because of dysregulation of the physiological cell death pathway.

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Pathways of p53-Dependent Apoptosis

LUIGI GRASSO* AND W. EDWARD MERCER*,†

*Departments of *Microbiology and Immunology*
and †Biochemistry and Molecular Pharmacology
Jefferson Medical College and Kimmel Cancer Institute
Thomas Jefferson University
Philadelphia, Pennsylvania 19107

I. Introduction

It has been known for some time that the growth of tissues (both normal and neoplastic) depends not only on the rate of cell birth, which is dictated by the length of the cell cycle and the growth fraction, but also on the rate of cell loss through terminal differentiation and/or cell death (reviewed by Baserga, 1985). When this balance is disturbed, abnormal cellular accumulations in the form of hyperplasia (an increase in cell number) and/or neoplasia may result. Programmed cell death, or apoptosis, is an important mode of cell death that occurs in response to a variety of agents, including those that induce DNA damage, such as ionizing radiation or anticancer chemotherapeutic drugs (reviewed by Fisher, 1994). Tremendous advances have been made in our under-

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standing of apoptotic cell death pathways and their relevance to the development of human cancer. Apoptosis is a genetically determined cell death program defined by characteristic morphological and biochemical changes that can be distinguished from necrosis, the other mode of cell death (reviewed by Wyllie, 1995). The induction of apoptosis is modulated by a variety of factors, including growth factors, intracellular mediators of signal transduction, nuclear proteins that regulate gene expression, and proteins that regulate DNA replication and repair processes. Evidence suggests that alterations in oncogenes and/or tumor suppressor genes can influence the rate and/or the susceptibility of cells to undergo a program of apoptotic cell death (reviewed by Williams and Smith, 1993; Fisher, 1994).

Since its discovery as a cellular protein bound to the large T antigen transforming protein of simian virus 40 (SV40) over a decade ago (Lane and Crawford, 1979), p53 protein has been the focus of intense scientific investigation. The gene encoding p53, located on chromosome 17p13.1, has been shown to be a very frequent target for genetic alterations in human cancers (Harris and Hollstein, 1993). Missense point mutations, deletions, and rearrangements of the p53 gene have been observed in a wide variety of different types of cancer (Hollstein *et al.*, 1991). The wild-type (wt) p53 protein has been implicated in many cellular processes, including cell cycle control in response to DNA damage, DNA replication and repair, differentiation, and apoptosis. Numerous reviews have been published dealing with the structure and function of p53 protein, and the reader should consult these for a comprehensive coverage of such topics (see, e.g., Levine *et al.*, 1991; Ullrich *et al.*, 1992; Oren, 1992; Donehower and Bradley, 1993; Harris and Hollstein, 1993; Prives, 1994). The present review only highlights some of the properties of p53 protein relevant to its role in cell cycle control, induction of apoptosis, and tumor development.

II. Dual Personality of the p53 Tumor Suppressor Protein

A. Biochemical Properties

1. *Structural Domains*

Human p53 protein is an oligomeric nuclear phosphoprotein. Although regions of considerable divergence exist among p53 protein coding regions of evolutionarily diverse species, all p53 proteins contain five highly conserved regions designated I–V (see Fig. 1). p53 protein

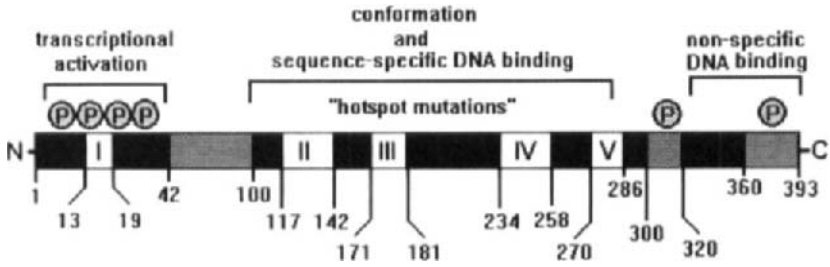

FIG. 1. The domain structure and phosphorylation sites of human p53 protein. The positions of conserved domains (I–V), transactivation and sequence-specific DNA binding domains, and the region of non-sequence-specific DNA binding are indicated. Shown also are the known sites of *in vivo* phosphorylation.

can be divided into three major functional domains, a charged N-terminal domain (residues 1–42), a central domain (residues 120–290), and a C-terminal domain (residues 310–390). The N-terminal region is important for p53-mediated transcriptional modulation, while the central domain confers sequence-specific DNA binding (reviewed by Prives, 1994). Most p53 mutations found in human cancers, the so-called "hotspot" mutations, usually fall within these conserved regions of the central domain (reviewed by Harris and Hollstein, 1993). These mutations tend to cluster at regions of conserved amino acid residues: 132–145, 171–179, 239–248, and 272–286 (reviewed by Levine *et al.*, 1991; Vogelstein and Kinzler, 1992). The C-terminal domain contains amino acid residues required for oligomerization and for binding to both single- and double-stranded regions of DNA and to mismatched single-stranded DNA loops. It is generally believed that the C-terminal region of p53 protein is involved in the recognition of damaged DNA (Nelson and Kastan, 1994; Jayaraman and Prives, 1995; Lee *et al.*, 1995).

It has now been firmly established that wtp53 protein functions as a modulator of transcription that can exert both positive and negative effects on the expression of a number of genes. Wtp53-mediated transcriptional activation occurs through its ability to function as a sequence-specific DNA-binding protein. For efficient DNA binding, p53 requires two copies of the 10-bp motif 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′, separated by 0–13 bp (Kern *et al.*, 1991; Funk *et al.*, 1992). The structure of the p53 sequence-specific DNA-binding domain (Cho *et al.*, 1994) and the oligomerization region (Clore *et al.*, 1994) have been visualized using X-ray crystallography and nuclear magnetic resonance spectroscopy, respectively. Wtp53 protein binds as a tetramer to its DNA recognition sequence (reviewed by Anderson and Tegtmeyer, 1995).

Crystallographic data suggest that the most frequently mutated amino acids in p53 protein (hotspot mutations) are those that either directly make contact with DNA (sequence-specific binding) or those critically important for maintaining the tertiary conformation necessary for DNA contact (Cho *et al.*, 1994).

2. *Target Genes*

Several target genes of wtp53 transactivation have now been identified. These include mouse muscle creatine kinase (Zambetti *et al.*, 1992), MDM2 (Wu *et al.*, 1993), WAF1 (El-Deiry *et al.*, 1993), thrombospondin-1 (Dameron *et al.*, 1994), GADD45 (Kastan *et al.*, 1992), cyclin G (Okamoto and Beach, 1994), Bax (Miyashita *et al.*, 1994b), and Fas (Owen-Schaub *et al.*, 1995). In addition to its transactivating function, overexpression of wtp53 (but not mutant p53) has been shown to inhibit expression of a wide array of cellular genes (Mercer *et al.*, 1990; Ginsberg *et al.*, 1991; Santhanam *et al.*, 1991; Subler *et al.*, 1992; Mack *et al.*, 1993), including those associated with cell cycle progression and DNA replication, such as proliferating cell nuclear antigen (Mercer *et al.*, 1991), B-myb, and DNA polymerase α (Lin *et al.*, 1992). Since none of these latter genes contain apparent p53 consensus DNA binding sequences, the mechanism(s) by which wtp53 represses their expression is unclear but may involve protein–protein interactions between wtp53 and positive-acting basal transcription factors (reviewed by Mercer, 1992). For example, *in vitro* experiments indicate that transcriptional repression of some genes by wtp53 is mediated through interactions of p53 protein with TATA-binding protein (TBP), a component of the basal transcription machinery of cells (reviewed by Mitchell and Tjian, 1989; Sharp, 1992). It has been shown that both free and DNA-bound p53 protein can bind to TBP and, conversely, that both free TBP and DNA-bound TBP can bind to p53 (Martin *et al.*, 1993). Some mutant p53 proteins can also bind to TBP, but in this case transcriptional repression is not observed (Martin *et al.*, 1995). p53 protein has also been shown to interact with the Sp1 transcription factor (Borellini and Glaser, 1993), the CCAAT-binding factor (Agoff *et al.*, 1992), and the TBP transcription factor holo-TFIID (Chen *et al.*, 1993). Studies by Thut *et al.* (1995) have demonstrated a direct and functional interaction between the transactivation domain of p53 and two subunits of the TFIID transcription complex, TBP-associated factors 40 and 60 ($TAFII_{40}$ and $TAFII_{60}$). The interaction of p53 protein with this wide array of basal transcription factors is likely to play an important role in the ability of p53 to modulate either positively or negatively the expression of different cellular genes.

B. Biological Properties

1. *Cell Cycle Control*

A number of studies have demonstrated that wtp53 protein activity is critically important for cell cycle control. The first suggestion that wtp53 protein might be involved in some aspect of cell cycle control was forthcoming from studies demonstrating that p53 protein was expressed in nontransformed cells upon mitogenic stimulation (Mercer *et al.,* 1982; Reich and Levine, 1984; Mercer and Baserga, 1985). Forced ectopic overexpression of exogenous wtp53 in various transformed cells carrying mutant p53 or completely lacking p53 was subsequently shown to inhibit cell proliferation (Mercer *et al.,* 1990; Baker *et al.,* 1990; Diller *et al.,* 1990; Martinez *et al.,* 1991). More specifically, using a human wtp53 cDNA transgene driven by the dexamethasone-inducible mouse mammary tumor virus promoter (Mercer *et al.,* 1990), or wtp53 cDNA constitutively expressed from the heterologous cytomegalovirus early promoter (Baker *et al.,* 1990; Diller *et al.,* 1990), or a temperature-sensitive mouse mutant p53 gene that adapts a mutant conformation at 37°C and a wild-type conformation at 32°C (Martinez *et al.,* 1991), overexpression of wtp53 protein was shown to block cell cycle progression predominantly in the G1 phase of the cell cycle. Studies also suggest that wtp53 plays some role in controlling the G2–M transition (Michalovitz *et al.,* 1990; Steward *et al.,* 1995; Guillouf *et al.,* 1995; Powell *et al.,* 1995; Paules *et al.,* 1995; Cross *et al.,* 1995).

2. *Checkpoint Control Function*

Substantial evidence now exists to support the notion that an important physiological function of wtp53 is to arrest cell cycle progression and/or induce apoptosis in response to extensive DNA damage, thereby monitoring the physical integrity of DNA and genomic stability (Hartwell and Kastan, 1994). For example, in response to DNA damage induced by ultraviolet and γ-irradiation or by anticancer chemotherapeutic drugs that produce DNA damage, wtp53 protein levels increase by a poorly understood mechanism. The increase in wtp53 protein levels results in transcriptional modulation of a number of p53 target genes that appear to mediate its "checkpoint control" function(s) (reviewed by Cox and Lane, 1995). Cells defective in p53-mediated checkpoint controls display a striking degree of genomic instability, revealed by a high rate of gene amplification and aneuploidy. This was first revealed in studies of mouse embryo fibroblasts derived from homozygous p53 knockout ($p53^{-/-}$) mice, which have been shown to be significant-

ly more prone to gene amplification, as measured by the frequency of *CAD* gene amplification (a gene encoding a single peptide chain containing *c*arbamyl phosphate synthetase, *a*spartate transcarbamylase, and *d*ihydroorotase) using an *N*-(phosphonacetyl)-L-aspartate (PALA) selection system assay, than normal mouse fibroblasts harboring wtp53 (Livingstone *et al.*, 1992). In addition, restoration of wtp53 function in tumor cells harboring endogenous mutant p53 was shown to induce G1-arrest and significantly reduce the frequency of PALA-induced amplification of the CAD gene (Yin *et al.*, 1992). A cause-and-effect relationship between wtp53 and the G1 arrest that occurs after DNA damage induced by γ-irradiation was clearly established by the demonstration that introduction of wtp53 (but not mutant p53) into p53-deficient cells restores G1 arrest (Kuerbitz *et al.*, 1992). In total, these studies demonstrated that wtp53 plays a role in cell cycle checkpoint control in response to metabolic perturbations or DNA damage.

