Bcl-2 and adenovirus E1B 19kDA protein prevent E1A-induced processing of CPP32 and cleavage of poly(ADP-ribose) polymerase.

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Short title: Bcl-2 prevents CPP32 activation.

July, 1996

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements of the degree of M.Sc.

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Abstract

The E1A oncoproteins of adenovirus type 5 are potent inducers of apoptotic cell death. To manifest growth promoting properties, therefore, E1A requires the co-expression of a suppressor of apoptosis. During normal viral infection, this function is provided by the E1B 19kDa protein. However, the cellular suppressor Bcl-2 can substitute for 19K during infection, and both proteins can effectively cooperate with E1A to facilitate transformation of primary cells in culture. How E1A induces apoptosis and at what point(s) on the pathway Bcl-2 and E1B 19K act at are not presently known. Here, we demonstrate that E1A-induced apoptosis is accompanied by specific endo-proteolytic cleavage of poly(ADP-ribose) polymerase (PARP), an event that is linked to the Ced-3/ICE apoptotic pathway in other systems. PARP cleavage was also observed in p53-null cells infected with 19K- virus expressing 13S E1A. In addition to PARP cleavage, expression of E1A caused processing of the zymogen form of CPP32, a Ced-3/ICE protease that cleaves PARP and is required for apoptosis in mammalian cells. These events were prevented when E1A was co-expressed with E1B 19K or BCL-2, which places these suppressors of apoptosis either at or upstream of processing of pro-CPP32.

Résumé

Les protéines oncogéniques E1A de l'adénovirus (type 5) sont de puissants inducteurs de la mort cellulaire programmée (apoptose). Afin de promouvoir la croissance cellulaire, E1A requiert donc la présence d'une autre protéine virale, E1B-19K, permettant d'inhiber l'apoptose. Son homologue cellulaire, la protéine Bcl-2, peut se substituer à E1B-19K lors de l'infection virale et permettre la survie cellulaire. Ces protéines possèdent donc une activité similaire permettant de contrer l'apoptose médiée par E1A, l'une et l'autre pouvant également coopérer avec E1A et promouvoir la transformation de cellules primaires en culture. Le(s) mécanisme(s) responsable(s) de l'apoptose médiée par E1A de même que le(s) mode(s) d'action de E1B-19K/Bcl-2 demeurent cependant obscurs. Le pésent travail démontre que l'apoptose médiée par E1A conduit à l'inactivation de la poly (ADP-ribose) polymérase (PARP) dans des fibroblastes en culture, un événement lié à l'activation du sentier Ced3/Ice dans plusieurs systèmes cellulaires. L'inactivation de PARP se produit indépendemment de la présence de p53. De plus, l'expression de E1A provoque l'activation de la forme latente de CPP32, une protéase de type Ced3/Ice ayant la capacité d'inactiver PARP et dont l'activité est requise à l'apoptose chez les cellules de mammifères. Ces modifications enzymatiques peuvent être complètement bloquées par la co-expression de E1B-19K ou de Bcl-2, de pair avec E1A. Les résultats obtenus suggèrent donc que l'intervention de ces inhibiteurs de l'apoptose pourrait se situer en amont ou directement au niveau de l'activation de la protéase CPP32.

Preface

In accordance with the regulations described in item 3 of the Guidelines concerning thesis preparation of McGill University Faculty of Graduate Studies and Research, as cited in full below, and as approved by the Department of Biochemistry, one published manuscript has been incorporated into this thesis.

> "Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.
>
> If this option is chosen, **connecting texts that provide logical bridges between the different papers are mandatory.** The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.
>
> The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". **The thesis must include:** A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.
>
> Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers. **Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis.**"

Chapter 2: Boulakia, CA, Chen, G, Ng, FWH, Teodoro, JG, Branton, PE, Nicholson, DW, Poirier, GG and GC Shore. (1996) Oncogene. 12:529-535.

In this publication, G. Chen and J. Teodoro supplied the cell lines, F. Ng supplied some reagents, and G. Poirier and D. Nicholson supplied the required antibodies. Other than these exceptions, all work described in this manuscript is entirely my own.

Chapter 1: Introduction

History of apoptosis.

Apoptosis was first characterized over thirty years ago in histochemical studies of cells undergoing hepatic ischemia. Microscopic examination of these tissues revealed two sub-populations of dying cells. The first was a traditional necrotic cell population, and featured morphology characteristic of this type of death, including swelling of organelles, clumping of chromatin into random masses, membrane rupture, and disintegration of large cellular populations, seen as patches, or large dead areas of tissue. The second population of cells had never previously been characterized, and appeared as small, rounded cytoplasmic masses, often containing pycnotic chromatin. Though these cells were clearly dying, their death appeared quite different than that of the necrotic cells, not only because of their morphology, but also because two features characteristic of necrosis were notably absent: these cells exhibited no inflammatory response, and their lysosomes did not appear ruptured. Also, these rounded, dying cells appeared on an individual basis, and not in large masses: the neighbour of a dying cell could appear quite healthy (Kerr, et, al., 1972; Kerr and Harmon, 1991).

Since this process was quite different than classical necrosis, it was considered a sub-category, and termed 'shrinkage necrosis', to delineate its unique morphological characteristics.

Closer microscopic investigation of the cells undergoing shrinkage necrosis revealed membrane enclosed fragments within cells, containing what appeared to be intact organelles as well as compacted chromatin. Hallmark features of these cells included the condensation of nuclear chromatin into a dense mass, condensation of cytoplasm, 'blebbing' of the plasma membrane into membrane-enclosed cytoplasmic fragments that could be taken up by neighbouring cells through phagocytosis, and little disruption of the neighbouring tissue architecture (Kerr and Harmon, 1991; Kerr, et, al., 1972).

Over the years, characterization of the biochemical properties of 'shrinkage necrosis' revealed it to be quite unique: *de novo* protein synthesis was an inherent part of it - certain specific proteins such as sulfated glycoprotein-2 (SGP2) were consistently found to be upregulated both at the transcriptional and translational level during the early stages of death through this process - so consistently, in fact, that this protein could be used as a marker for this type of death (May and Finch, 1992; Michel, et, al., 1992). A second hallmark of shrinkage necrosis also became evident: the double stranded cleavage of nuclear DNA into fragments of multiples of approximately 185 base pairs (bp) (Afanas'ev, et, al., 1986). This cleavage appeared to occur at intervals between nucleosomes, since each nucleosomal unit was approximately this length, and was the cause of the chromatin condensation morphology (Wyllie, et, al., 1984). DNA cleavage was found to be an active cellular process, caused by the activation of an endogenous endonuclease (nuc-1 in *c. elegans*) (Ellis and Horvitz, 1986), and offered a

simple assay for shrinkage necrosis, where one could extract DNA from the cells in question, and separate it electrophoretically on an agarose or polyacrylamide gel to look for the characteristic DNA laddering pattern resulting from these cleavages.

The biochemical features of necrosis were quite different, and did not include *de novo* protein synthesis, or in fact any active involvement of the cell. In fact, necrosis seemed quite random, especially when compared to the ordered death of shrinkage necrosis. The two processes seemed so different, shrinkage necrosis comparing more easily to a form of 'reverse mitosis' than its namesake, that a new name was given to it, highlighting its distinction, as well as its' unique putative role *in vivo* - apoptosis, from the Greek words *apos*, meaning 'to detach', and *ptosis*, or 'petals' and eluding to the dying of leaves on a deciduous tree to protect its' branches from the weight of the winter snow (Kerr, et, al., 1972).

'Cellular self destruct'.

Apoptosis has been likened to this shedding of leaves in many ways - it appears to be an organized, genetically regulated cellular self destruct mechanism, that, when triggered, kills sub-populations or individual cells to decrease the risk to the rest of the organism. It is used to combat disease and infection and to regulate T cell number (Terai, et, al., 1991), to kill the cells between fingers during embryogenesis (digit formation) (Baer, 1994), as well as to regulate the size of organs (androgen-dependent prostate

tissue involution) (Evans and Chandler, 1987). It has been implicated in countless diseases, either through inappropriate inhibition leading to uncontrolled growth and tumour mass formation (Hodgkin's' Disease, B-cell lymphomas, etc.) (Kerr, et, al., 1994; Kadin, 1994; Hollowood and Macartney, 1991), or inappropriate triggering leading to uncontrolled death in populations of cells (AIDS (Gougeon, et, al., 1993), Alzheimer's (Smale, et, al., 1995), alopecia (Norris, et, al., 1995), amyotrophic lateral sclerosis (Lou Gherig's Disease, or ALS) (Yoshiyama, et, al., 1994), cellular damage due to cardiac stroke (Krajewski, et, al., 1995), sunburn (Schwarz, et, al., 1995), etc.). It has also been found to be a response to radiation therapy as well as to many forms of chemical cancer therapy (Milas, et, al., 1994). Control of the apoptotic process is also 'hijacked' by many viruses, both to counteract normal defense systems that trigger the process to defend the organism (Chiou, et, al., 1994), and for the killing of the cells at a later phase in the virus cycle (Rao, et, al., 1992).

'The golden egg'.

Much research has been oriented towards the biochemical pathways of apoptosis, or programmed cell death, as it is also termed. The elucidation of a 'death pathway' could lead to countless targets for drug design for combating most of today's major diseases, and has become a lucrative investment choice for many pharmaceutical firms as well as for scientists interested in linking their research to applied medical fields in order to

gain relevancy, or because they must in order to satisfy a public interested in research based on disease.

The research has also been a focus of attention because of the interesting characteristics of this new death pathway; it has attracted many basic research scientists interested in the mechanisms of cell life and death.

Bcl-2.

A gene first isolated as part of a translocation event in human B-cell follicular lymphoma (Silverman, et, al., 1990), Bcl-2 came to the forefront because, unlike previously characterized oncogenes, it appeared to work by allowing cells to live longer rather than by making cells divide faster (Hockenbery et, al., 1990). The end result was the same - more cells - but the mechanism of action appeared quite different. Instead of promoting growth and replication, the oncogene simply prevented the cells from dying, offering the cellular equivalent to a fountain of eternal youth. However, this fountain, like so many others, was flawed: the cells could live longer, and potentially forever, but they were not rendered immune to the mutations that accumulate with age. As well, these cells were kept alive, but often at the cost of the organism - there was often a good reason for the triggering of an auto-destruct process.