3. *p53-Mediated Apoptosis and Tumor Suppression*

Different cell types vary profoundly in their susceptibility to p53-dependent induction of apoptotic cell death. Using a temperature-sensitive mouse mutant p53 construct expressed as mutant p53 at 37°C and wtp53 at 32°C, Oren and co-workers (Yonish-Rouach *et al.*, 1991) discovered that overexpression of wtp53 protein alone could induce rapid apoptotic cell death in a p53-null mouse myeloid cell line designated M1. Subsequent studies revealed that overexpression of wtp53 in other p53-null tumor cell types also resulted in apoptotic cell death (Shaw *et al.*, 1992; Ryan *et al.*, 1993; Wang *et al.*, 1993). On the contrary, in other p53-deficient tumor cell lines, restoration of wtp53 activity was insufficient to induce apoptosis but rendered the cells more susceptible to apoptotic cell death induced by DNA-damaging agents such as ionizing radiation or chemotherapeutic drugs (Lowe *et al.*, 1993; Brishow *et al.*, 1994; Fisher, 1994). These observations suggest that an intrinsic property of some cell types can influence the ability of wtp53 to trigger an immediate apoptotic response.

The tumor-suppressing function of wtp53 may be linked to its ability to induce apoptotic cell death. The generation of $p53^{-/-}$ mice (Donehower *et al.*, 1992) allowed a direct examination of the role that p53 plays in tumor development *in vivo*. Mice homozygous for inactivated p53 alleles appeared normal at birth but exhibited a high frequency of spontaneous malignant tumors. For example, by 6 months of age, over 70% of homozygous animals developed lethal tumors, and they succumbed to these tumors by 10 months. The tumor spectrum of these animals revealed mostly tumors of lymphoid origin (Donehower *et al.*, 1992; Harvey *et al.*, 1993). In addition, heterozygous mice carrying one

wild-type allele and one inactivated allele ($p53^{+/-}$) were also susceptible to the development of spontaneous tumors, although these tumors developed with a much longer latency period. Some of the tumors that arose in heterozygous animals had lost the remaining wtp53 allele. These experiments demonstrated that wtp53 function plays an important role in suppression of tumorigenesis *in vivo*.

Numerous studies have also demonstrated that wtp53 is required for the induction of apoptosis by ionizing radiation and by some DNA-damaging drugs *in vivo* (Lowe *et al.*, 1993; Clarke *et al.*, 1993). For example, Lowe and collaborators (1993) reported that tumors generated from cells expressing wtp53 in immunocompromised mice contained a high proportion of apoptotic cells and typically regressed after treatment with γ-irradiation or Adriamycin. On the contrary, tumors generated from p53-deficient cells treated in the same way continued to enlarge and contained fewer apoptotic cells. Interestingly, Merratt *et al.* (1993) reported that the loss of wtp53 in homozygous $p53^{-/-}$ animals exposed to whole-body ionizing radiation rendered the epithelial cells of the small intestine and colon more resistant to radiation-induced apoptosis. However, apoptosis associated with normal tissue homeostasis was not found to be significantly different between homozygous $p53^{-/-}$, heterozygous $p53^{+/-}$, or $wtp53^{+/+}$ mice, suggesting that wtp53 is not involved in this process.

Mutations in the p53 gene are common in diverse tumor types, and germline p53 mutations confer a predisposition to the development of various malignancies in individuals with Li–Fraumeni syndrome (Malkin *et al.*, 1990). Based on our current understanding of wtp53 function, it seems likely that enhanced tumor development stems from a reduced capacity to arrest cell cycle progression so that sublethal DNA damage can be repaired and/or from the inability to eliminate by the process of apoptosis those cells that sustain extensive DNA damage. Defects in the capacity to repair damaged DNA and induce apoptosis in cells are likely to play a key role in the development of malignant human tumors as a result of an increase in genetic alterations involving oncogenes and tumor suppressor genes (reviewed by Hartwell and Kastan, 1994). Figure 2 illustrates the relationship between p53 status, growth arrest, DNA repair, and apoptosis in tumorigenesis.

III. Oncogene-Driven p53-Dependent Apoptosis

Major advances in our understanding of the factors involved in p53-dependent apoptosis have emerged from the study of DNA tumor virus-

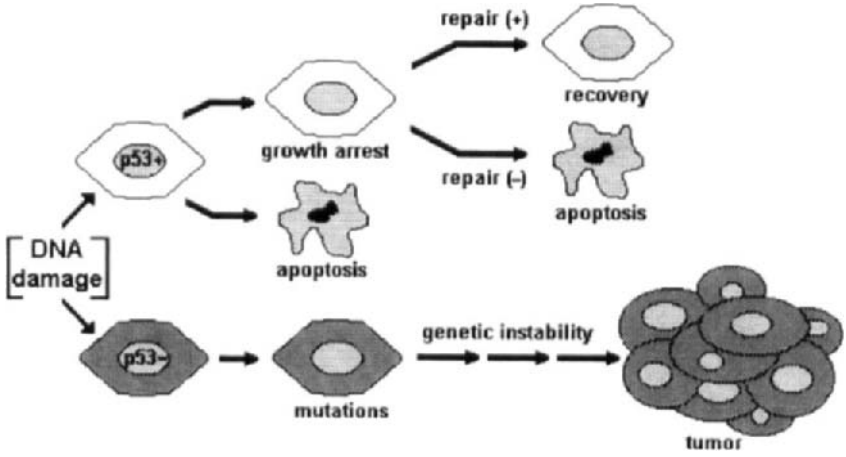

FIG. 2. Relationship between p53 status, growth arrest, DNA repair, and apoptosis in tumorigenesis. DNA damage in cells that harbor wtp53 can result in growth arrest and/or immediate apoptotic cell death. Growth arrest is generally considered to be a protective response that allows cells time to repair (+ repair) the damage before parental cells can pass it on to their progeny. However, if DNA damage is extensive and cannot be repaired (− repair), apoptotic cell death rather than recovery is triggered. DNA damage in p53-deficient cells (p53) does not produce G1 arrest but instead may become "fixed" as mutations as cells continue to proliferate. The accumulation of mutations and genomic instability in progeny cells may in turn contribute to the development of tumors.

es. In several distinct classes of DNA tumor viruses, specific viral oncoproteins have evolved to target and inactivate wtp53 and another tumor suppressor gene product, retinoblastoma tumor suppressor protein (pRB), and retinoblastoma (RB)-related proteins such as p107 and p130 (reviewed by Levine *et al.*, 1991). In addition, some DNA tumor viruses also encode genes that protect cells from p53-dependent apoptosis (see later discussion).

A. SV40 Large T Antigen

Studies in transgenic mice expressing wild-type or certain mutant SV40 large T oncoproteins have revealed disruption of growth control and tumorigenesis in a wide variety of cell types in different tissues (reviewed by Hanahan, 1989; Adams and Cory, 1991). SV40 large T antigen contains two separate domains that target p53, pRB, and the RB-related proteins p107 and p130 (reviewed by Fanning and Knippers, 1992). For example, expression of large T antigen in the choroid plexus

epithelium of the brain, a nondividing epithelial cell layer that forms the blood–cerebral spinal fluid barrier, results in aggressive nonclonal development of tumors (Chen and Van Dyke, 1991). Using a battery of mutants of large T antigen, Van Dyke and co-workers investigated the regions of T antigen required for tumorigenesis in the choroid plexus (Saenz-Robles *et al.*, 1994; McCarthy *et al.*, 1994; Chen *et al.*, 1992; Symonds *et al.*, 1993). One of these mutants, designated T121, encoded a truncated version of T antigen that had lost the ability to bind pRB and RB family members but retained the capacity to bind p53 protein. This mutant T antigen protein was sufficient to induce slow-growing tumors in the brains of mice harboring a normal p53 gene (Saenz-Robles *et al.*, 1994). The contribution of wtp53 to tumor initiation was examined using homozygous and heterozygous p53 knockout mice carrying the T121 mutant T antigen gene (Symonds *et al.*, 1994). This study revealed that the suppressed growth of T121-induced tumors compared to tumors induced by wild-type T antigen was most likely due to the presence of wtp53, since aggressive tumor growth was restored when the T121 mutant T antigen was expressed in homozygous p53-null mice. In addition, in p53-heterozygous mice, loss of the wtp53 allele resulted in the emergence of rapidly growing lethal tumors. Analysis of rapid-growing versus slow-growing tumors revealed a significant difference in the percentage of cells undergoing apoptotic cell death. This study provided evidence that p53-dependent apoptosis occurring in response to oncogenic events is important in limiting tumor growth and provided a plausible explanation for how mutations in p53 could promote the development of human tumors, which is widely believed to be an oncogene-driven process (reviewed by Weinberg, 1989; Bishop, 1991).

B. Adenovirus E1A and E1B Gene Products

In has been known for a number of years that the adenovirus E1A gene products promote both viral and host cell DNA replication (Kaczmarek *et al.*, 1986; Stabel *et al.*, 1985), in part by binding to the host cell cycle regulatory proteins: pRB (Whyte *et al.*, 1988), RB-related proteins p107 and p130 (Egan *et al.*, 1988; Whyte *et al.*, 1989; Giordano *et al.*, 1991), the cyclin-dependent kinase (cdk) complexes cyclin A–cdk2 and cyclin E–cdk2 (Faha *et al.*, 1993), and p300 (Stein *et al.*, 1991). Stimulation of host cell DNA replication co-segregates with the induction of apoptosis (White *et al.*, 1991) through wtp53-dependent pathways (Debbas and White, 1993; Lowe *et al.*, 1993). E1A alone is sufficient to transform mouse fibroblasts derived from $p53^{-/-}$ knockout

mice but not fibroblasts derived from normal $p53^{+/+}$ mice, presumably because the latter undergo apoptotic cell death (Lowe *et al.,* 1994). In mouse fibroblasts harboring wtp53, E1A expression induces p53 protein accumulation (Lowe and Ruley, 1993), which may mimic the cellular response to DNA damage (Kastan *et al.,* 1992; Kuerbitz *et al.,* 1992; Lu and Lane, 1993).