Studies with Bcl-2 have shown that its overexpression can prevent the apoptotic death of cells induced by almost any agent, including viral

infection (Levine, et, al., 1993), all sorts of drug treatment (Miyashita and Reed, 1992; Chen, et, al., 1995), UV irradiation (Martin, et, al., 1995), glucocorticoid treatment (Miyashita and Reed, 1992), most forms of endogenous cellular signal (Baffy, et, al., 1993; Yang, et, al., 1995), and oxidative damage (Korsmeyer, et, al., 1995), to name a few. This discovery implicated Bcl-2 downstream in the apoptotic pathway, past the convergences of these widely varied and biochemically divergent routes (Reed, 1994).

Bcl-2 knockouts.

One can gain much insight into the workings and importance of a protein by looking at what happens in animals deprived of it. Homozygous loss-of-function mutations (knockouts), traditionally in mice, offer this insight by providing mice lacking the gene in question. Phenotypically, knockout mice for Bcl-2 (Veis, et, al., 1993) appeared normal at birth. However, upon maturation, these mice quickly showed symptoms consistent with the proposed function of Bcl-2 - that of preventing selected cells from dying. They acquired polycystic kidney disease, a general immune function failure due to the death of mature T and B cells through apoptosis, hair hypopigmentation due to inappropriate apoptosis in melanocytes, as well as distortion of the small intestine, due to the apoptotic death of crypt cells. These mice could, however, function in a relatively normal fashion, and progressed through development with this mild phenotype, including a seemingly normal nervous system, intestines, and skin, suggesting

possible redundancy in the role of Bcl-2, and the probability of a Bcl-2 gene family (Veis, et, al., 1993).

Bcl-2 gain-of-function mutations resulted in mice with the same phenotype as that responsible for the discovery of the gene - a susceptibility to cancer, particularly b-cell lymphomas (Katsumata, et, al., 1992).

Bcl-2 family members and a possible regulatory cascade.

Bcl-2 has rapidly become the head of a large gene family. Family members contain three homologous regions (White, 1996), termed BH1 BH2, and BH3, for Bcl-2 homology region 1, 2 and 3, respectively; the family members known to date are shown in Figure 1. Interestingly, Bcl-2 homologues appear to belong to two subgroups: those that, like Bcl-2 appear to repress or inhibit apoptosis which, to date include Bcl-X_L (Boise, et, al., 1993), A1 (Lin, et, al., 1993) and Mcl-1 (Kozopas, et, al., 1993); and those that have the opposite effect and accelerate or instigate apoptosis, including Bcl-X_S (Boise, et, al., 1993),Bax (Oltvai, et, al., 1993), Bak (Farrow, et, al., 1995), Bad (Yang, et, al., 1995), and Bik1 (Boyd, et, al., 1995).

Bcl-2 has been shown to homodimerize (Hanada, et, al., 1995), as well as heterodimerize with other family members (Yang, et, al., 1995; Farrow and Brown, 1996), summarized as known to date in fig. 2, with instigators of apoptosis in grey and repressors in white. This complexity of dimerization implies a possible regulatory cascade, where levels of homodimerization vs.

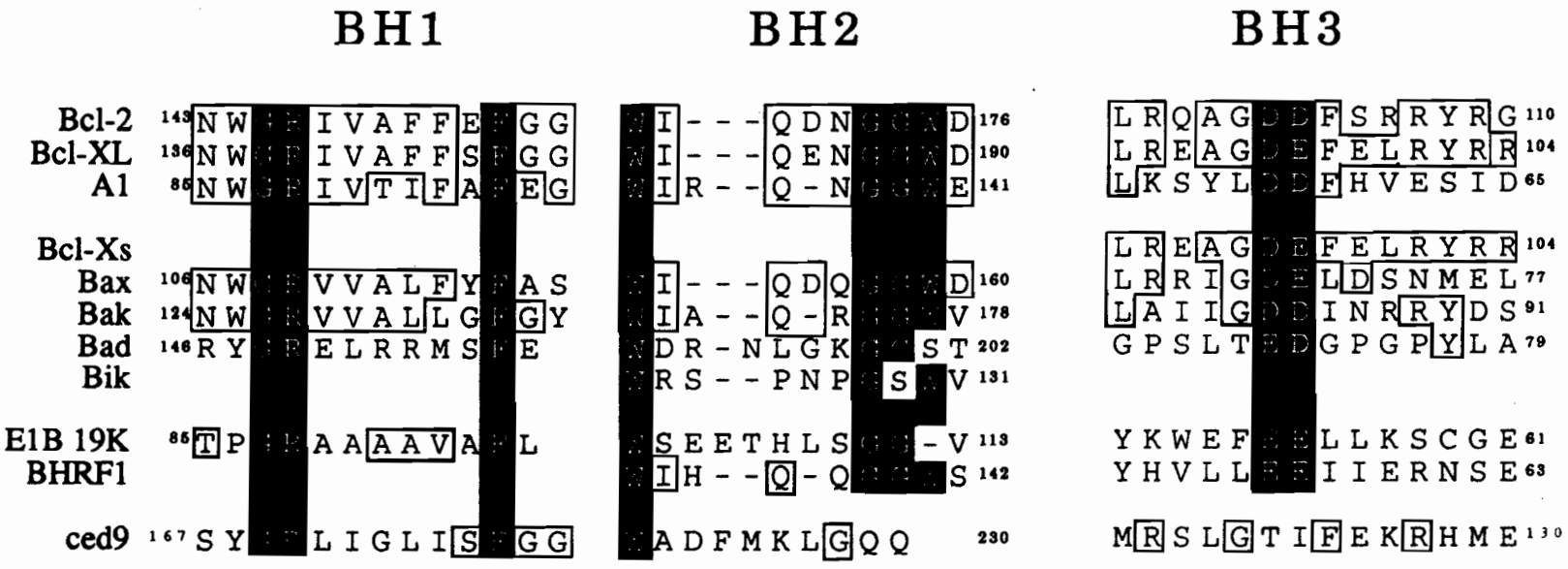

Figure 1: Bcl-2 family members and homologies

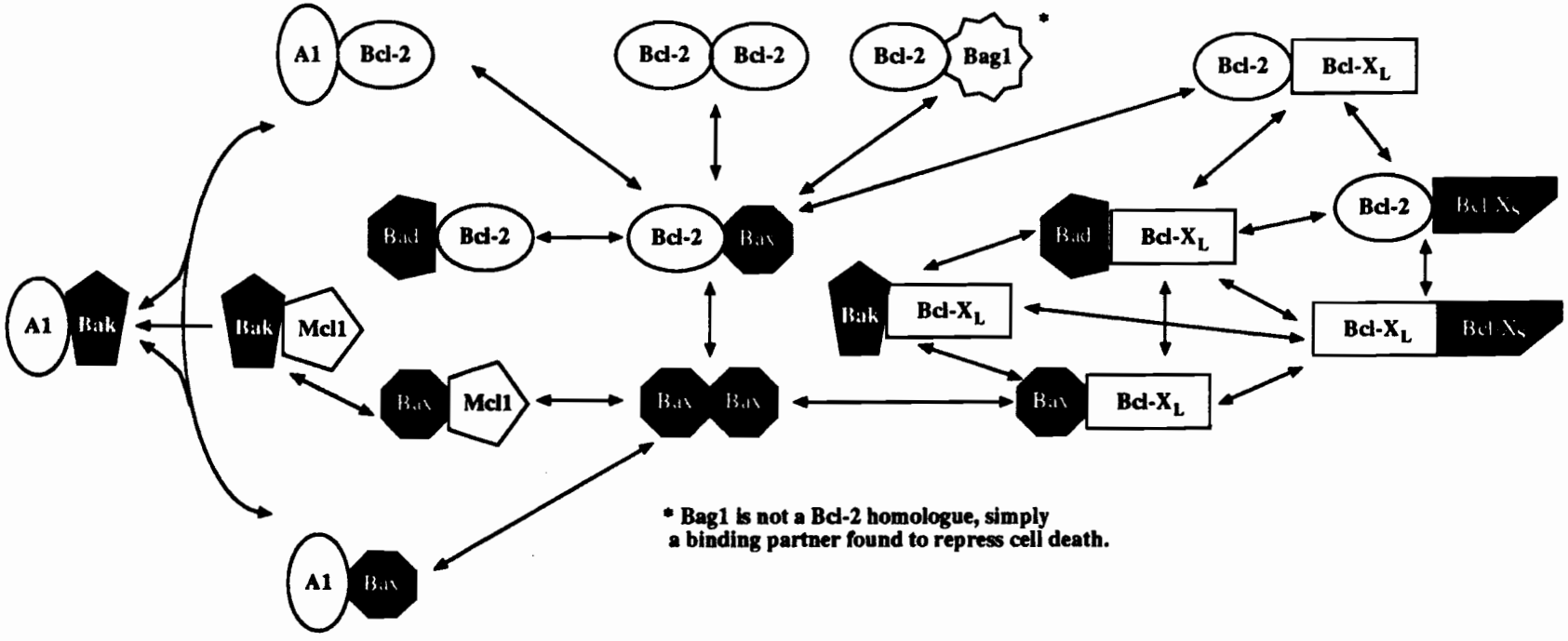

Figure 2: Bcl-2 family member dimerizations.

heterodimerization with different partners would mean the difference between life and death of the cell. Because of the complexity of the cascade, the incompleteness of the protein family, and the lack of known function for any of the family members, it is as yet unclear as to which of the family members simply act as regulators of the family, and which have a function or target elsewhere - even whether death is actively turned on by a Bax-like protein, or actively turned off by one like Bcl-2 is unclear. However, it has been shown that the levels of Bcl-2 are only relevant when compared to the levels of other Bcl-2 like proteins such as Bax and Bad, and that the reverse is also true - that is, Bax overexpression can block Bcl-2 action, and Bcl-2 overexpression can block Bax (Oltvai, et, al., 1993; Chen, et, al., 1996).

Interestingly, a knockout of Bax (Knudson, et, al., 1995) proved viable, with a phenotype that included hyperplasia in thymocytes and B cells, and excess granulosa cells in the ovaries. Bax deficient males proved to be infertile due to disordered seminiferous tubules, no sperm maturation, giant multinucleated cells, and rampant cell death in the male reproductive system. This duality of cell death and prevention of cell death, depending on the tissue, may be important when taken in combination with the dimerization of Bcl-2 family members shown in fig. 2 - Three possibilities can be seen: The knocking out of Bax may upregulate other Bcl-2-like proteins in a tissue specific manner; Bax may occur both as an activator, and a repressor of death, depending on the tissue; or Bax may be a regulator of apoptosis only, without an active role in the death process apart from sequestering and dimerizing with other Bcl-2 family members.

Bcl-X knockout mice (Motoyama, et, al., 1995) proved lethal at embryonic day 13, suggesting that the two Bcl-X gene products may be the most important Bcl-2 family members for suppression and regulation of death during development.

Bcl-2 mimics and viral family members.

Family members of Bcl-2 appear in viral lines - Epstein Barr Virus BHRF1 (EBV BHRF1) (Lee and Yates, 1992), African swine fever virus HMW5-HL (ASFV HMW5-HL) (Neilan, et, al., 1993) and Adenovirus early 1b promoter region 19K protein (E1B 19K) (Chiou, et, al., 1994). These proteins are triggered early in the infection cycle, and appear to be used by the virus to prevent cellular apoptosis triggered by the presence of the virus. In this way, the virus can replicate and take control of the cellular systems without the cellular self-destruct mechanism being activated.

E1B 19K has been used in functional complementation studies with Bcl-2, and has been shown to block Bax induced apoptosis (Han, et, al., 1996). E1B 19K has also been shown to dimerize, or at least interact with Bak (Farrow, et, al., 1995) and Bax (Han, et, al., 1996), suggesting that it acts at the same general portion of the apoptotic pathway as Bcl-2. Also, Bcl-2 complementation of E1B 19K has shown that it can substitute for the latter in the viral replication cycle (Chiou, et, al., 1994).

The adenovirus E1A region also contains transformation properties that appear to depend on both the complexing of adenovirus proteins with various cellular proteins, and the ability to repress apoptosis. The same adenovirus proteins that, in the presence of E1B 19K, trigger transformation, also trigger apoptosis when the E1B 19K protein is not expressed (Evan, et, al., 1995).

Bcl-2 structure.

Bcl-2 is a membrane protein, with one putative transmembrane region, at its' carboxyl terminus (Tanaka, et, al., 1993). This transmembrane region contains a predicted signal anchor sequence consistent with targeting to the mitochondrial outer membrane, and inserts into this membrane, with the bulk of the protein facing the cytosol, in *in vitro* import assays (Nguyen, et, al., 1994). Deletion of the signal anchor sequence of Bcl-2, converting the protein to a soluble, cytosolic form, was found to diminish its apoptotic repression activity (Nguyen, et, al., 1994).

Immunofluorescence staining of Bcl-2 in cells overexpressing it shows a pattern of expression consistent with mitochondrial localization, as well as staining in the endoplasmic reticulum (ER) and nuclear membranes (Krajewski, et, al., 1993). However, it is as yet unclear whether or not the protein exists in the latter two regions at normal metabolic levels - this may be due to overexpression of the protein, and 'spillage' of excess protein into other cellular membranes, since the protein has no recognizable ER or

nuclear targeting information. This 'spillage' may be important, however, and may contribute to the proteins' role when overexpressed in the case of a chromosomal translocation - this possible change in localization may be responsible for Bcl-2 death repression activity.

The crystal structure to Bcl-2 has not yet been elucidated, however, the x-ray and NMR structure to the Bcl-2 family member Bcl-X_L has recently been determined (Muchmore, et, al., 1996), and has shed some light on the possible functional role of this family. Bcl-X_L exist in two hydrophobic α-helices, surrounded by amphipathic helices, and resembles the structure of many bacterial toxins including the membrane insertion domain of diphtheria toxin, a molecule thought to dimerize to form a pH dependent membrane pore (Muchmore, et, al., 1996).

One can easily see a regulation system where Bcl-2 family pore formation may be a regulator of apoptosis that would depend on the family members dimerized - if Bax is homodimerized, the pore exists and allows a given substance (or the whole mitochondrial electrochemical gradient in cooperation with an inner mitochondrial membrane uncoupler) to dissipate, contributing to or causing the death of the cell. If Bcl-2 is in excess of Bax, this dimerization may not occur, the cell favouring Bcl-2:Bax heterodimers, which may not allow such a pore to form.

Of course, this hypothesis is largely speculative, with only a structural homology between Bcl-2 family members and diphtheria to go on, but it

offers an alluring answer to the question of function of Bcl-2. This explanation does nothing, though, to address the upstream or downstream components of the death pathway, only Bcl-2 family function.

Mitochondrial electrochemical gradient and reactive oxygen species.

This recent structural tie to function increases the mounting evidence linking Bcl-2 to various reactive oxygen species (ROS), and the enzymes that regulate them. ROS are predominantly found in the mitochondria, as a 'side product' of the electron transfer chain, and thus are proximal to Bcl-2 (Hockenbery, et, al., 1993). Three enzymes are involved in the homeostasis of ROS - Superoxide dismutase, involved in the conversion of O_2^- to hydrogen peroxide (H_2O_2), and glutathione peroxidase and catalase, which convert H_2O_2 to water (Hockenbery, et, al., 1993).

Superoxide dismutase 1 (SOD1) knockout mice develop symptoms and pathology similar to ALS, and appear to be a consistent mouse model for this disease (Rosen, et, al., 1993). Overexpression of Bcl-2 in SOD null cells increased resistance to H_2O_2-induced apoptosis, and upregulated the levels of catalase and peroxidase. Bcl-2 overexpression has also been found to prevent apoptosis induced by glutathione depletion (Kane, et, al., 1993); this depletion usually causes an increase in ROS and lipid peroxides before the death of the cell, these increases were prevented in the Bcl-2 overexpressing lines. Bcl-2 has also independently been found to generally decrease the generation of reactive oxygen species (Kane, et, al., 1993). All of these

findings put together appear to link Bcl-2 quite intimately to the ROS pathway.

This hypothesis is compelling, but this theory is not without its' opponents and opposite findings: Bcl-2 can prevent or inhibit cell death both in cells lacking respiration (Jacobson et, al., 1993), and in the presence of near-anaerobic (low oxygen) conditions (Jacobson and Raff, 1993), two environments diminishing the probability of ROS existence, hence marginalizing their role in the death process. This may mean that Bcl-2 acts downstream of the ROS, or, more likely, in a parallel pathway, rather than directly at their level.

A second hypothesis is that Bcl-2 family members are involved in the regulation of calcium, or are themselves mitochondrial calcium channels. An increase in intracellular Ca^{++} has been found to precede the rise in ROS when a cell is undergoing apoptosis, and experiments where the intracellular Ca^{++} levels have been raised artificially have resulted in the death of the cell in a mechanism that is both Bcl-2 repressible and that involves a rise in ROS (Lam, et, al., 1994).

Evolutionary conservation and model systems.

Much genetic work is done in *Caenorhabiditis elegans (C. elegans),* due to the organisms' easily manipulatable nature, fixed pattern of cell divisions, migrations, and deaths, short generation times, large number of offspring,

invariable anatomy, and small size (Ellis and Horvitz, 1986). Embryogenesis in this small nematode includes the apoptotic death of a total of 131 (approximately 13%) of its cells (Hengartner and Horvitz, 1994). Using genetic studies with mutant nematodes, nine genes were found to be involved in this apoptotic process, and were named ced-1 through ced-9, for cell death abnormal proteins one through nine (Ellis and Horvitz, 1986). The gene nuc-1 was also found to be involved in the death process, proven by its' mutation, resulting in a nuclease deficient nematode resistant to apoptosis (Ellis and Horvitz, 1986). One of the ced proteins, ced-9, was found to have significant structural homology to Bcl-2 (Hengartner and Horvitz, 1994). The functional role of this protein also seemed to parallel that of Bcl-2: overexpression of ced-9 resulted in an average of nine to eleven less than the required amount of cells dying during maturation; knocking out of the protein caused inappropriate cell death in other, neighboring cells, including widespread death in post-embryonically derived neurons of the ventral nerve cord (Hengartner, et, al., 1992). Bcl-2 was found to efficiently replace ced-9 in complementation studies in the *C. elegans* system (Hengartner and Horvitz, 1994), not only implying that the two proteins act in the same manner, but also suggesting the importance of the Bcl-2-type genes, due to their evolutionary conservation over two such evolutionary diverse creatures as *C. elegans* and *H. sapiens*.

Two other ced proteins, ced-3 and ced-4, were found to be absolutely required for the apoptotic death process in both embryonic and post-embryonic development of the nematode (Ellis and Horvitz, 1986). Both

genes appeared to have a role in activating death through apoptosis, which made the mammalian homologues to these proteins very interesting potential candidates for studying the pathways and mechanisms of death.

ICE.

The mammalian homologues to ced-4 have not yet been cloned, however, ced-3 now belongs to yet another rapidly growing family of proteins (Yuan, et, al., 1993), summarized in fig. 3 (Kumar and Harvey, 1995). This family is named for its' first cloned member, Interleukin-1ß converting enzyme (ICE), a cysteine protease used during inflammatory response to cleave interleukin-1ß into its active form (Thornberry, et, al., 1992). Again, to check for evolutionary conservation, complementation experiments overexpressing ced-3 or ICE in mammalian cells induced apoptotic events that could be inhibited by Bcl-2 (Kumar, 1995). All other mammalian family members, including nedd-2 (Kumar et, al., 1994), Tx (Faucheu, et, al., 1995), Ich1 (Wang, et, al., 1994), Ich2 (Kamens, et, al., 1995), ICE_{rel}-II (Munday, et, al., 1995), ICE_{rel}-III (Munday, et, al., 1995), Mch-2 (Fernandes-Alnemri, et, al., 1995a), Mch-3 (Fernandes-Alnemri, et, al., 1995b), Cmh-1 (Lippke, et, al., 1996), and CPP-32 (Fernandes-Alnemri, et, al., 1994), were also found to induce apoptosis, to varying levels, either *in vitro* or when overexpressed in cell culture.

Fig 3 - Ced-3 family of proteins and their preferred cleavage sequences to date.

Name	Aliases	Targets	Inhibitors	Consensus sequence
Ced-3			Baculovirus p35	
ICE		ICE, CPP32, Interleukin-1ß	Baculovirus p35, CrmA, YVAD-FMK**	YVAD
CPP32ß	Yama, Apopain, prICE	Poly(ADP-ribose) polymerase (PARP)	Baculovirus p35, DEVD-FMK**	DEVD
Mch2		nuclear lamins		
Mch3	ICE-LAP-3, CMH-1			
Mch4				
ICE relII				
ICE rel II	Tx, Ich2		Baculovirus p35	
Ich-1			Baculovirus p35	
Nedd-2				
FLICE	MACH			DEVD
Granzyme B*		CPP32		
Undetermined ICE family member		DNA dependent protein kinase U1 ribonucleoprotein protein kinase cδ actin and other cytoskeletal proteins		

*-not a family member, but a serine protease found to cleave CPP32.
**-tetrapeptide-fluoromethyl ketone.