Furthermore, E1A stimulates host cell DNA replication but fails to transform primary baby rat kidney cells, most likely because of the induction of apoptosis (Debbas and White, 1993). Overexpression of dominant negative mutant p53, which abrogates wtp53 function, or introduction of apoptosis inhibitors such as adenovirus E1B 19K protein, rescues cells from E1A-induced apoptosis and promotes the establishment of transformed cell lines (Rao *et al.,* 1992; White *et al.,* 1991, 1992). The E1B 19K protein was originally identified as an inhibitor of DNA fragmentation and cell death in human cells productively infected with adenovirus. In addition to preventing E1A-mediated apoptotic cell death (White *et al.,* 1991), the E1B 19K protein has also been shown to block cell death induced by different agents such as tumor necrosis factor-α and the Fas antigen (Gooding *et al.,* 1991; Hashimoto *et al.,* 1991; White *et al.,* 1992), both of which are potent inducers of apoptosis in some cell types (Laster *et al.,* 1988; Itoh *et al.,* 1991). Sabbatini *et al.,* (1995a) have shown that the E1B 19K protein enhances wtp53-mediated transcriptional activation and alleviates wtp53-mediated transcriptional repression. These investigators suggested that the ability of E1B 19K protein to alleviate p53-mediated transcriptional repression may protect cells from apoptosis resulting from transcriptional repression of cellular genes necessary for cell survival. The mechanism by which this is accomplished is at present unclear since the E1B 19K protein does not bind directly to wtp53 and is localized in cytoplasmic membranes, nuclear membranes, and the nuclear lamina (White *et al.,* 1984; White and Cipriani, 1990), whereas wtp53 protein is predominantly localized in the nucleus. Therefore, E1B 19K protein interactions with the transcriptional modulating properties of wtp53 are likely to be indirect. Taken together, these studies support the hypothesis that decreased induction of apoptosis rather than enhanced cell proliferation is an important component of the processes leading to viral oncogene-driven immortalization and transformation of primary cells.

Lowe *et al.* (1993) have shown that ionizing radiation and several anticancer drugs trigger apoptosis. In this study, wtp53 was identified as a factor required for apoptosis. Treatment of E1A-expressing cells with low doses of either γ-irradiation or anticancer drugs such as 5-fluorouracil, etoposide, and Adriamycin rapidly induced apoptosis E1A-

expressing cells while having little or no effect on the viability of normal cells or E1A-expressing cells that lacked p53. Therefore, it appears that the growth-promoting oncoproteins such as E1A may contribute to p53-dependent apoptosis by lowering the apoptotic threshold of cells. This finding has potential clinical implications since the deregulated growth of most human tumors is most likely an oncogene-driven process (Weinberg, 1989; Bishop, 1991). Therefore, a "window of therapeutic opportunity" may exist for the treatment of tumors that retain wtp53 activity or in which wtp53 activity can be restored.

C. Human Papillomavirus E7 and E6 Oncogenes

Oncogenic human papillomaviruses (HPVs) such as type 16 and type 18 encode two genes, *HPV-E7* and *HPV-E6,* which have been implicated in malignant transformation and apoptosis. In a scenario very similar to that of adenovirus E1A and E1B (55K) genes, the *HPV-E7* gene product binds to the pRB protein and RB-related proteins (Dyson *et al.,* 1989) and the *HPV-E6* gene product binds to wtp53 protein and targets it for degradation via a ubiquitin-dependent proteolytic system (Scheffner *et al.,* 1990). *In vitro* studies of human diploid fibroblasts expressing *HPV-E6* have shown that these cells exhibit altered cell cycle regulation and an increased frequency of amplication of the *CAD* gene due to their inability to arrest in G1 phase of the cell cycle following exposure to PALA (White and Gooding, 1994). In addition, introduction of the *HPV-E6* gene into tumor cells that harbor a wtp53 gene has been shown to abrogate G1 arrest induced by exposure to γ-irradiation (Slebos *et al.,* 1994). The *HPV-E6* gene has also been shown to sensitize human mammary epithelial cells to apoptosis induced by the anticancer drug mitomycin C (Xu *et al.,* 1995).

In vivo animal studies have revealed that transgenic mice expressing *HPV-E7* in the photoreceptor cells of the retina exhibited extensive apoptosis but did not develop retinoblastomas in a wtp53 background; however, in a p53-null background retinoblastoma tumors were observed (Howes *et al.,* 1994). This finding is strikingly reminiscent of the results reported by Saenz-Robles *et al.* (1994) in tumors of the choroid plexus of transgenic mice expressing a truncated SV40 large T antigen transgene. Pan and Griep (1994) demonstrated that expression of the HPV-E7 oncoprotein in the developing lens of transgenic mice induced cell proliferation in inappropriate regions of the lens and inhibited differentiation. The ultimate fate of cells so affected was elimination through a process of apoptosis. Interestingly, the capacity for E7 to mediate this response was dependent on an intact pRB binding region,

thus implying that the function of pRB (or RB-related proteins) was essential for normal lens differentiation and that the failure of these proteins to function triggered apoptotic cell death. Conversely, the HPV-E6 oncoprotein inhibited E7-mediated apoptosis. Taken together, these results suggest that the activities of E6 and E7 modulate wtp53-dependent apoptosis. Pan and Griep (1995) investigated the pathways that mediate E7 induction and E6 inhibition of apoptosis during different stages of development in the lens of transgenic animals. E7-transgenic animals null for p53 were only partially rescued from apoptosis, indicating that both p53-dependent and p53-independent pathways mediate E7-induced apoptosis in the developing lens. The *E6* transgene expressed in a p53-null background displayed an additive effect to reduce the level of E7-mediated apoptosis. This observation suggests that the E6 oncoprotein, in addition to targeting p53-dependent pathways of apoptosis, also targets p53-independent apoptotic pathways. Interestingly, during early stages of lens development apoptosis appeared to be highly p53 dependent, whereas at later stages apoptosis occurred through both p53-dependent and p53-independent pathways. The observation that E6 and E7 affect lens cells through both p53-dependent and p53-independent pathways suggests that the activities of these viral oncoproteins *in vivo* are likely to be more complex than initially envisioned.

D. Epstein–Barr Virus

Epstein–Barr virus (EBV) oncoproteins also target p53-dependent pathways of apoptosis. For example, the EBV-encoded EBNA-5 protein required for EBV-mediated transformation of B cells can complex with both wtp53 and pRB and thus may be capable of inactivating wtp53 and pRb functions in a manner similar to that of SV40 large T antigen (Szekely *et al.*, 1993). The immediate early protein BZLF1 of EBV has been shown to bind to p53 and pRB proteins both *in vitro* and *in vivo* and to inhibit the sequence-specific DNA-binding and transcriptional activity of wtp53 protein (Zhang *et al.*, 1994). Interestingly, another EBV-encoded protein, BHRF-1, which has weak homology to the anti-apoptotic protein Bcl-2 (Cleary *et al.*, 1986), can block aopototic cell death caused by growth factor deprivation in human and murine hematopoietic cells (Henderson *et al.*, 1993). Taken together, these findings indicate the DNA tumor viruses have evolved with a strategy to target the activities of tumor suppressor genes such as p53 and *RB* and to circumvent the apoptotic cell death that accompanies oncogene-driven processes that deregulate cell cycle control pathways.

IV. Coupling of p53-Dependent Cell Cycle Regulatory Pathways and Apoptosis

In normal mammalian cells, cell cycle progression is precisely regulated by the sequential formation, activation, and subsequent inactivation of a series of protein kinase complexes, each containing a catalytic subunit (the cdk), a cyclin protein, and various accessory proteins (reviewed by Sherr, 1994; Hunter and Pines, 1994). The biochemical activity of cyclin–cdk complexes coordinates key cell cycle transitions at the G1–S phase and G2–M phase boundaries to ensure the initiation and completion of sequential events required for the orderly flow of cells from one cell cycle phase to the next. The $p21^{WAF1/CIP1}$ protein encoded by the *WAF1* gene is a potent inhibitor of cdk activity (El-Deiry *et al.*, 1994; Dulic *et al.*, 1994). p53-dependent transcriptional activation of WAF1 has been shown to play a key role in p53-mediated checkpoint control of the G1–S phase transition in response to DNA damage. Forced ectopic expression of $p21^{WAF1/CIP1}$ is sufficient to produce G1 arrest in p53-deficient cells (El-Deiry *et al.*, 1994; Harper *et al.*, 1993).

Homozygous *WAF1* knockout mice ($p21^{-/-}$) were shown to be partially defective in G1 arrest following exposure to ionizing radiation (Deng *et al.*, 1995). However, in response to metabolic perturbations produced by PALA treatment, G1 arrest in $p21^{-/-}$ cells appeared to be nearly as defective as in $p53^{-/-}$ cells. In a colorectal tumor cell line in which both alleles of the *WAF1* gene were disrupted by homologous recombination, exposure to ionizing radiation or the anticancer drug Adriamycin failed completely to trigger G1 arrest (Waldmann *et al.*, 1995). Taken together, these data strongly suggest that $p21^{WAF1/CIP1}$ is an important downstream mediator of wtp53-induce G1 arrest in response to DNA damage.

A. Myc-Mediated Apoptosis Is Dependent on wtp53 Function

As mentioned previously, the regulation of normal cell proliferation involves a delicate balance between the actions of genes that promote growth, such as proto-oncogenes, and those that suppress growth, such as tumor suppressor genes. Studies have revealed a relationship between cell cycle regulatory pathways and pathways leading to apoptosis. Deregulated expression of the oncogene c-*myc* has been observed in a variety of human tumors, and overexpression of c-Myc protein in experimentally manipulated cells and transgenic mice can lead to malignant transformation and tumorigenesis (for review, see Bishop, 1991; Spencer and Groudine, 1991, Marcu *et al.*, 1992). Forced ectopic ex-

pression of c-Myc in some cases is required and sufficient to stimulate resting Go cells to enter S phase (Evan *et al.*, 1992; Wagner *et al.*, 1993).

In some cell types, such as Rat-1 cells, deregulated expression of c-Myc results in rapid apoptotic cell death in cells deprived of growth factors (Evan *et al.*, 1992). Using this model, Harrington and co-workers (1994) demonstrated that Myc-mediated apoptosis could be prevented by specific growth factors. Growth factor-mediated protection from apoptosis was not linked to the ability of the growth factors to promote cell proliferation, since growth factors that on their own were not mitogenic afforded protection from apoptotic cell death. Wagner *et al.* (1994) investigated the mechanism of Myc-mediated apoptotic cell death by introducing a conditionally active c-*myc* transgene into primary mouse embryo fibroblasts null for p53, and into fibroblasts without endogenous p53 expression but ectopically expressing a temperature-sensitive mutant p53 transgene. These studies revealed that expression of wtp53 was required for susceptibility to Myc-mediated apoptosis. In addition, although ectopic expression of wtp53 blocked cells in G1 phase of the cell cycle, G1 arrest mediated by isoleucine starvation in the absence of wtp53 did not confer susceptibility of cells to Myc-mediated apoptosis. The observations that isoleucine starvation arrested cells in G1 phase and that p53-null cells were refractory to Myc-induced apoptosis elicited by this condition suggest that, even in cells growth arrested in a p53-independent manner, wtp53 activity is still required for susceptibility to Myc-induced apoptosis (Wagner *et al.*, 1994). Interestingly, in this study Myc-mediated apoptosis was not correlated with the ability of wtp53 to transactivate the *WAF1 / CIP1* gene. This study provided evidence for the existence of regulatory pathways that couple deregulated expression of a growth-promoting cellular oncogene, c-*myc,* with p53-dependent apoptosis and also provided the first clue that an intrinsic property of wtp53 distinct from its ability to induced G1 arrest might be involved in the apoptotic process.

How does deregulated expression of Myc induce apoptosis? Early studies of Baserga and co-workers (Kaczmark *et al.*, 1985) demonstrated that c-Myc acts as a competence factor in mouse fibroblasts. For example, microinjection of c-Myc protein into quiescent G0 mouse fibroblasts has been shown to induce entry into S phase but only in the presence of progression factors such as insulin or insulin-like growth factor-I (IGF-I). BALB/c 3T3 mouse fibroblasts, which are exquisitely sensitive to growth factors contained in serum, require both competence factors such as platelet-derived growth factor (PDGF) and progression factors such as insulin or IGF-1 for a mitogenic response (reviewed by Mercer, 1993). Constitutive expression of c-Myc in this cell line abrogates the requirement for PDGF and, in the presence of in-

sulin or IGF-1, sustains the growth of these cells, at least for a short time (Travali *et al.*, 1991). Wagner and collaborators (1994) have reported that BALB/c 3T3 cells that express wtp53 are not susceptible to Myc-mediated apoptosis when deprived of serum growth factors, suggesting that in this cell line wtp53-dependent apoptotic pathways have been altered. Given the dependence of these cells on growth factors, it is remarkable that deregulated expression of c-Myc does not trigger an apoptotic response. It is likely that an unidentified genetic lesion(s) may have occurred during the establishment of this cell line that render it resistant to Myc-mediated p53-dependent apoptosis. It is interesting to note that Harvey and Levine (1991) have reported that mutations or deletions of the p53 gene were present in all clonal fibroblast cell lines established by a 3T3 passage schedule from BALB/c embryos. Thus, the BALB/c 3T3 clone A31 line may be exceptional in that it retains wtp53 and is refractory to Myc-induced apoptosis when deprived of serum growth factors (Wagner *et al.*, 1994). This could also explain why deregulated expression of c-Myc is able to abrogate the requirement for the competence factor PDGF without inducing apoptosis (Travali *et al.*, 1991).

The ability of Myc to induce apoptosis appears to require dimerization with Max (Amati *et al.*, 1993) and the N terminus of Myc protein (Evan *et al.*, 1992), which contains domains necessary for transcriptional activation (reviewed by Marcu *et al.*, 1992), suggesting that Myc/Max-mediated modulation of transcription may be involved. Yet Myc-mediated apoptosis does not require *de novo* protein synthesis since apoptosis occurs even in the presence of the protein synthesis inhibitor cyclohexamide (Wagner *et al.*, 1994). It is possible that, under conditions of serum starvation, the synthesis of the protein products of Myc-induced target genes may somehow escape inhibition by cyclohexamide. Alternatively, Myc protein may modulate other cellular genes involved in apoptotic pathways. There is a precedence for this latter idea since constitutive expression of myc in BALB/c 3T3 cells has been shown to suppress cyclin D1 expression and enhance expression of cyclins E and A (Jansen-Durr *et al.*, 1993; Philipp *et al.*, 1994). The studies of Dalla-Favera and co-workers (Gu *et al.*, 1994) have shown that Myc can interact with the RB-related protein p107, which has been implicated in transcriptional modulation and cell cycle control. Furthermore, Moses and co-workers (Dagnino *et al.*, 1995) have shown that p107 can suppress transcriptional activity directed by a number of different promoters in transient transfection assays. Although the mechanism(s) of Myc-mediated p53-dependent apoptosis in some cell types is unclear at present, there are hints that the ability of Myc to modulate transcription may be involved.

B. The pRB/E2F Connection

The product of the retinoblastoma tumor suppressor gene, pRB protein, has also been implicated in cell cycle control and apoptosis. In this case, pRB expression appears to protect cells from apoptotic cell death. For example, one of the first clues that pRB might be involved in protecting cells from apoptosis stemmed from the observation that mice carrying a homozygous knockout of the *RB* gene die prenatally, with extensive apoptotic cell death in cells of the nervous system (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). In normal cells, the pRB protein has been strongly implicated as a negative regulator of cell cycle progression, and its function in this capacity is modulated by cyclin–cdks, which phosphorylate it in a cell-cycle-dependent manner (reviewed by Sherr, 1994; Hunter and Pines, 1994; Sang *et al.*, 1995). The hypophosphorylated form of pRB, pRB^{105}, is the predominant form found in Go and early G1 phase cells. As cells progress toward S phase, sequential phosphorylation occurs, giving rise to a hyperphosphorylated form, pRB^{110}. The pRB^{105} form is growth suppressing and, when overexpressed in cells, is capable of arresting cells in G1 phase (Goodrich *et al.*, 1991). A direct link has been established between p53 and phosphorylation of pRB protein. Specifically, p53-dependent transactivation of the *WAF1* gene following exposure of human diploid fibroblasts to ionizing radiation results in increased levels of $p21^{WAF1/CIP1}$ protein associated with decreased phosphorylation of pRB, thereby rendering the latter constitutively active in growth suppression (Dulic *et al.*, 1994). Forced ectopic expression of $p21^{WAF1/CIP1}$ in p53-deficient cells is sufficient to inhibit cyclin–cdk activity, leading to accumulation of the growth-suppressing hypophosphorylated form pRB^{105} and growth arrest but not apoptosis (Zhang *et al.*, 1995).

Haas-Kogan and co-workers (1995) have investigated the involvement of pRB in apoptosis induced by exposure to ionizing radiation. Using a high-level *RB* expression plasmid transfected into the human osteosarcoma cell line SAOS-2, which is deficient in both pRB and p53, these investigators demonstrated that ectopic expression of pRB protected cells from radiation-induced apoptosis. Specifically, exposure of parental SAOS-2 cells to ionizing radiation resulted in apoptotic cell death in a time- and dose-dependent manner; however, derivative cell lines that expressed wild-type pRB exhibited a marked increase in viability and decreased apoptosis following exposure to a gradient of radiation doses. On the contrary, derivative cell lines that expressed mutant pRB did not exhibit protection from radiation-induced apoptosis. This study clearly demonstrated that the presence of a functional pRB

protein could protect cells from apoptotic cell death. Although the mechanism by which pRB protects cells from radiation-induced apoptosis is not yet clear, these investigators speculated that pRB may prevent apoptotic cell death by promoting a state of Go–G1 arrest in which cells may be less susceptible to apoptosis.

RB knockout mice presumably express a functional wtp53 protein and, given the potential link between p53-induced G1 arrest and the accumulation of the growth-suppressing form of pRb, it was possible that the loss of pRB function in cells that overexpress wtp53 protein might trigger apoptosis. Haupt *et al.* (1995) examined the possibility that pRB could protect HeLa cells from p53-mediated apoptosis. Co-transfection of wtp53 and a functional pRB expression plasmid resulted in significant protection of HeLa cells from p53-mediated apoptosis. This study suggests that *RB* plays a major role in the decision of whether cells respond to activated wtp53 by undergoing growth arrest or apoptosis. Moreover, it reveals a direct functional link between two tumor suppressor gene products, pRB and p53, both of which are involved in negative cell cycle regulation and apoptosis.

What pRB function protects cells from entering the apoptotic cell death pathway? Some clues have been forthcoming from studies of the relationship between pRB protein and the transcription factor E2F1. The E2F1 transcription factor is suspected of activating genes required for progression into S phase (reviewed by Nevins, 1992). For example, a number of cellular genes, such as dihydrofolate reductase, thymidine kinase, B-*myb*, and DNA polymerase α, contain E2F binding sites (reviewed by Farnham *et al.*, 1993). These genes are coordinately regulated during the cell cycle, being induced in mid- to late G1 at a time when active E2F1 protein accumulates (DeGregori *et al.*, 1995). It is widely believed that E2F1 activity contributes to the timing of activation of the promoters for these genes.

E2F1 is a known target of pRB and can enter into multicomponent protein–protein complexes containing cyclin A–cdk2, cyclin E–cdk2, or pRB, all of which participate in cell cycle control (reviewed by Sherr, 1994). Qin *et al.* (1994) demonstrated that forced ectopic overexpression of E2F1 in quiescent Go fibroblasts resulted in premature entry into S phase followed by apoptotic cell death. E2F1-mediated apoptosis was suppressed by coexpression of wild-type pRB or a transdominant negative mutant of p53. In contrast, coexpression of a naturally occurring "loss-of-function" pRB mutant or wtp53 failed to suppress E2F1-mediated apoptosis. This study strongly suggests that deregulated E2F1 activity leads to both growth-promoting and apoptotic signals. The involvement of wtp53 in the latter was firmly established by the studies

of Wu and Levine (1994), who demonstrated that p53-induced growth arrest could be converted to apoptosis by overexpressing E2F1. It is likely that other members of the RB family, such as p107 and/or p130, may act in a similar manner; however, this possibility remains to be tested.

V. Downstream Effector Genes of p53-Dependent Apoptosis: The Bcl-2/Bax Connection

The first negative regulator of apoptosis to be identified (Vaux *et al.*, 1988) was the cytoplasmic membrane protein encoded by *bcl-2,* the gene frequently translocated to an immunoglobulin locus in human follicular lymphomas (reviewed by Korsmeyer, 1992). Enforced ectopic expression of Bcl-2 in normal and neoplastic lymphoid cells delays or inhibits apoptosis induced by many DNA-damaging drugs and ionizing radiation (Sentman *et al.*, 1991; Strasser *et al.*, 1994; Miyashita and Reed, 1992). As mentioned previously, restoration of p53 in the murine myeloid cell line M1 induces rapid apoptosis (Yonish-Rouach *et al.*, 1991), and in this model apoptosis has been shown to be correlated with a decrease in the steady-state level of Bcl-2 mRNA and protein (Miyashita *et al.*, 1994a; Selvakumaran *et al.*, 1994). The effects of wtp53 on *bcl-2* gene expression are thought to be mediated in part by a *cis*-acting p53 negative response element located in the 5′ untranslated region of the *bcl-2* gene (Miyashita *et al.*, 1994a). Interestingly, although the p53 negative response element identified by Reed and coworkers (Miyashita *et al.*, 1994a) contains a TATAA sequence that has been implicated in wtp53-mediated transcriptional repression of other genes (see Prives and Manfredi, 1993), deletion of this sequence does not diminish the ability of wtp53 to repress transcription of heterologous reported gene constructs.

Enforced ectopic expression of Bcl-2 in a cell line with inducible wtp53 has been shown to partially block p53-dependent apoptotic cell death, at least in some hemopoietic cell lines (Wang *et al.*, 1993). In addition, rodent cells transformed by adenovirus E1A and a temperature-sensitive mutant p53 are transformed at 37°C but undergo rapid apoptosis at 32°C when p53 protein is converted to its wild-type conformation (Chiou *et al.*, 1994a,b). Ectopic expression of human Bcl-2 in this model completely suppressed wtp53-mediated apoptosis and caused cells to remain in a growth-arrested state. Shen and Shenk (1994) have suggested that a possible mechanism by which E1B 19K protein, and perhaps Bcl-2, suppresses wtp53-dependent apoptosis could be through inhibition of p53-mediated transcriptional repression.

By breeding homozygous p53 knockout mice with *bcl-2* transgenic mice and comparing the effect of loss of p53 and gain of Bcl-2 on lymphocyte survival, Strasser and co-workers (1994) found that the loss of p53 rendered both B and T lymphocytes resistant to radiation-induced apoptotic cell death, as did constitutive expression of Bcl-2. Unexpectedly, mitogenically activated lymphocytes and cycling T-lymphoma cells from p53-deficient mice exhibited significant apoptotic cell death following exposure to ionizing radiation or DNA-damaging drugs. These findings suggest that p53 may not be the only mediator of apoptotic cell death provided by DNA damage.