ICE family member functions.

ICE family members are all cysteine proteases. All seem to cleave at the C terminal end of an aspartic acid residue, though each appears to have its own consensus sequence around the aspartic acid for specificity ((Thornberry, et, al., 1992), summarized in fig. 3 (Takahashi and Earnshaw, 1996; Marshall and Wyllie, 1996). ICE family members also cleave other proteins containing this consensus sequence, such as Poly(ADP-ribose) polymerase (described below), interleukin 1-ß (a cytokine), and some nuclear lamins, a family of intermediate filament proteins that provide structure and support for the inner nuclear membrane and may be responsible for the "chromatin condensation" morphology apparent in apoptotic nuclei (Lazebnik, et, al., 1995; Takahashi and Earnshaw, 1996).

ICE family members exist as zymogens - they exist in a non-active form, requiring activation by proteolytic cleavage (Thornberry, et, al., 1992). That is, though cysteine proteases themselves (when active), they require proteolysis in order to become active. This suggests that these proteases exist within "hierarchies of auto- and trans- cleavage" (Fraser and Evan, 1996), either for amplification, or modulation and regulation of the apoptotic signal. If each of these members then in turn had a regulator, one can easily see the potential for a single, united, complex regulatory pathway, involving Bcl-2 family members and ICE family members, either acting in parallel, directly on each other, or up or downstream of one another.

ICE family member knockouts.

A knockout of Interleukin-1ß converting enzyme resulted in mice that developed much like wild-type, and appeared relatively healthy and fertile, though with deficiencies in inflammatory response. Apoptosis occurred normally in these mice, indicating either that ICE itself had no real role in apoptosis, or that there is functional redundancy and that other ICE family members could replace ICE in apoptosis. Interestingly, though, ICE family members could not replace ICE in conversion of interleukin-1ß: These mice have barely detectable levels of the mature (cleaved) form of this protein, or of interleukin-1α (Li, et, al., 1995).

CPP32.

A good example of a well characterized ICE family member is CPP32. It exists as a 32kDa pro-enzyme, and contains, within its sequence, 2 potential ICE-like cleavage sites, cleaving it into two fragments, of 12 and 17 kDa in size, which heterodimerize (Fernandes-Alnemri, et, al., 1994). Two of these heterodimers homodimerize to form a $(17,12)_2$ structure, forming the active enzyme (fig. 4). This active enzyme can then proteolytically cleave after aspartic acid, within the consensus sequence DEVD (Tewari, et, al., 1995). This consensus sequence appears in many proteins, including Poly (ADP-ribose) polymerase (PARP), which can be cleaved by purified CPP32 *in vitro* (Tewari, et, al., 1995). In addition to possible interactions with itself or other ICE proteins, CPP32 action may be mediated through the

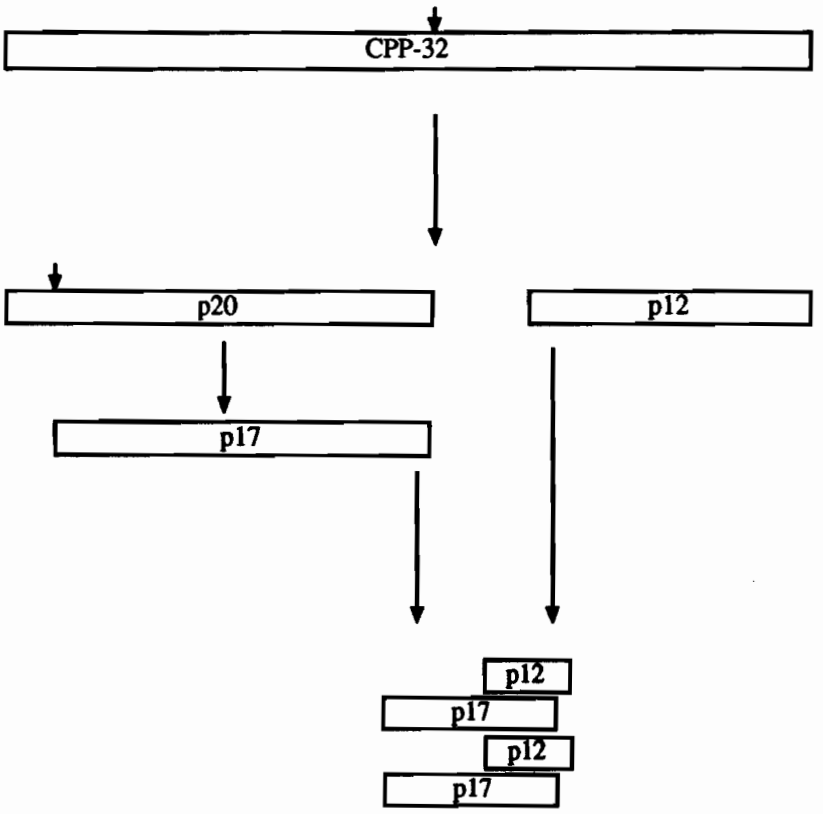

Fig. 4: CPP-32 Cleavage events

inactivation, through proteolytic cleavage, of this protein.

PARP.

PARP is a very abundant (1 copy/80kb of DNA), nuclear localized, 116 kDa protein, containing two zinc finger DNA binding sites at its amino-terminus, a central automodification domain, and a catalytic domain at its' carboxyl end (Bürkle, et, al., 1992). The catalytic domain of PARP converts NAD to nicotinamide and protein-linked ADP-ribose polymers. It appears to be relatively inactive in normal cells, but activates when cells are treated with agents that cause single and double strand breaks (Bürkle, et, al., 1992). Poly (ADP-ribosyl)ation of a protein appears to facilitate removal of that protein from DNA, and is hypothesized to help DNA repair enzymes access a break site (de Murcia, et, al., 1994).

PARP is cleaved by CPP32, into a 25 kDa fragment containing the amino terminal DNA-binding zinc finger domains, and an 85kDa fragment containing the automodification and catalytic domains. This causes the lack of activation of the catalytic domain by exogenous nicked DNA, probably because of the lack of a DNA binding site (Kaufmann, et, al., 1993; Lazebnik, et, al., 1994).

It is thought that this cleavage event causes the zinc fingers of PARP to still bind nicked DNA, but, without a catalytic domain, instead of helping with the removal of unwanted proteins near damaged areas, causes this 25kda

cleavage product to become an unwanted protein in these areas, inhibiting, rather than aiding, the action of other DNA repair enzymes.

The PARP cleavage event has been well correlated with apoptosis, and has become a diagnostic tool for confirming the execution phase of programmed cell death.

PARP overexpressing cells appear normal (Miranda, et, al., 1995), and it is hypothesized that PARP exists in excess even in a normal cell, and that DNA strand breaks are required to activate the resident enzyme.

The knocking out of all poly (ADP-ribosyl)ation, by the knocking out of poly (ADP-ribose) transferase (Wang, et, al., 1995), a enzyme similar to PARP and involved in addition of ADP-ribose to nuclear proteins in response to DNA strand breaks, yielded mice that were healthy and fertile. These knockout mice, although able to repair DNA, had problems with proliferation of primary fibroblasts and thymocytes following damage by gamma-irradiation, and were especially susceptible to epidermal hyperplasia with age (Wang, et, al., 1995). This phenotype suggests a subtle role for PARP in DNA repair, and a function for this protein only when the organism is responding to externally instigated stress. This is consistent with the finding that this protein is only activated in the presence of DNA strand breaks.

Bcl-2 and p53.

Binding and promoter studies, both *in vivo* and *in vitro*, have linked Bcl-2 to many other proteins implicated in the apoptotic process, The most interesting, and most researched of these proteins is p53, a growth suppressor so intimately associated with the apoptotic process that many classify apoptosis in two sub-categories - the p53 dependent, and the p53 independent (Chen, et, al., 1995; Bates and Vousden, 1996; Hollstein, et, al., 1991).

p53 is a transcription factor and the most commonly mutated gene in human tumours. It induces growth arrest through activation of the p21/Waf-1 pathway, inhibiting cell-cycle dependent kinases.

Though the involvement of p53 in apoptosis is well documented, its actual role is not clear. p53 is absolutely required for activation by certain inducers of death, and has been shown both to induce Bax expression and inhibit the Bcl-2 promoter (Miyashita, et, al., 1994, Miyashita and Reed, 1995, Miyashita, et, al., 1994). Bcl-2 overexpression has also been shown to block p53-induced apoptosis.

Experimental goals.

The goal of this thesis is to determine the relative place on the apoptotic pathway for the Bcl-2 regulatory cascade with respect to the ICE cleavage

steps. Because there are so many different ICE family members, and that we could not address all of them, especially since new family members are being found every day, the first step was to look at a product of ICE activity: PARP cleavage and inactivation. By overexpressing Bcl-2 or E1B 19K proteins in cells, then subjecting these cells to an apoptotic trigger, such as infection with adenovirus lacking the E1B region, one can look at the effect of this death trigger on PARP inactivation, using western analysis of electrophoretically separated lysates from these cells. If PARP is cleaved both in control and Bcl-2 overexpressing cells, the Bcl-2 repression of apoptosis occurs downstream of PARP cleavage and therefore of ICE activity. We found that Bcl-2 acted upstream of the PARP cleavage event, which caused us to ask whether Bcl-2 acts upstream of the ICE cleavage cascade. The experiment was thus taken one step further, to look at the activation of CPP32, an enzyme responsible for the cleavage of PARP. The results indicate that the repression of apoptosis by Bcl-2 occurs upstream of this event as well - that Bcl-2 (and its viral homologue) are acting directly at, or upstream of, the CPP32 cascade, but definitively not after it, and that at least one step of this putative cascade occurs downstream of Bcl-2's activity.

These experiments are relevant because of the diversity of the agents from which Bcl-2 rescues death: Bcl-2 is an element far downstream in the apoptotic pathway; CPP32 is even further. This may imply that a Bcl-2 analogue, or a protein that acts in the same manner as Bcl-2, may be a global negative regulator of the ICE family or proteases, offering a target of

action for drug design possibly more effective than simply targeting a single ICE family member.

Chapter 2: Bcl-2 and adenovirus E1B 19kDa protein prevent E1A-induced processing of CPP32 and cleavage of poly(ADP-ribose) polymerase.

Bcl-2 and adenovirus E1B 19 kDA protein prevent E1A-induced processing of CPP32 and cleavage of poly(ADP-ribose) polymerase

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The E1A oncoproteins of adenovirus type 5 are potent inducers of apoptotic cell death. To manifest growth promoting and transforming properties, therefore, E1A requires the co-expression of a suppressor of apoptosis. During normal viral infection, this function is provided by the E1B 19 kDa protein. However, the cellular suppressor Bcl-2 can substitute for 19K during infection, and both proteins can effectively cooperate with E1A to facilitate transformation of primary cells in culture. How E1A induces apoptosis and at what point(s) on this pathway Bcl-2 and E1B 19K act are not presently known. Here, we demonstrate that E1A-induced apoptosis is accompanied by specific endo-proteolytic cleavage of poly(ADP-ribose) polymerase (PARP), an event that is linked to the Ced-3/ICE apoptotic pathway in other systems. PARP cleavage was also observed in p53-null cells infected with 19K$^-$ virus expressing 13S *E1A*. In addition to PARP cleavage, expression of E1A caused processing of the zymogen form of CPP32, a Ced-3/ICE protease that cleaves PARP and is required for apoptosis in mammalian cells. These events were prevented when *E1A* was co-expressed with *E1B 19K* or *BCL-2*, which places these suppressors of apoptosis either at or upstream of processing of pro-CPP32.

Keywords: apoptosis; E1A; PARP; CPP32; Bcl-2; E1B 19K

Introduction

Both genetic and biochemical approaches have identified members of a novel class of cysteine proteases as important regulators of apoptosis (Hengartner and Horvitz, 1994; Kumar, 1995; Earnshaw, 1995). Ced-3 was the first of these to be recognized, and was originally identified as the product of one of two genes essential for programmed cell death in the nematode, *C elegans* (Ellis and Horvitz, 1986). Cloning of *ced-3* revealed homology to a previously characterized cysteine protease in mammals, interleukin-1β converting enzyme (ICE) (Yuan *et al.*, 1993). Subsequently, additional proteases related to Ced-3 were identified, including mouse Nedd2 (Kumar *et al.*, 1994) and its human homolog Ich-1_L (Wang *et al.*, 1994), ICE_{rel}II/Tx/ICH-2 (Munday *et al.*, 1995; Faucheu *et al.*, 1995; Kamens *et al.*, 1995), ICE_{rel}III (Munday *et al.*, 1995), Mch2 (Fernandes-Alnemri *et al.*, 1995), prICE (Lazebnik *et al.*, 1994) and CPP32/Yama/Apopain (Fernandes-Alnemri *et al.*, 1994). Ectopic expression of cDNAs encoding all of these proteins have been shown to trigger apoptosis in cultured cells. Conversely, inhibition of prICE, which has been detected as an activity in extracts from cells that are committed to undergo apoptosis, blocks apoptosis *in vitro*, and prevents the site specific cleavage and consequent inactivation of one of the targets of prICE, the DNA repair enzyme, poly(ADP-ribose) polymerase (PARP). Recent evidence now suggests that several of these proteases have the potential to cleave PARP (Fernandes-Alnemri *et al.*, 1995; Gu *et al.*, 1995; Martin and Green, 1995; Nicholson *et al.*, 1995). CPP32, however, is the most likely candidate for executing the analogous Ced-3 function in mammals (Nicholson, 1995; Tewari *et al.*, 1995). Together, the findings imply the existence of a family of cysteine proteases, each of which may have the potential to influence cell death through the inactivation/activation of specific target proteins. Collectively, the proteases constitute the Ced-3/ICE family.

The multiplicity and possible functional redundancy (Li *et al.*, 1995), of these family members suggests the potential for complex patterns of regulation of cell death, including cell-type specificity, different upstream regulators and downstream targets, and the potential for a regulatory cascade involving different family members. Of note, however, is the fact that the active enzymes are derived by processing of an inactive proenzyme (reviewed by Kumar, 1995). Whether or not regulators of apoptosis influence this step remains to be determined. It is established, however, that Bcl-2 can effectively block cell death caused by ectopic expression of *nedd2/ich-1$_L$* cDNA (Miura *et al.*, 1993), which places this important suppressor of apoptosis (Oltvai and Korsmeyer, 1994; Reed, 1994) on the Ced-3/ICE pathway. In contrast, little is yet known about the activation pathways of Ced-3/ICE proteases and how they are accessed by positive apoptotic signals.

In this regard, the study of viruses may provide important model systems, because both the genes that trigger apoptosis, and some of their downstream targets, are known. The immortalizing properties of the E1A region of adenovirus, for example, are manifested only if the outcome of E1A expression that induces cell death is blocked (Rao *et al.*, 1992). The ability of E1A to induce DNA synthesis, cell

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Received 11 September 1995; revised 23 October 1995; accepted 27 October 1995

growth, and transformation is linked to its ability to form complexes with various cellular proteins (Yee and Branton, 1985; Harlow *et al.*, 1986; Moran and Matthews, 1987; Egan *et al.*, 1988, 1989; Whyte *et al.*, 1988a,b, 1989; Barbeau *et al.*, 1994; Eckner *et al.*, 1994), and it may be that one or more of these cellular targets also influence apoptosis (White *et al.*, 1991; Mymryk *et al.*, 1994). To escape E1A-induced apoptosis (Rao *et al.*, 1992), adenovirus employs two proteins that are encoded by the E1B region: E1B 19kDa protein (19K), a general suppressor of apoptosis that is functionally interchangeable with Bcl-2 (Rao *et al.*, 1992; White *et al.*, 1992; Boyd *et al.*, 1994; Nguyen *et al.*, 1994), and E1B 55 kDa protein, which binds p53 and impairs its ability to regulate gene expression (Yew *et al.*, 1994). p53 is absolutely required for the induction of apoptosis by the 243-residue product of 12S E1A mRNA (Debbas and White, 1993; Lowe and Ruley, 1993). However, the alternatively spliced 13S transcript of E1A yields a 289 residue product that can bypass p53, and trigger apoptosis via one or more additional viral proteins whose synthesis is presumably activated by the 289 residue E1A (Teodoro *et al.*, 1995).

As a first step in linking E1A and its targets to the common pathway that executes apoptosis, we show here that E1A induces processing of CPP32 and the appearance of PARP-cleavage in infected cells, resulting in the production of an 85 kDa fragment of PARP, an event which is intimately linked to the induction of apoptosis in other systems (Kaufman, 1989; Kaufman *et al.*, 1993; Lazebnik *et al.*, 1994; Nicholson *et al.*, 1995; Tewari *et al.*, 1995). Both E1B-19K and Bcl-2 suppress these E1A-induced events and protect the cells against apoptosis. 13S E1A can induce PARP cleavage in p53-null cells, indicating that activation of this pathway can bypass a requirement for p53 under these conditions.

Results and discussion

In this study, we have employed a mutant adenovirus type 5, designated pm2/3, which does not express E1B 19K because it lacks the appropriate translational start site (McLorrie *et al.*, 1991). As previously documentated, cultured cells infected with pm2/3 rapidly undergo changes which are characteristic of apoptosis. These include cell shrinkage and extensive membrane blebbing, nuclear pyknosis and chromatin condensation, DNA fragmentation, and loss of cell vaibility (Nguyen *et al.*, 1994; Teodoro *et al.*, 1995). As well, expression of E1A in the absence of E1B products causes pre-transformed primary cell foci to ultimately degenerate and undergo apoptosis (Rao *et al.*, 1992). The advantage of using virus for studies of E1A-induced apoptosis, rather than transformed foci, however, is twofold. Firstly, 19K-defective virus causes cells to rapidly engage programmed death, with little opportunity for this event to be influenced by collateral genetic changes to the cell which are unrelated to E1A gene expression. Secondly, virus encoding 13S E1A can induce apoptosis in cells lacking the expression of p53, via cooperation between the 289-residue product of 13S E1A and another viral protein (Teodoro *et al.*, 1995). This provides the opportunity to investigate the apoptotic pathway in a p53-null background. Both p53-dependent and p53-independent apoptosis caused by 19K-deficient virus is the result of specific functional domains within E1A (White *et al.*, 1991, 1992; Teodoro *et al.*, 1995).

Bcl-2 and E1B 19K prevent E1A-induced PARP cleavage

PARP is a DNA repair enzyme that contributes to genome integrity (Juarez-Salinas *et al.*, 1979; Bürkle *et al.*, 1992) and provides a critical defense against cellular stress (Wang *et al.*, 1995). Importantly, PARP is cleaved and inactivated at the onset of apoptosis by one or more Ced-3/ICE proteases (Kaufmann *et al.*, 1993; Lazebnik *et al.*, 1994; Nicholson *et al.*, 1995). In addition to contributing to DNA repair, PARP can influence indirectly the poly(ADP-ribos)ylation of other substrates (eg, the Ca^{2+}/Mg^{2+}-dependent nuclease implicated in internucleosomal DNA fragmentation during apoptosis, Tanaka *et al.*, 1984) and consumption of NAD and its precursor ATP (Kaufmann *et al.*, 1993), an energy source that is required for apoptosis *in vivo* (Wyllie *et al.*, 1984; Kaufmann, 1989). Apoptotic inactivation of PARP involves a single cleavage of the polypeptide, after aspartate 216 (Lasebnik *et al.*, 1994), which separates the NH_2-terminal zinc finger DNA-binding domain of PARP from the COOH-terminal automodification and poly(ADP-ribos)ylating domains. This results in liberation from the 113 kDa full-length enzyme of an 85 kDa fragment which retains immunoreactivity with the monoclonal anti-PARP antibody, C-2-10 (Kaufmann *et al.*, 1993).

In Figure 1, the C-2-10 antibody was used to assess PARP cleavage to the 85 kDa fragment in human KB cells following mock infection or infection with $19K^-$ adenovirus, employing stable cell lines that express a neomycin resistance gene (*neo*), either alone or co-expressed with *BCL-2*. PARP cleavage and cell viability assays were conducted on the same samples of cells. Results very similar to those presented in Figure 1 were obtained for different *neo*- and *BCL-2*-expressing cell lines. Cleavage of PARP to the 85 kDa fragment was pronounced in *neo* cells following infection and the extent of PARP cleavage with time after infection closely correlated with the time course of cell death (Figure 1, compare upper and lower graphs). In contrast, *neo* cells that had received a mock infection subsequently exhibited very little PARP cleavage (compare lanes 4 and 7 of the immunoblot in Figure 1) and loss of cell viability. KB cells stably expressing *BCL-2* (Nguyen *et al.*, 1994), on the other hand, were protected against cell death induced by $19K^-$ virus and showed a corresponding decrease in the extent of PARP cleavage (Figure 1, compare lanes 5–7 with 11–13). Likewise, CHO LR73 cells stably expressing E1B 19K (Chen *et al.*, 1995) exhibited a markedly extended viability compared to *neo* cells following infection with mutant virus (Figure 2A) and had a corresponding decrease in cleavage of PARP to the 85 kDa fragment (Figure 2B).