Mutations in the p53 gene are frequently associated with tumor progression (Fearon and Vogelstein, 1990; Sidransky *et al.,* 1992), which may reflect diminished tumor cell death due to apoptosis as a component of malignant progression. Interestingly, in a survey of hematopoietic malignancies, a significant number of tumors were found to have either p53 mutations or Bcl-2 overexpression but not both (Gaidano, 1991; Magrath, 1992). This may reflect a common pathway in which these genes act, so that there is no selective advantage to interfere with both since *bcl-2* is thought to act downstream of wtp53 (reviewed by Reed, 1994).

The existence of a Bcl-2-related gene product, Bax, that accelerates apoptotic cell death has been reported (Oltvai *et al.,* 1993; Boise *et al.,* 1993). The Bax protein is able to homodimerize and form heterodimers with Bcl-2 protein. The interaction of Bcl-2 with Bax appears to play an important role in the ability of Bcl-2 to delay or inhibit apoptosis (Yin *et al.,* 1994; Seto *et al.,* 1994). In contrast to the effect of wtp53 on Bcl-2 expression, restoration of wtp53 function in myeloid M1 cells has been shown to be accompanied by an increase in Bax mRNA and protein. The *bax* gene promoter region contains four motifs with homology to consensus p53 binding sites, and co-transfection experiments using p53-deficient tumor cells have shown that wtp53 (but not mutant p53) expression can transcriptionally activate a reporter gene that utilizes the *bax* gene promoter to drive transcription of the chloramphenicol acetyltransferase gene (Miyashita and Reed, 1995). Based on gel retardation assays, it was shown that wtp53 (but not mutant p53) bound to oligonucleotides corresponding to the *bax* promoter. Taken together, the results of this study suggests that the *bax* gene is a wtp53 primary response gene that appears to be involved in p53-dependent induction of apoptosis.

It is conceivable that p53-mediated effects on *bcl-2* and *bax* gene expression might alter the ratio of Bcl-2 and Bax proteins, thereby enhancing the susceptibility of cells to apoptosis. Figure 3 illustrates the relationship between wtp53 and the Bcl-2 and Bax proteins in cell survival and apoptosis. This idea is supported further by studies of Bax

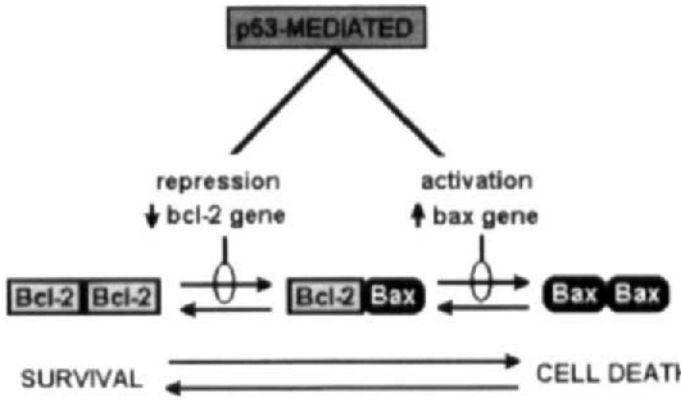

FIG. 3. Role of Bcl-2 and Bax proteins in p53-dependent apoptosis. In some cell types the choice between survival and apoptotic cell death may depend on the balance between Bcl-2 and Bax proteins. The formation of Bcl-2–Bcl-2 homodimers promotes cell survival, whereas the formation of Bax–Bax homodimers promotes cell death. Wtp53 may tilt the balance to favor the formation of Bax–Bax homodimers through its ability to repress transcription of the *bcl-2* gene and activate transcription of the *bax* gene.

protein levels in tissues from p53 knockout mice, which revealed markedly reduced levels of Bax in some tissues, such as prostate epithelium, central and peripheral neurons, and small intestine, but not in others (Miyashita *et al.*, 1994b). This finding suggests that other unknown tissue-specific factors might influence the extent to which wtp53 mediates the expression of Bax *in vivo*. Thus, it will be important to determine the molecular basis for p53-independent tissue-specific expression of Bax and its contribution to apoptotic cell death.

VI. Growth Factors, Cytokines, and Survival Factors

A. IGF-I–IGF-I Receptor Interactions

Growth factors and cytokines are continuously required to suppress apoptosis (Raff, 1992; Williams *et al.*, 1990) and are dominant to the effects of some oncogenes that induce cell death (Askew *et al.*, 1991; Evan *et al.*, 1992; Harrington *et al.*, 1994; Yonish-Rouach *et al.*, 1991). In the absence of growth factors and cytokines, some oncogenes, such as *bcl-2* (Nunez *et al.*, 1991; Vaux *et al.*, 1988), v-*src* (Canman *et al.*, 1995), and *raf* (Cleveland *et al.*, 1994), can suppress apoptotic cell death. In contrast, deregulated expression of c-Myc can accelerate apoptotic cell death when cells are deprived of survival factors (Askew *et al.*, 1991;

Evan *et al.*, 1992; Yonish-Rouach *et al.*, 1991). The effects of growth factors on E2F1-mediated apoptosis are in contrast to those on Myc-mediated apoptosis. Harrington and co-workers (1994) demonstrated that IGF-I and to a lesser degree PDGF (but not epidermal growth factor or fibroblast growth factor) could protect cells from Myc-induced apoptosis. In contrast, forced ectopic expression of E2F1 beyond a threshold was shown to override the signals transmitted by survival factors (Hiebert *et al.*, 1995).

How can growth factors such as IGF-I and PDGF protect cells from apoptotic cell death? *In vitro* experiments have clearly shown that the interaction of IGF-I with its receptor could protect cells from apoptosis induced by the anticancer drug etoposide (Sell *et al.*, 1995). In this study, exposure of BALB/c 3T3 cells, which require both PDGF and IGF-I for a mitogenic response (see reviews by Mercer, 1993; Baserga, 1995) to etoposide, induced rapid apoptotic cell death. Addition of IGF-I had only a slight suppressing effect on etoposide-induced apoptosis. However, in a derivative BALB/c 3T3 cell line that overexpressed the IGF-I receptor, enhanced survival following exposure to etoposide was observed. Baserga and collaborators (Resnicoff *et al.*, 1994, 1995) investigated the role of the IGF-I receptor in the process of apoptotic cell death *in vivo*. This study revealed that a decrease in the number of IGF-I receptors resulted in massive apoptosis in several transplantable tumors from either from humans or rodents. Conversely, overexpression of the IGF-I receptor protected cells from apoptosis. These investigators concluded that the IGF-I receptor, activated by its ligand, IGF-I, plays a critical role in protecting cells from apoptotic cell death. Interestingly, PDGF, which also protects cells from Myc-induced apoptosis, albeit to a lesser degree than IGF-I (Harrington *et al.*, 1994), has been shown to stimulate promoter activity of the IGF-I receptor gene (Rubini *et al.*, 1994). These studies strongly suggest that the interaction of IGF-I with its receptor is important in protecting cells from apoptosis and that overexpression of the IGF-I receptor potentiates this activity. Apoptosis induced by forced ectopic expression of E2F1 may also be linked to the alterations in the interaction between IGF-I and its receptor, since an E2F-like activity has been shown to repress expression of IGF-I promoter (Porcu *et al.*, 1994). Thus, disrupting the interaction between IGF-I and its receptor might be functionally equivalent to deprivation of survival factors.

B. Cytokines in Hematopoietic Cell Survival

The growth and survival of hematopoietic cells in culture is highly dependent on the presence of specific cytokines (reviewed by Raff,

1992). Cytokines such as interleukin-6 (IL-6), interleukin-3 (IL-3), and erythropoietin (EPO) can inhibit p53-mediated apoptosis in hematopoietic cells (Yonish-Rouach *et al.*, 1991, 1993; Johnson *et al.*, 1993; Gottlieb *et al.*, 1994). Exposure of myeloid M1 cells transfected with wtp53 to IL-6 effectively protects cells from p53-dependent apoptosis (Yonish-Rouach *et al.*, 1991). The protective effect of IL-6 appears to be specific for p53-dependent apoptosis, since IL-6 fails to protect parental M1 cells from cell death induced by serum deprivation (Yonish-Rouach *et al.*, 1993). Furthermore, the protective effect of IL-6 does not appear to involve the shutoff of wtp53 expression but may involve signaling pathways downstream of wtp53. Wtp53 also modulates apoptosis in normal IL-3-deprived hematopoietic cells (Blandino *et al.*, 1995). Hiebert and co-workers (1995) have shown that overexpression of E2F1 in the absence of IL-3 in an IL-3-dependent myeloid cell line designated 32D.3 induced rapid apoptosis. In this model, however, low levels of ectopic E2F1 expression were tolerated in the presence of IL-3. Studies from the Benchimol laboratory (Lin and Benchimol, 1995) have shown that murine erythroleukemia cells that carry a temperature-sensitive p53 transgene undergo apoptosis or G1 arrest when wtp53 protein is overexpressed. Interestingly, apoptosis but not G1 arrest was blocked by the action of cytokines such as EPO, c-Kit ligand, or IL-3. This suggests that p53-mediated apoptosis can be uncoupled from G1 arrest, at least in this model. This observation is also consistent with the idea that apoptosis and G1 arrest represent separate functions of p53. Another important observation from this study was revealed by analysis of two variant clones that exhibited G1 arrest but not aoptosis when the wtp53 protein was overexpressed. One of these clones secreted an EPO-like factor and the other secreted a factor with IL-3 activity, suggesting that autocrine production of cytokines is able to block p53-mediated apoptosis. If a similar scenario occurs in tumor-derived cell lines, this could explain why different cell types vary in their apoptotic response to overexpression of wtp53 protein. In total, it is tempting to speculate that the ability of different cytokines and/or growth factors to suppress apoptosis in some cell types may be linked to disruption of the inducing signal that triggers the apoptotic response.

VII. Role of p53-Mediated Modulation of Transcription

As already discussed in a previous section of this review, at the molecular level, wtp53 can both activate transcription of genes through its sequence-specific DNA binding activity and suppress transcription of

certain genes that do not contain binding sequences. What function of wtp53 is required for oncogene-driven apoptosis? Although the function of wtp53 as a transcriptional activator is tightly linked to its ability to arrest cell cycle progression, a role for p53-mediated transcriptional activation in the induction of apoptosis is controversial (Raff, 1992; Lane, 1993; Caelles *et al.*, 1994). Studies suggest that, at least in some cell types, p53-mediated transcriptional activation is not required to activate the apoptotic process. For example, Karin and co-workers (Caelles *et al.*, 1994) have demonstrated that in certain cells ultraviolet radiation-induced apoptosis is dependent on wtp53 but does not require transcriptional activation by p53. In addition, Oren and collaborators (Haupt *et al.*, 1995) have shown that the transcriptional activating function of wtp53 is not required to activate the apoptotic process, at least in HeLa cells. In this latter study it was shown that apoptosis could be induced by a truncated p53 protein deficient in transcriptional activation; however, the apoptotic response was weaker than that of full-length wtp53 protein. This observation has led to the suggestion that dual pathways of p53-mediated apoptosis exist. In this model, one p53-dependent pathway is transactivation dependent and the other is transactivation independent but could involve p53-mediated transcriptional repression. Figure 4 illustrates the proposed pathways of transcription-dependent and transcription-independent p53-mediated apoptosis. It will be of interest to determine whether this model of p53-mediated apoptosis holds true for apoptosis induced in other cell types by different agents, specifically p53-dependent Myc-mediated apoptosis and apoptosis induced by DNA-damaging agents.

While it is clear that in some cell types p53-mediated transcriptional activation is required to trigger oncogene-driven apoptosis (see, e.g., the paper of Sabbatini *et al.*, 1995b), apoptosis can be induced in other cell types by p53 proteins that are defective in transactivating function (Caelles *et al.*, 1994; Haupt *et al.*, 1995). In some cell types, p53-mediated apoptosis may be a result of transcriptional repression. It is likely that some cell types may already be poised to undergo apopotic cell death at the slightest insult. As already discussed, forced ectopic expression in some cell types induces rapid apoptosis, whereas in other cell types it appears simply to lower the threshold for an apoptotic response to occur. It is clear that in some cell types the balance between death genes (e.g., *bax*) and survival genes (e.g., *bcl-2*) plays an important role in modulating apoptotic cell death (reviewed by Reed, 1994). Wtp53 protein has been implicated in transcriptional activation of Bax and transcriptional repression of Bcl-2 (Miyashita and Reed, 1995). One can easily envision a scenario in which some transformed cells may

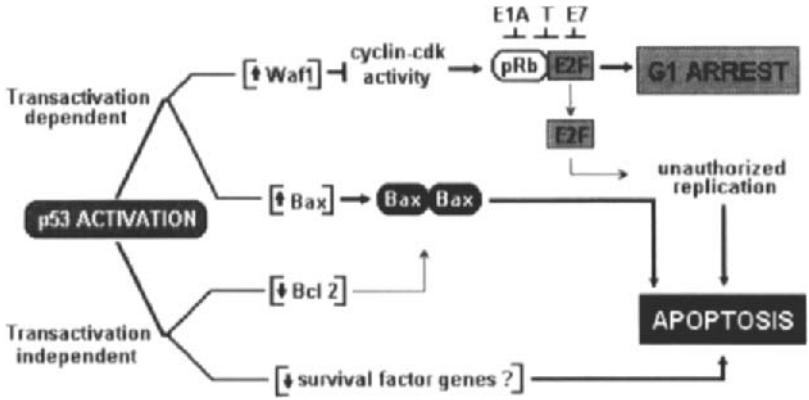

FIG. 4. Transcription-dependent and transcription-independent pathways of p53-mediated growth arrest and apoptosis. In the transcription-dependent pathway leading to growth arrest, p53-induced transactivation of the *WAF1* gene results in elevated levels of $p21^{Waf1/Cip1}$ protein and inhibition of G1 phase cdk activity. Inhibition of cdk activity leads to the accumulation of the hypophosphorylated (growth-suppressing) form of pRB protein, which sequesters the E2F1 transcription factor so that it is no longer able to transcriptionally activate E2F-dependent genes required for progression into S phase and DNA replication. Transcriptional activation of the *bax* gene results in elevated levels of Bax–Bax protein homodimers, which ultimately triggers an apoptotic response. Expression of viral oncoproteins such as adenovirus E1A, SV40 large T antigen, or HPV-E7, which are capable of liberating E2F1 from pRB sequestration, may promote an apotic response in cells by allowing unauthorized DNA replication to occur. In the transcriptional-independent pathway of p53-mediated apoptosis, repression of *bcl-2* gene expression also favors the formation of Bax–Bax protein homodimers and in some cell types may also repress the expression of survival genes.

rely on maintaining a delicate balance between the steady-state levels of Bcl-2 (or Bcl-related proteins) and Bax to avoid apoptotic cell death. Under these conditions, p53-mediated transcriptional repression of Bcl-2 in the absence of transcriptional activation of Bax may be sufficient to upset the Bcl-2–Bax balance to trigger an apoptotic response. It is also possible to envision a similar scenario in which the interactions of growth factors and their receptors tips the Bcl-2–Bax balance in favor of protection from oncogene-driven apoptosis.

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Viral Inhibitors of Apoptosis

ANTHONY G. UREN AND DAVID L. VAUX

The Walter and Eliza Hall Institute of Medical Research
Royal Melbourne Hospital
Victoria 3050, Australia

I. INTRODUCTION

Physiological cell death is a process by which cells actively participate in their own destruction. This form of cell death, often referred to as "apoptosis" or "programmed cell death," is highly conserved across metazoan phyla and is widely used to remove specific cells to a variety of ends. Induction of apoptosis occurs during development to shape the developing organism, in homeostasis of actively proliferating tissues to maintain cell number, and in response to mutagens and infection by viruses and other pathogens.

Apoptotic cell death is characterized by the condensation of chromatin to the margins of the nucleus and blebbing of the cytoplasmic membrane (Kerr *et al.*, 1972). Ultimately the cell is fragmented into apoptotic bodies that are engulfed by neighboring cells or phagocytes. By comparison, necrotic cell death is a passive process generally caused by damage or injury to the cell and is characterized by formation of microvesicles, swelling of the cell and its mitochondria, early breakage of the cytoplasmic membrane, and leakage of the cytoplasm into the intercellular space.

When infected by viruses, cells of multicellular organisms often dis-

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play the characteristic appearance of apoptosis, suggesting the physiological death mechanisms of the host have been activated. Presumably the infected cell detects foreign nucleic acid and foreign proteins, or changes in metabolism and macromolecular synthesis initiated by the virus. As a response the cell commits suicide by apoptosis. The induction of apoptosis in virus-infected SF9 cells results in a lower viral titer than is seen when the cells die of necrosis (Lee *et al.,* 1993). Apoptotic cells are fragmented into membrane-bound "apoptotic bodies," which are efficiently engulfed by surrounding cells to be degraded in their lysosomes, whereas lytic viral infection results in the leakage of cytoplasm and hence the release of viral particles. The degradation of DNA into fragments that accompanies apoptosis may also serve to degrade viral genomes (Clouston and Kerr, 1985).

A number of viral proteins that have been shown to prevent apoptosis *in vitro,* such as the baculovirus gene p35 and the cowpox response modifier A (*crmA*) gene of cowpox virus, are also known to be required for optimal virulence during *in vivo* infection (Clem and Miller, 1993; Pickup *et al.,* 1986). Similarly, the artificial inhibition of virus-induced apoptosis by the overexpression of antiapoptosis genes such as *bcl-2* and E1B 19kD allows for more persistent productive infections by Sindbis virus and human immunodeficiency virus (Levine *et al.,* 1993; Antoni *et al.,* 1995). These results demonstrate that apoptosis can be an effective mechanism for combating viral infection. Thus it is not surprising that many viruses encode a variety of genes that inhibit cell death.

The molecular effectors of apoptosis are highly conserved across metazoan phyla. Figure 1 outlines the main families of apoptotic genes identified to date. The mammalian antiapoptotic gene *bcl-2* is highly homologous to the nematode gene *ced-9.* Gain-of-function mutations in *ced-9* prevent programmed cell death during *Caenorhabditis elegans* development (Hengartner and Horvitz, 1994). When expressed as a transgene, *bcl-2* is able to complement a loss of *ced-9* function in *C. elegans* (Vaux *et al.,* 1992) and is able to protect against apoptosis induced by viral infection of insect cells (Alnemri *et al.,* 1992). A highly conserved family of apoptotic cysteine proteases have also been observed in *C. elegans,* insects, and mammals (reviewed in Steller, 1995). The prospect that all animals use the same mechanisms for cell death argues for an origin of apoptosis very early in metazoan evolution. At face value it would seem unlikely that apoptosis arose in single-celled organisms, since any cell that kills itself does not pass on its genetic material. However, it is possible that apoptosis originated in single-celled organisms as a defense against viral infection. By committing suicide, virus-infected cells may prevent the spread of virus particles to related

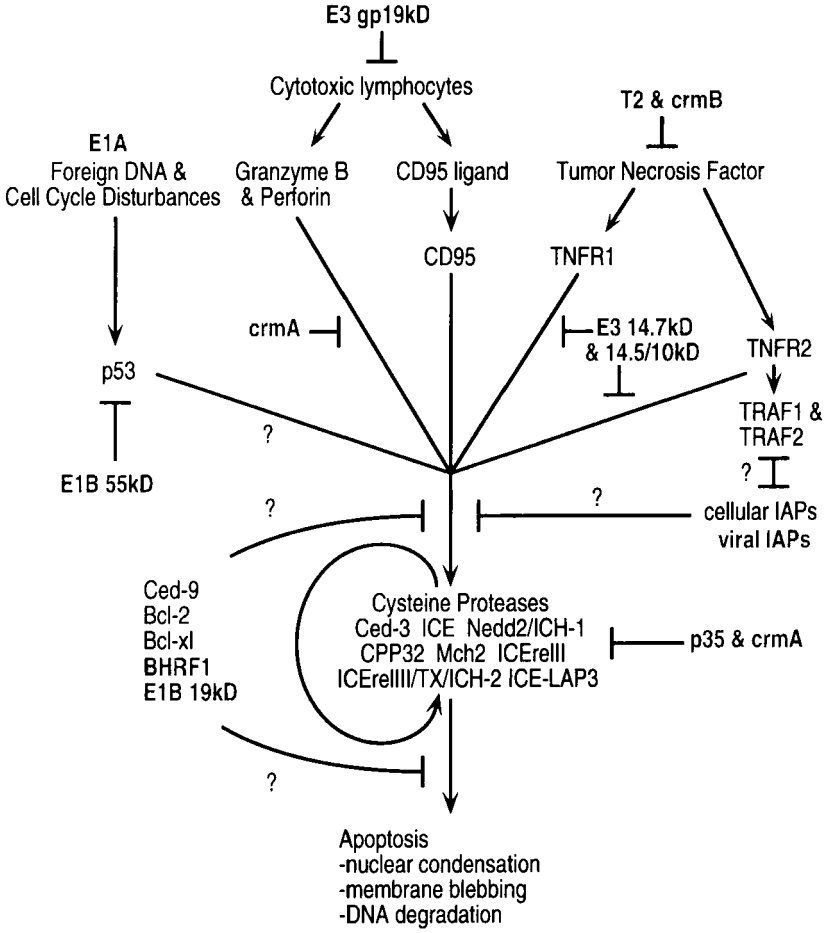

FIG. 1. The placement of viral antiapoptotic proteins with respect to cellular apoptosis pathways. Cellular genes are marked in black, viral genes are marked in grey. A variety of signals are known to induce apoptosis in response to viral infection. Viral proteins such as adenovirus E3 proteins, T2, CrmB, and perhaps CrmA appear to interfere with these signals. Induction of apoptosis generally involves the cleavage and activation of a conserved family of cysteine proteases. Viral proteins such as p35 and CrmA bind and inhibit the activity of these proteases, thus suppressing host cell apoptosis. The Bcl-2/Ced-9 family and the IAP family of apoptosis inhibitors are able to inhibit apoptosis mediated via these cysteine proteases, by as-yet unidentified mechanisms.

cells, which carry the same cell death genes. Some strains of *Escherichia coli* infected by certain viruses are able to commit suicide, and this protects other bacteria from infection (reviewed in Shub, 1994). Thus it seems possible that cell suicide genes may have been selected

for in single-celled organisms, but only if these genes increased the overall fitness of the population of cells carrying them.

While the evidence for virus-induced apoptosis in single-celled organisms remains sparse, there are numerous examples in more complex organisms. In most cases apoptotic responses to viral infection are initiated within the infected cell; that is, the death is cell-autonomous. Presumably the infected cell detects molecules such as viral nucleic acids or changes in metabolism caused by the virus and, in response, the cell kills itself. In some cases, however, an apoptotic response to viral infection may also be initiated from outside the infected cell (i.e., nonautonomous cell death or "cell murder"). An example of this is the killing of virus-infected cells by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells (reviewed in Martz and Howell, 1989).