Together, these results suggest that induction of apoptosis by E1A is linked to the pathway leading to cleavage of PARP, and that Bcl-2 and E1B 19K intersect and block this pathway at a point upstream of PARP cleavage.

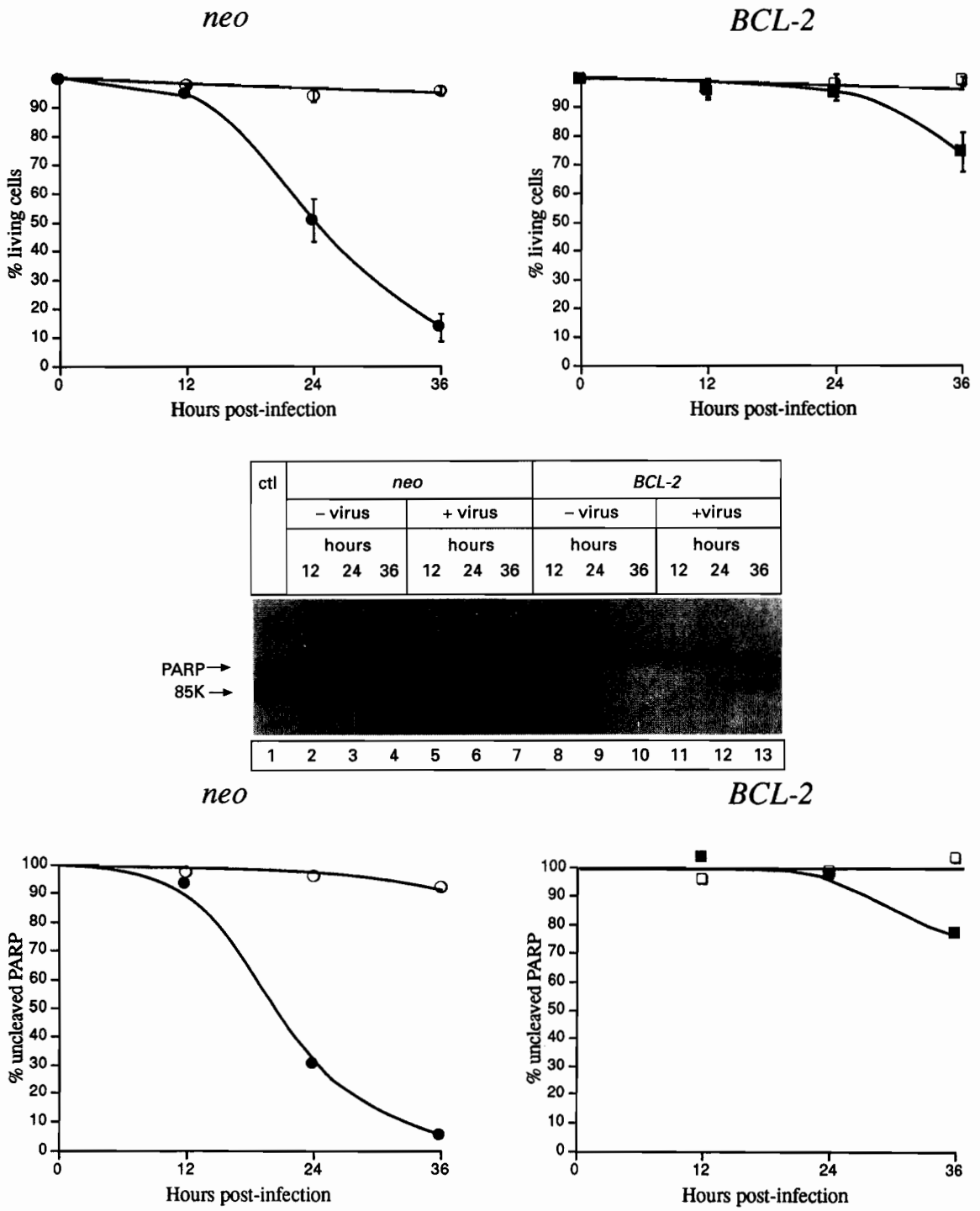

Figure 1 Bcl-2 suppresses E1A-induced apoptosis and associated cleavage of PARP. **(top)** KB cell lines expressing *neo* either alone or with human *BCL-2* were either mock-infected (○ and □, respectively) or infected with pm2/3 virus (● and ■, respectively). Cells were assayed for viability at the indicated times by measuring exclusion of Trypan Blue. Shown are the averages of three determinations, with bars representing standard deviation. **(middle)** Infected (+virus) and mock infected (−virus) cells from **top** were collected at the various time points and subjected to SDS–PAGE and immunoblotting with C-2-10 monoclonal antibody against PARP. Arrows denote full-length PARP (113 kDa) and the 85 kDa proteolytic cleavage product of PARP (85k). Lane 1 is a control (ctl) and shows the 85 kDa frament generated by cleavage of PARP between Asp216 and Gly217 in HL60 cells stimulated to undergo apoptosis by VP16 (Kaufmann *et al*., 1993). **(bottom)** The relative intensities of the upper and lower PARP bands shown in **middle** were quantified using a Macintosh Quadra 610 and NIH Image v.1.57 image analysis software. Results are presented as percent PARP contained in the upper band at the various time points. Lanes 2–4 (mock infection of *neo* cells) (○); lanes 5–7 (infection of *neo* cells with pm2/3 virus) (●); lanes 8–10 (mock-infection of *BCL-2* cells) (□); lanes 11–13 (infection of *BCL-2* cells with pm2/3 virus) (■)

Induction of PARP cleavage in p53-null cells

The 19K$^-$ adenovirus that was employed for the experiments described in Figures 1 and 2 expresses the 13S form of *E1A* and, consequently, can induce apoptosis in a p53-null background via cooperation with another viral protein (Teodoro *et al.*, 1995). To determine if apoptosis under these conditions also manifests cleavage of PARP to the 85 kDa fragment, a mouse embryo fibroblast cell line, 10(1), which contains chromosomal deletions of both p53 alleles (Harvey and Levine, 1991) was examined following infection with 19K$^-$ virus. Induction of apoptosis in these cells by 13S E1A has previously been documented (Teodoro *et al.*, 1995). As shown in Figure 3, infection with 19K$^-$ virus resulted in a time course of cell death (Figure 3A) which was accompanied by PARP cleavage (Figure 3B).

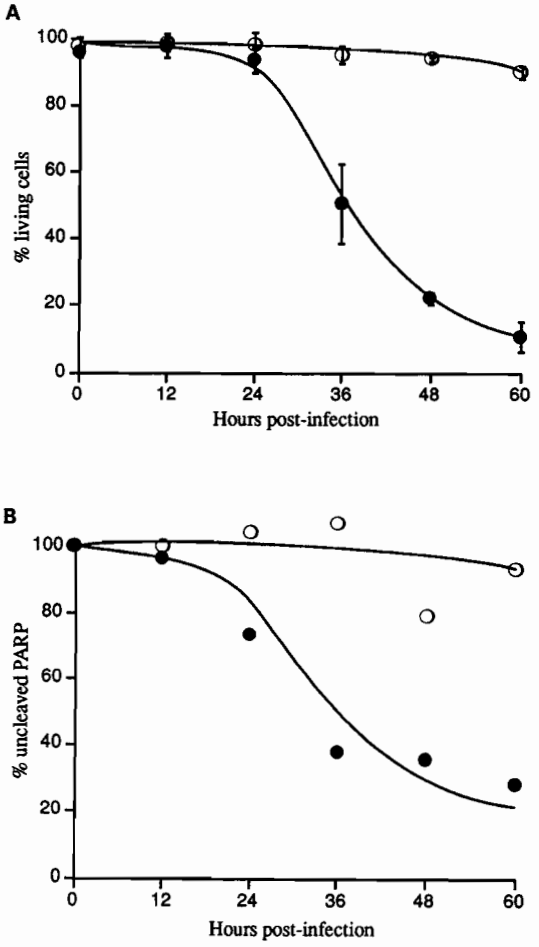

Figure 2 Prevention of E1A-mediated apoptosis and PARP cleavage by E1B 19 kDa protein. CHO LR73 cell lines stably expressing *neo* either alone (●) or with *E1B 19K* (○) were infected with pm2/3 virus. (**A**) Cells were assayed for viability at the indicated times by measuring exclusion of Trypan Blue. Shown are the averages of three determinations, with the bars indicating standard deviation. (**B**) Samples of the cells at each time point in **A** were subjected to SDS–PAGE followed by immunoblotting with monoclonal C-2-10 anti-PARP antibody. Relative band intensities were quantified and graphed as described in Figure 1

Processing of CPP32 during E1A-induced apoptosis

CPP32 is a Ced-3/ICE protease family member that cleaves PARP and is necessary for apoptosis in mammalian cells (Nicholson *et al.*, 1995). Like the other family members, CPP32 cleaves after an aspartate residue for which specificity is determined by the P1–P4 context of the scissile bond in the substrate (Nicholson *et al.*, 1995). In non-apoptotic cells, CPP32 resides as an inactive 32 kDa zymogen which, upon stimulation of apoptosis, is converted to its active form. Processing of pro-CPP32 yields two subunits, 17 kDa and 12 kDa in size, of which two each are required to form the holoenzyme (Nicholson *et al.*, 1995). As demonstrated for ICE (Thornberry *et al.*, 1995), processing of pro-CPP32 to the p17 and p12 subunits may be autocatalytic, and occurs at aspartates 28 and 175 (Nicholson *et al.*, 1995).