These apoptotic defense mechanisms generate a strong selective pressure for viruses that have evolved means of preventing or circumventing host cell death. As a result, many viruses have integrated host genes into their genomes and adapted them toward the prevention of apoptosis. Thus, as with viral control of cell proliferation and metabolism, a variety of viral controls of apoptosis are employed to optimize viral fitness. As the molecular basis of apoptosis has become better characterized, an increasing number of viral genes that manipulate apoptosis have been identified. In some cases, the identification of viral regulators of apoptosis has preempted and even aided in the discovery of their cellular homologs, which also regulate apoptosis. This article reviews a number of regulators of apoptosis encoded by viruses from a variety of host organisms, detailing their discovery, their role in viral infection and replication, and in some cases the use of these genes as tools for the molecular dissection of apoptosis.

II. Viral Proteins That Resemble Bcl-2

A. BHRF1

The Epstein–Barr (EBV) gene *BHRF1* was one of the earliest virus antiapoptosis genes to be identified. When the mammalian antiapoptotic gene *bcl-2* was originally cloned, it showed closest sequence homology with the predicted open reading frame (ORF) of an EBV genomic sequence (Cleary *et al.*, 1986). The corresponding transcript was later cloned and found to encode a 17-kDa component of the restricted early antigen complex termed BHRF1 (Pearson *et al.*, 1987). Like *bcl-2, BHRF1* was shown to protect B cells against apoptosis induced by serum depletion and exposure to ionomycin (Henderson *et al.*, 1993).

As a constitutively expressed transgene in Chinese hamster ovary (CHO) cells, *BHRF1* is also able to protect against DNA-damaging agents and infection by adenovirus lacking the E1B 19kD gene (Tarodi *et al.*, 1994). *BHRF1* is not however, essential for *in vitro* replication of EBV (Lee and Yates, 1992; Marchini *et al.*, 1991).

Infection with EBV efficiently converts resting human B cells into actively cycling, immortal lymphoblastoid cell lines, and this may in part explain the close association between EBV infection status and Burkitt's lymphoma. It has been proposed that the anti-cell death properties of *BHRF1* may contribute to this immortal phenotype by conferring independence of growth factors and aiding in resistance to antitumor cytokines of the immune system.

B. E1B 19kD

Expression of the E1A transcript of adenovirus promotes progression of the host cell through the cell cycle (Braithwaite *et al.*, 1983). The virus uses E1A to activate E2F transcription factors, which promote the synthesis of host cell enzymes needed for viral replication. In doing so, E1A provokes a p53-dependent apoptotic response from the host cell. To counter this defense mechanism, adenoviruses also encode two inhibitors of apoptosis, both encoded by the E1B transcript. The shorter product of this transcript, E1B 19kD, resembles Bcl-2 and appears to be required to prevent adenovirus-induced apoptosis, since E1B 19kD-deficient mutants tend to leave host cells more susceptible to cell death (White *et al.*, 1991; Rao *et al.*, 1992). Transfection of E1A into primary quiescent rodent cells induces apoptosis, which can be blocked by expression of either E1B 19kD or Bcl-2 (Rao *et al.*, 1992; Debbas and White, 1993). Apoptosis induced by p53 is also effectively prevented by E1B 19kD or Bcl-2; however, other effects of p53, such as cell cycle arrest, are not affected by these antiapoptosis proteins. E1B 19kD can also protect against treatment with CD95 antibody and tumor necrosis factor (TNF) (Gooding *et al.*, 1991a; White *et al.*, 1992; Hashimoto *et al.*, 1991), nerve growth factor withdrawal (Martinou *et al.*, 1995), and vaccinia virus infection (Ink *et al.*, 1995).

Bcl-2 is able to block the apoptotic response to infection with adenovirus mutants lacking E1B 19kD (Chiou *et al.*, 1994). Close comparison of the E1B 19kD and Bcl-2 amino acid sequences reveals limited homology (Tarodi *et al.*, 1994) that, coupled with their functional equivalence, suggests a common origin for these proteins. Both E1B 19kD and Bcl-x_L, a Bcl-2 structural and functional homolog (Boise *et al.*, 1993), are able to bind the death-promoting protein Bak (also a member of the Bcl-2 family) (Farrow *et al.*, 1995). The binding (and perhaps

inactivation) of anti-Bcl-2 proteins such as Bak, Bax, and Bad (Oltvai *et al.*, 1993; Yang *et al.*, 1995; Farrow *et al.*, 1995; Chittenden *et al.*, 1995; Kiefer *et al.*, 1995) by E1B 19kD may be the mechanism by which it antagonizes cell death signals.

As in EBV and adenoviruses, a Bcl-2 homolog has also been identified in African swine fever virus (Neilan *et al.*, 1993). Given the strong conservation of Bcl-2 mechanisms throughout evolution, it seems likely that other viral proteins will be found that either imitate Bcl-2 or interfere with its interactions.

III. Viral Proteins That Inactivate p53

The tumor suppressor gene p53 is commonly mutated or inactivated in human cancers. It was originally identified as a protein associated with the simian virus 40 T antigen (Linzer and Levine, 1979; Lane and Crawford, 1979) and was later also shown to bind the adenovirus E1B 55kD protein (Sarnow *et al.*, 1982). p53 is a tetrameric transcription regulator that, when upregulated may either cause cell cycle arrest or induce apoptosis. A number of viruses encode proteins that antagonize p53, thus preventing p53-mediated cell cycle arrest and p53-triggered apoptosis.

Adenovirus E1B 55kD is encoded within the E1B transcript by an ORF that overlaps the ORF of E1B 19kD. Like E1B 19kD, E1B 55kD is also able to inhibit E1A-induced apoptosis, although it is not as effective as E1B 19kD (Rao *et al.*, 1992; Debbas and White, 1993). Overexpression of E1B 55kD also compensates for a loss of E1B 19kD when transforming primary baby rat kidney cells with adenovirus (Zhang *et al.*, 1992). E1B 55kD binds p53 and is able to interfere with its transcriptional activation without displacing it from its DNA binding site (Yew *et al.*, 1994). It is possible that this is the means by which E1B 55kD inhibits the p53-dependent induction of apoptosis by the E1A transcript. However, protein synthesis (and hence transcriptional activation) may not be required for p53-mediated apoptosis (Caelles *et al.*, 1994). Alternately, p53 may induce apoptosis by its ability to inhibit transcription of particular cell survival genes. Thus E1B 55kD may promote cell survival by permitting expression of p53-suppressed genes.

IV. Inactivators of Apoptotic Cysteine Proteases

Apoptotic cysteine proteases are a highly conserved component of cell death pathways. The *C. elegans* protease Ced-3 is required for pro-

grammed cell death during nematode development. A number of homologous mammalian cysteine proteases have been identified, some of which have been shown to have a role in apoptosis. The current list of mammalian homologs includes interleukin-1β converting enzyme (ICE), Nedd2, CPP32, Mch2, TX/ICH-2/ICE_{rel}II, ICE_{rel}III, and ICE-LAP3, although new members of this family are still being identified (for review see Kumar and Harvey, 1995). These proteases have a reactive cysteine residue within their active site (hence the name cysteine protease) and cleave their substrates after aspartate residues. The central role of these proteases in apoptosis makes them a convenient target for virus proteins that inhibit apoptosis.

A. CrmA

Cowpox response modifier A (*crmA*) was originally identified as a region of the cowpox genome required for the formation of hemorrhagic pocks during coxpox infection (Pickup *et al.*, 1986). Virus mutants lacking the *crmA* transcript are unable to replicate efficiently and the pock lesions formed are white rather than hemorrhagic red, due to an inflammatory infiltrate (Palumbo *et al.*, 1989). The amino acid sequence of CrmA resembles that of serine protease inhibitors (serpins). The finding that CrmA was able to prevent the processing of interleukin-1β (IL-1β) by the cysteine protease ICE suggested that CrmA might be a regulator of cytokine activity (Ray *et al.*, 1992). Thus inhibition of IL-1β production was thought to be the means by which CrmA limited the host inflammatory response to cowpox infection.

When the *C. elegans* cell death gene *ced-3* was cloned, it was found to encode a cysteine protease similar to ICE (Yuan *et al.*, 1993). The subsequent observation that transient overexpression of ICE induces apoptosis that is inhibitable by CrmA indicated that CrmA might also be a viral inhibitor of apoptosis (Miura *et al.*, 1993). Studies of association kinetics demonstrate that CrmA inhibits ICE by binding to it (Komiyama *et al.*, 1994).

When introduced into cells, CrmA is able to block apoptosis initiated by a variety of stimuli, presumably by its inhibition of ICE or ICE-like apoptotic cysteine proteases. Injection of CrmA into neurons can protect against nerve growth factor withdrawal (Gagliardini *et al.*, 1994). Expression of CrmA protects cultured cells against CD95 ligation (Enari *et al.*, 1995; Los *et al.*, 1995; Tewari and Dixit, 1995), TNF (Miura *et al.*, 1995; Talley *et al.*, 1995; Tewari and Dixit, 1995), and CTL killing (Tewari *et al.*, 1995b). The ability of CrmA to inhibit these apoptotic stimuli implicates the involvement of ICE or ICE-like cysteine proteases in each of these systems. CrmA at high concentrations *in vitro*

can also inhibit the proteolytic activity of CPP32 (Tewari *et al.*, 1995a; Nicholson *et al.*, 1995). Furthermore, CrmA has also been shown to bind and inhibit the cytotoxic T-cell serine protease granzyme B, although not as effectively as it binds and inhibits ICE (Quan *et al.*, 1995).

One of the closest cellular homologs of CrmA is the serine protease inhibitor (serpin) plasminogen activator inhibitor-2 (PAI-2). Preliminary results suggest that PAI-2, and perhaps other serpins, may have a role in cell death regulation (Dickinson *et al.*, 1995; Gan *et al.*, 1995; Jensen *et al.*, 1994). The structural and functional similarity between CrmA and PAI-2 suggests a conserved mode of action between serine and cysteine proteases and their inhibitors (Komiyama *et al.*, 1994). The existence of a number of virus-encoded serine protease inhibitors, such as *SERP1* of myxoma virus, a gene required for optimal viral virulence (Lomas *et al.*, 1993; Macen *et al.*, 1993), raises the possibility that CrmA was originally derived from a viral serine protease inhibitor and has subsequently evolved to become an inhibitor of cysteine proteases.

B. p35

The antiapoptosis protein p35 is a product of the *Autographa californica* nuclear polyhedrosis virus (AcNPV), a strain of baculovirus. It was originally identified in an AcNPVp35 mutant strain that was unable to prevent the apoptotic response of cells to infection *in vitro* (Clem *et al.*, 1991). Reinsertion of p35 into these p35-null mutants inhibited virus-induced apoptosis, increased viral yields in culture, and increased the strain's virulence *in vivo* (Hershberger *et al.*, 1992; Lerch and Friesen, 1993; Clem and Miller, 1993). A functional homolog of p35 has been identified in another baculovirus strain, *Bombyx mori* nuclear polyhedrosis virus (BmNPV) (Kamita *et al.*, 1993).

As well as preventing infection-induced apoptosis, p35 expressed as an isolated transgene was also found to protect SF9 cells against actinomycin D (Crook *et al.*, 1993). The p35 protein has also been shown to protect against developmental and γ-irradiation-induced apoptosis in the *Drosophila* eye (Hay *et al.*, 1994) and against apoptosis in *Drosophila* caused by the ectopic expression of the *hid* (head involution defect) gene (Grether *et al.*, 1995). It can also function in other phyla, as it complements *ced-9* loss-of-function mutations in the nematode *C. elegans* (Sugimoto *et al.*, 1994) and has been demonstrated to protect against a variety of apoptotic stimuli in mammalian cells. It protects mammalian neurons from glucose, calcium ionophores, and serum withdrawal (Rabizadeh *et al.*, 1993) and from nerve growth factor withdrawal (Marti-

nou *et al.*, 1995) and protects a breast cancer cell line against CD95 ligation and TNF treatment (Beidler *et al.*, 1995).

Immunochemical staining demonstrates that p35 is predominantly localized to the cytosol of infected cells (Hershberger *et al.*, 1994). The target of p35 in all these organisms is the highly conserved family of apoptotic cysteine proteases, such as ICE in mammals and Ced-3 in *C. elegans*. The ability of p35 to inhibit ICE may explain its ability to block CD95- and TNF-mediated cell death. It has been shown that p35 binds to apoptotic cysteine proteases and is cleaved at an aspartate residue and, in the process, remains irreversibly bound to the proteases, thus inactivating them (Bump *et al.*, 1995; Xue and Horvitz, 1995). The cleavage of p35 appears to be essential for its inhibitory function. The binding of p35 to cysteine proteases thus implicates cysteine proteases as being mediators of an apoptotic response to stimuli as diverse as viral infection, γ-irradiation, growth factor withdrawal, and CD95/TNF ligation. To date no cellular homologs of p35 have been identified.

V. Inhibitor of Apoptosis Proteins

Inhibitor of apoptosis (IAP) proteins are another group of baculovirus proteins that suppress apoptotic responses to baculoviral infection. *Cydia pomonella* granulosis virus IAP was originally identified based on its ability to complement a loss of p35 function in AcNPV (Crook *et al.*, 1993). Subsequently a homolog from *Orgyia pseudotsugata* NPV was also isolated using the same complementation assay (Birnbaum *et al.*, 1994). As well as being able to suppress host cell apoptosis, both these genes, like p35, are able to independently block apoptosis induced by actinomycin D. Both genes encode approximately 30-kDa products containing a C-terminal ring finger motif and two cysteine–histidine-rich repeat motifs at their N-terminus, termed baculovirus IAP repeats (BIRs).

Three other insect virus homologs of these genes have been found in *Autographa californica* NPV (termed AcIAP) (Crook *et al.*, 1993) *Chilo iridescent* virus (CIV) (Handermann *et al.*, 1992), and *Bombyx mori* NPV; however, AcIAP does not appear to have any effect on apoptosis and the CIV and *Bombyx mori* NPV IAP homologs remain uncharacterized. Complete sequencing of the mammalian African swine fever virus has also revealed an ORF containing a BIR-like repeat (Yanez *et al.*, 1995), which suggests that the use of IAPs as a defense against host cell apoptosis is a strategy employed by the viruses of a wide array of metazoan organisms.

More recently, a number of cellular homologs of these genes have been identified in both *Drosophila* and mammals, most of which contain three BIR motifs rather than two (Rothe *et al.*, 1995; Hay *et al.*, 1995; Uren *et al.*, 1996; Liston *et al.*, 1996) and one of which contains no ring finger motif (Roy *et al.*, 1995). A number of these cellular IAPs have also been shown to inhibit apoptosis (Hay *et al.*, 1995; Uren *et al.*, 1996; Liston *et al.*, 1996).

VI. Virus-Encoded Cytokine Regulators

The cytokines TNF and γ-interferon have both been shown to have antiviral properties, some of these being mediated by apoptosis. As mentioned previously, some viruses inhibit these activities by intracelluar means, such as the inhibition of TNF-induced apoptosis by adenovirus E1B 19kD. A number of poxviruses also interfere with cytokine-mediated toxicity using extracellular means, by directly interfering with ligation of cytokines to their cell surface receptors.

The T2 proteins encoded within the terminal repeats of leporipoxviruses have amino acid sequences that demonstrate striking homology with the ligand-binding domain of the p75 TNF receptor. The T2 protein of Shope fibroma virus is a secreted, soluble glycoprotein able to specifically bind TNF-α and -β (Smith *et al.*, 1991). This interaction competes with ligation of TNF to its receptors and hence effectively protects the infected cell from TNF ligation. T2 mutant myxoma virus strains replicate normally in tissue culture but are greatly attenuated when inoculated into myxoma-susceptible rabbits (Upton *et al.*, 1991). Interestingly, myxoma T2 protein protects TNF-hypersensitive L929-8 cells from TNF treatment with rabbit TNF-α but not human or mouse TNF-α. Thus myxoma T2 is specifically evolved to protect against the TNF of its natural host, the South American rabbit (Schreiber and McFadden, 1994).

The finding that particular strains of vaccinia virus also carry fragmented ORFs that appear to be remnants of T2-like genes suggested that orthopox viruses may also encode T2-like proteins (Howard *et al.*, 1991; Upton *et al.*, 1991). Subsequently, a gene located within the terminal repeats of the cowpox virus genome, cytokine response modifier B (*crmB*), has also been shown to have significant homology with the ligand binding domain of the p75 TNF receptor. Like T2, it is also a secreted, soluble protein that is able to bind both TNF-α and TNF-β in a competitive manner (Hu *et al.*, 1994). Variola virus (smallpox) also appears to encode a CrmB-like protein (Shchelkunov *et al.*, 1993; Massung

et al., 1993). Interestingly, the C termini of CrmB and T2, while showing no apparent similarity to either the p75 or p55 TNF receptors, are all homologous to each other, thus implying this region of the proteins also has a conserved function (Hu *et al.,* 1994).

Binding of γ-interferon to its receptor is known to cause growth arrest and in some cases cell death that demonstrates many of the characteristics of apoptosis. It is produced by activated T cells and NK cells and is also important for the activation of macrophages and the induction of major histocompatibility complex (MHC) markers. As with the p75 TNF receptor, a number of poxviruses also encode secreted, soluble proteins with sequence similarity to the γ-interferon receptor (Upton *et al.,* 1992; Mossman *et al.,* 1995a,b). The M-T7 protein of myxoma virus has been shown to bind and sequester γ-interferon, and hence inhibit antiviral activities mediated by its receptor (Upton *et al.,* 1992). Hence it is also possible, that like T2 and CrmB, these proteins may play a role in *in vivo* cytokine-mediated cell death responses.

VII. Other Viral Inhibitors of Apoptosis

In addition to the previously mentioned classes of viral proteins, there are a number of other viral proteins with less thoroughly defined antiapoptotic properties. One of these is the herpes simplex virus 1 (HSV1) γ_1*34.5* gene. HSV1 contains two copies of this gene, one within each terminal repeat region, and the loss of both copies of this gene renders the virus unable to prevent host cell apoptosis (Chou and Roizman, 1994). The closest cellular homologs of this gene known are *gadd34* and *MyD116,* which were discovered by their increased expression in response to induction of apoptosis by γ-irradiation and induction of terminal differentiation by IL-6, respectively. Preliminary results suggest these genes are involved in growth arrest and apoptosis induced by DNA damage (Zhan *et al.,* 1994).

Adenovirus infection of mouse cells increases sensitivity to TNF-mediated cytolysis. This sensitivity is suppressed, however, by proteins encoded by the E3 transcript (Gooding *et al.,* 1988). Three E3-encoded proteins, E3 14.7kD and a complex of E3 14.5kD–E3 10kD, are able to inhibit TNF-mediated lysis in cell culture (Gooding *et al.,* 1988, 1991b). The protection of E3 14.7kD against TNF has also been demonstrated *in vivo.* Recombinant vaccinia viruses expressing the TNF-α gene have an attenuated phenotype when infecting mice. However, similar recombinant viruses expressing both TNF and adenovirus E3 14.7kD protein have their virulence partially restored when infecting BALB/c (Tu-

fariello *et al.*, 1994a) and severe combined immunodeficiency mice (Tufariello *et al.*, 1994b). The mode of action of these proteins is unclear, although it is known that the 14.7kD and 10.4kD–14.5kD proteins inhibit the release of arachidonic acid induced by TNF (reviewed in Wold, 1993). Eicosinoids produced from arachidonic acid are mediators of inflammation, hence the E3 proteins may block inflammatory responses to adenovirus infection.

Another protein of the adenovirus E3 transcript, E3 gp 19kD, indirectly inhibits CTL cytolysis (Rawle *et al.*, 1989). This protein is localized to the endoplasmic reticulum and binds class I antigens of the MHC, thus retaining them in the endoplasmic reticulum and preventing presentation of antigens at the cell surface. It is thought that CTLs are consequently unable to recognize and lyse infected target cells, due to a lack of antigen presentation (reviewed in Wold and Gooding, 1989).

The Chinese hamster ovary host resistance (*CHOhr*) gene of cowpox appears to be involved in the replication of cowpox virus in CHO cells (Spehner *et al.*, 1988). Vaccinia virus, which lacks an equivalent of *CHOhr,* quickly induces apoptosis when infecting CHO cells. However, recombinant vaccinia virus expressing a copy of the *CHOhr* gene is able to delay the infected cell's apoptotic response and hence replicate in CHO cells (Ink *et al.*, 1995). Similarly, the E2 glycoprotein of Sindbis virus appears to be required to prevent apoptosis in infected cells. Mutation of a single amino acid within this protein results in a strain with a neurovirulent phenotype that, unlike avirulent strains, induces apoptosis in cell lines expressing Bcl-2 (Ubol *et al.*, 1994). The mode of action for both CHOhr and E2 glycoprotein are as yet unknown.

Some of these genes may encode proteins that specifically interact with cellular death mechanisms. Others may act more indirectly by transcriptionally regulating cell death genes, or even by directing cell metabolism and second messenger levels away from conditions favorable for apoptosis.

VIII. Conclusions

Viruses manipulate the host cell cycle and macromolecular synthesis in order to facilitate their own replication. Multicellular organisms have evolved means of detecting these changes and responding by induction of apoptosis. It is clear that viral inhibition of this response improves viral fitness. It is not always clear, however, to what extent particular viral proteins are involved in *in vivo* inhibition of apoptosis. Proteins such as BHRF1 and E1B 19kD appear to act as general sup-

pressors of apoptosis. Other virus-encoded proteins, however, may act on more than one level. A variety of viral regulators of p53 have been identified, but it is difficult to evaluate the relative importance of their effects on apoptosis versus their effects on the cell cycle. CrmA inhibits apoptosis via its inactivation of cysteine proteases, thus presumably allowing more time for viral replication. The ability of CrmA to prevent inflammation and pock formation, however, might be more dependent on its ability to inhibit production of mature IL-1β.

Numerous human pathogens, such as EBV, HPV, adenovirus, herpes simplex virus, and poxviruses, all manipulate host cell apoptosis. Infections of many of these viruses currently remain untreatable or incurable. The tumorigenicity of a number of viruses has been linked to their ability to prevent cell death, since the antiapoptotic proteins encoded by them contribute to the immortal phenotype of cancer cells. Furthermore, some of these viral proteins (such as the BHRF1 protein) also confer upon cells resistance to chemotherapeutic agents that act by induction of apoptosis. An improved understanding of viral regulators of apoptosis has contributed to our understanding of the molecular basis of cellular apoptotic pathways. Ideally, this knowledge will eventually also be useful in devising new strategies for the treatment of virus infection and associated oncogenesis.

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