Immunoblotting with an antibody raised against the recombinant p17 subunit of CPP32 was used to assess the effect of infection with 19K$^-$ virus on CPP32 in KB cells expressing either *neo* alone or *neo* plus *BCL-2* (Figure 4). One difficulty with such experiments in cultured cells is the likely possibility that, once CPP32

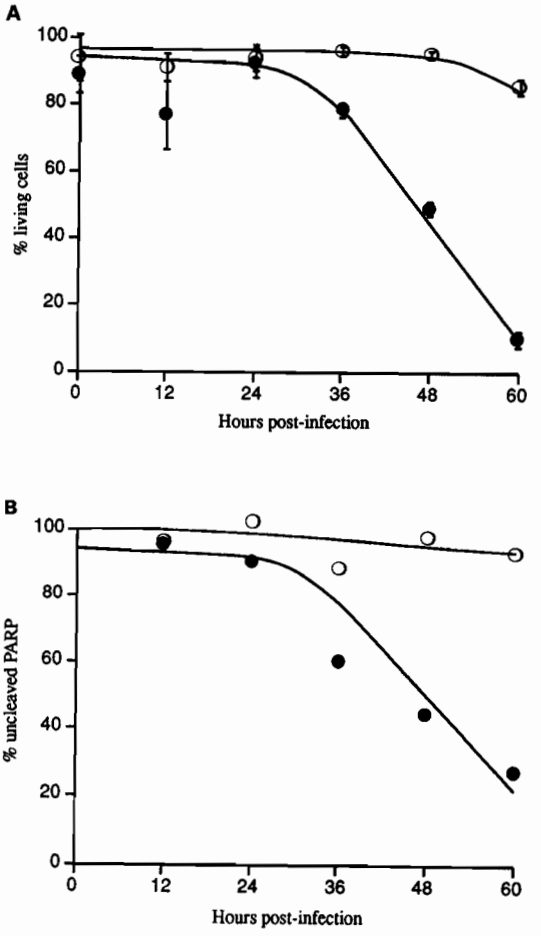

Figure 3 Appearance of PARP cleavage in p53-null cells undergoing apoptosis induced by adenovirus expressing 13S E1A. 10(1) cells were either mock infected (○) or infected with adenovirus pm2/3 (●). At the indicated times, samples were analysed either for viability (**A**) or for PARP cleavage (**B**), as described in Figure 1

is activated in a particular cell, the cell may immediately execute apoptosis and quickly die, rendering the components of the holoenzyme (ie, p17 and p12) difficult to detect. The pre-execution (latency) period, in which CPP32 presumably exists in the inactive pro-enzyme form, however, may be variable in length and would account for the gradual percent loss of cell viability for the population as a whole (see Figure 1). Immediately following infection of KB cells with 19K$^-$ adenovirus, the 32 kDa form of pro-CPP32 was observed and then it disappeared with time in *neo*-expressing cells (Figure 4, lanes 1–4), but was retained in *BCL-2*-expressing cells (Figure 4, lanes 5–8). Loss of pro-CPP32 in *neo* cells correlated with the appearance, albeit weakly, of an ~17 kDa immunoreactive band (Figure 4, lanes 3 and 4) which presumably corresponds to the p17 subunit of the holoenzyme. Of note, an ~29 kDa band was observed in Figure 4 (denoted by an asterisk) which would be consistent with a processing intermediate of CPP32 via cleavage at aspartate 28 (Nicholson *et al.*, 1995). Interestingly, this product was present in the cells at the time of viral infection (Figure 4, lanes 1 and 5). As shown in Figure 5, E1B 19K was very similar to Bcl-2 (Figure 4) in preventing processing of pro-CPP32 in response to infection with 19K$^-$ virus.

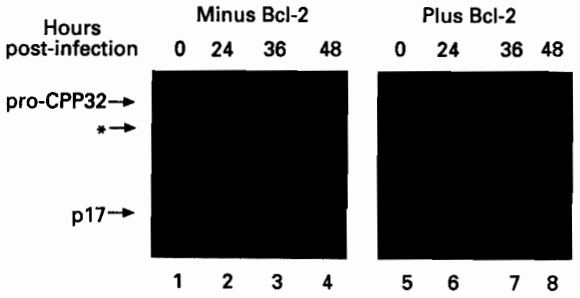

Figure 4 Bcl-2 prevents E1A-induced processing of CPP32. KB cell lines expressing *neo* either alone (lanes 1–4) or with *BCL-2* (lanes 5–8) were subjected to infection with pm2/3 virus. Cells were collected at the various time points and subjected to SDS–PAGE and immunoblotting with rabbit polyclonal antibody raised against the recombinant p17 subunit of human CPP32 purified from expressing bacteria. The blot was visualized by electrochemiluminescence (Amersham). Arrows denote full-length CPP32 (pro-CPP32) and the ~17 kDa (p17) and ~29 kDa (asterisk) products

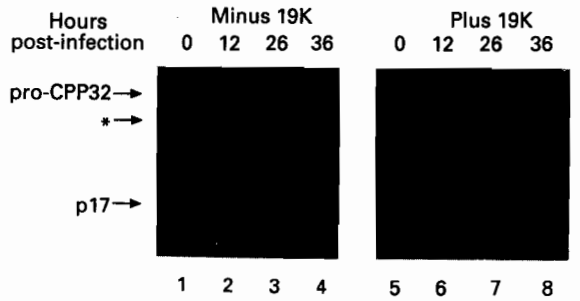

Figure 5 E1B 19 kDa protein prevents E1A-induced processing of CPP32. Analysis was as described in Figure 4, except that KB cells either expressing (lanes 5–8) or not expressing (lanes 1–4) E1B 19K cDNA were used

Conclusions

Adenovirus E1A proteins function as both positive and negative regulators of transcription, through interactions with different classes of transcriptional adaptor proteins whose gene-targets regulate DNA synthesis and cell growth. However, the additional consequence of E1A expression–apoptosis–may also be linked to some of these same targets of E1A that stimulate cell growth and transformation. This presumably explains why the manifestation of the growth-promoting properties of E1A requires the co-expression of a suppressor of apoptosis. Although E1B 19K has evolved specifically to counter the cytotoxic properties of E1A during viral infection, 19K is also an effective suppressor of apoptosis when expressed ectopically under diverse circumstances. In most cases, it can substitute for the cellular suppressor, Bcl-2. Like E1A, therefore, E1B 19K may also function within a common cellular pathway that regulates cell survival. The present results establish that this is the case and show that E1A is linked to activation of the Ced-3/ICE (CPP32) and PARP cleavage pathway. Bcl-2 and E1B 19K intersect and block this E1A-induced pathway, which places these negative regulators of apoptosis either at, or upstream of, processing of pro-CPP32. Importantly, apoptosis and PARP cleavage also occurred as a consequence of 13S *E1A* expression in p53-null cells. In this situation, however, E1A must cooperate with another early viral protein to cause death. Since PARP cleavage was also observed in the p53-null background, this as yet unidentified protein presumably enables E1A to access the Ced-3/ICE pathway independently of p53. One possibility may be that it can functionally substitute for a cellular activator of the Ced-3/ICE pathway that would otherwise require p53 for its expression.

Materials and methods

Cells and virus

Stable cell lines were established following transfection of KB and CHO LR73 cells with Rc/RSV vectors expressing *neo* either alone or with human *BCL-2* (KB cells, Nguyen *et al.*, 1994) or *EIB 19K* of adenovirus type 5 (CHO LR73 cells, Chen *et al.*, 1995). Cells were cultured on 60 mm dishes in αMEM supplemented with 10% fetal bovine serum, steptomycin, penicillin and G418. After reaching 80% confluency, the medium was replaced with 0.5 ml of fresh medium containing either no virus (mock-infection) or 100 pfu/cell of mutant (pm2/3) adenovirus type 5 that lacks expression of E1B 19 kDa protein (equivalent to pm1716/2072 in McLorrie *et al.*, 1991). Following incubation for 1 h at 37°C, 5 ml of fresh medium was added and the cells cultured and subsequently collected at various time points.

Cell viability

At various time points following infection, adherent and non-adherent cells were collected, washed, suspended in phosphate-buffered saline (PBS), and aliquots mixed with an equivalent volume of 0.4% Trypan Blue. The percentage of cells that excluded the dye was determined. At least three individual culture dishes were analysed per time point.

PARP cleavage

Analyses were performed on the same cultures for which cell viability was determined. After rinsing with PBS, cells were harvested by scraping in ice-cold PBS containing 0.5 μg/ml aprotinin and pepstatin and 1 mM phenylmethysulfonylfluoride. The cells were collected by centrifugation and suspended and sonicated on ice in 50 mm Tri HCl, pH 6.8, 6M urea, 6% β-mercaptoethanol, 3% sodium dodecyl sulfate (SDS) and 0.0003% bromophenol blue, at a concentration of 2×10^6 cells/ml. After warming to room temperature, aliquots were subjected to 10% SDS polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose, and probed with mouse monoclonal C-2-10 antibody against PARP (Kaufmann *et al.*, 1993). The blot was developed with horseradish peroxidase conjugated secondary antibody and visualized by Electrochemiluminescence (Amersham). Bands were quantified from a scanned image of the autoradiogram, using a Macintosh Quadra 610 and NIH Image v. I.57 image analysis software (US National Institutes of Health).

Acknowledgements
We are grateful to Stanley Korsmeyer for providing *BCL-2* cDNA.

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Chapter 3: Discussion.

Findings.

Our findings increase the mounting evidence that Bcl-2 and E1B 19K work in the same manner, or at the very least on the same biochemical pathway. We also show that the activation of the ICE family proteins and its resulting cleavage of PARP is an inherent part of E1A-induced death. The results of the last chapter also indicate that Bcl-2 and E1B 19K intersect and block this E1A-induced pathway, which places these negative regulators of apoptosis either at, or upstream of, processing of pro-CPP32.

This E1A-induced apoptosis and PARP cleavage occur in a p53-independent manner, since they also occur in p53-null cells. It is likely that, in this case, E1A is cooperating with another viral protein to induce death, since the killing of cells by E1A has been found by other groups to be p53-dependent (Sabbatini, et, al., 1995). Since PARP cleavage was also observed in the p53-null background, this as yet unidentified protein presumably enables E1A to access the Ced-3/ICE pathway independently of p53. One possibility may be that it can functionally substitute for a cellular activator of the Ced-3/ICE pathway that would otherwise require p53 for its expression.

A summary of these results, combined with what is known about the rest of the apoptotic pathway, is illustrated in figure 5 (Fraser and Evan, 1996; Farrow and Brown, 1996).

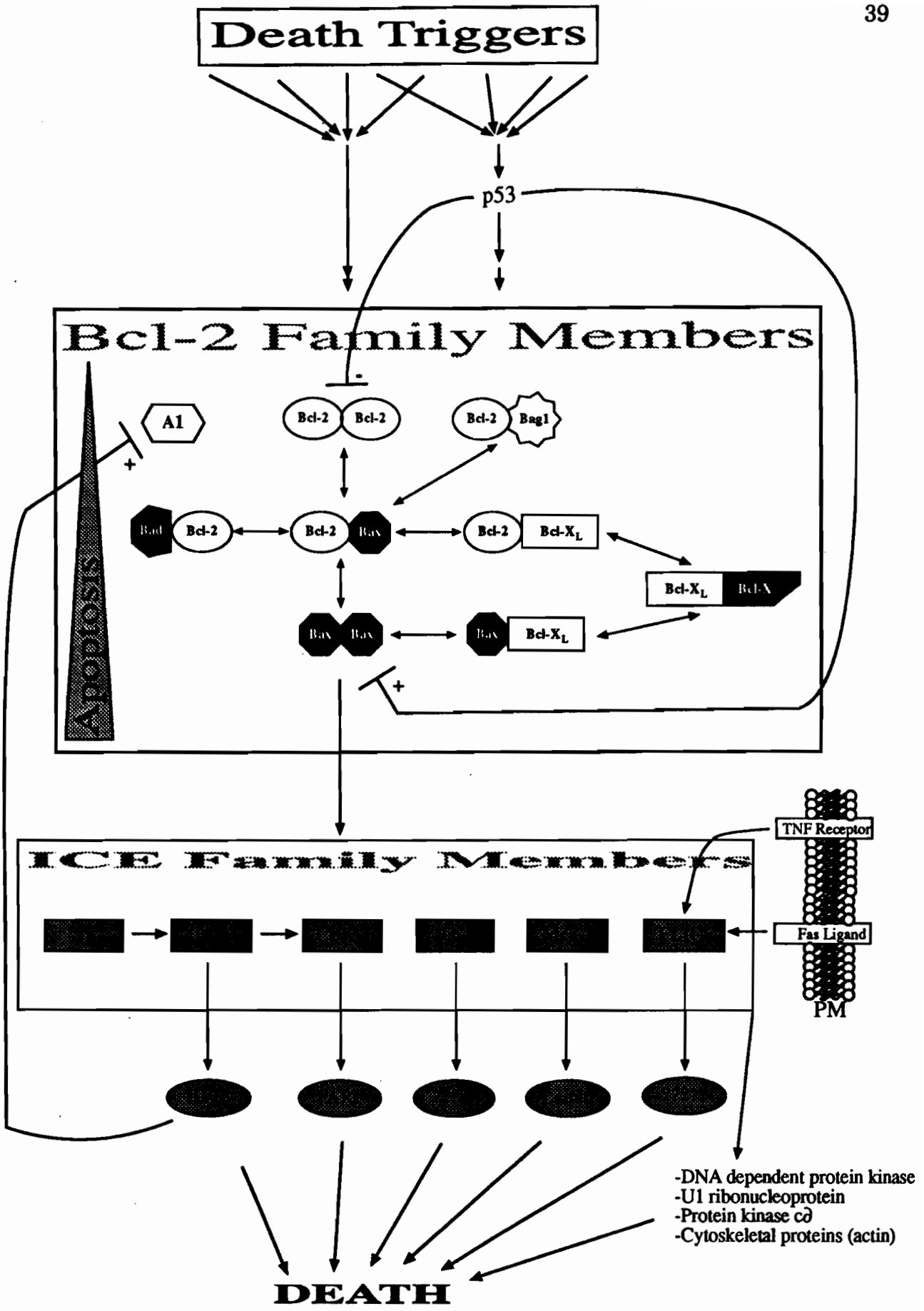

Fig. 5 - The apoptotic pathway to date.

Limitations of the cell culture system used in our experiments.

Although a powerful technique, there are many limitations to the cell culture system. It is often the case that discoveries found in cell culture do not apply in animal systems - such may be the case of Bcl-2 localization in tissue culture overexpression studies. One is also limited to a specific cell type, or a series of cell types growing in isolation of one another, a situation that rarely occurs *in vivo*. Also, the cell culture system does not effectively mimic physiological distribution systems - a monolayer of cells, covered with media containing all the essential growth factors, oxygen, and food, does not accurately represent the three dimensional structure of blood distribution through tissues, and an effective drug in culture is often found to have very little effect *in vivo*, due to distribution problems.

Problems also occur in the opposite direction - because of upscaling to a living model rather than working in true *vitro*. Although a very powerful technique, transfection is time-consuming, and investigation of a large number of potential proteins in this manner can be very costly. Also, some proteins do not express well in culture, and others may cause induction of compensatory feedback loops that mask the direct effects of the overexpression. Drug design is also limited *in vivo* - one must solve both the problem of entry into the cell *and* effectiveness of the drug at the same time. Also, cross-linking studies, or purification of novel proteins, from a tissue culture system, requires large numbers of cells, and with it, large quantities of the death triggering agent, drug, or other system you are

subjecting the cells to. Another important factor to consider is that a large, time consuming experiment can be destroyed by the contamination of a single, yet essential, tissue culture plate. Also, variations in the confluency of the cells, due to variations in the cell doubling time and the difficulty of exact calculation of the cell density, can make the difference between a positive and a negative result.

Solutions - other techniques.

These problems and limitations with the cell culture system have led labs in different directions. Many labs are now pursuing mouse transgenics, and animal models of disease. Although a very exciting and new field, this also has its limitations (Viney, 1995). Mouse transgenics are costly, and nothing is more frustrating than spending tens of thousands of dollars, and hundreds of person-hours, only to find that the protein knockout or overexpression in question is fatal or has no recognizable phenotype. Also, interaction studies between many different proteins are difficult to do - multiple knockouts are still in their infancy. Another important limitation is the pliability of the living system - evolution has forced a large amount of redundancy in every living thing - knocking out one protein is sure to be compensated in a living model by overexpression of a similar protein family member.

Other labs have gone in the "opposite" direction, and have created cell-free models, and test-tube reconstitutions of biochemical pathways. These also

have their limitations - the isolation of the pathway, the questionable relevancy in a system which normally has much feedback regulation and triggering of multiple parallel pathways in response to a signal from one pathway, and, in the case of drug studies, the artificial, even and saturable distribution of a homogenous mixture in a test-tube.

These cell-free systems have their advantages as well, in the ease of manipulation and use, the rapidity of results once the system is working, and the possibility of asking questions about the workings of a system in isolation of regulatory pathways, membrane permeabilities, distribution, and localization. A cell free system is the simplest way of getting an answer, and very powerful, as long as its limitations are kept in mind. A working cell-free system has become the first step in elucidating any mechanism - once you have a hypothesis *in vitro*, you can move to cell culture or animal models to find evidence supporting the model. Once you have a drug that works in the test-tube, you work towards modifying that drug so that it can enter the cell, or so that it is targeted to the right cells, in order to improve its efficacy. Once you have a biochemical pathway in order, you look at its regulation.

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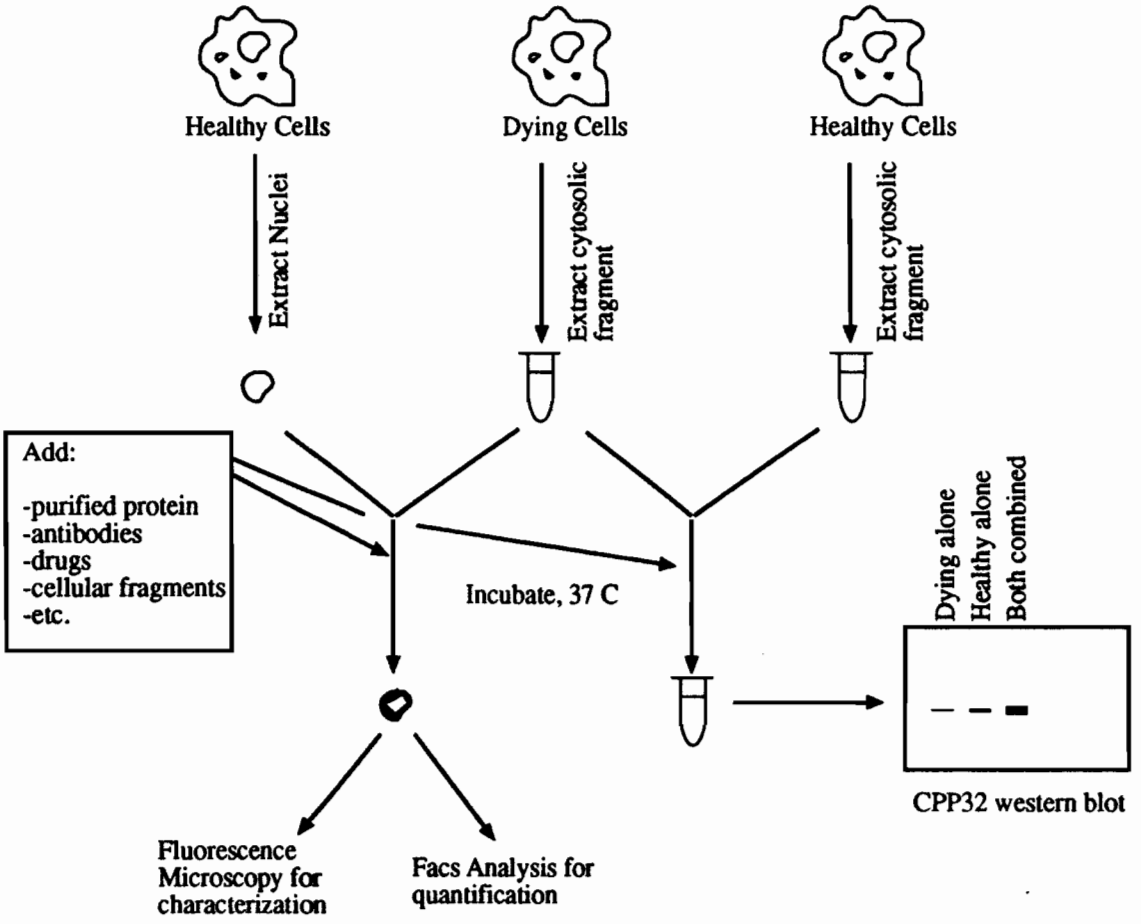

Figure 6 - in vitro assay flow sheet

cleavage could only mean transfer of the apoptotic signal to the healthy nuclei. This biochemical marker system was, by extension, used in nuclear-free systems - the nuclei were no longer necessary, if one could express purified, radiolabelled PARP *in vivo*, and measure its' cleavage when incubated in the cytosolic fragment.

It became clear to us, however, that this PARP cleavage assay was only really doing one thing: measuring the activity of CPP32, or other ICE family proteins that could cleave PARP. A more relevant assay became the extent of induction of CPP32; that is, measuring the extent of induction of CPP32 processing in a healthy cytosol by a 'primed' cytosolic fragment.

Much work went into optimizing the conditions of this assay, the results can be summarized in figure 7, and a seemingly simple protocol, shown in *Appendix 1: Additional Materials and Methods*. Primed cytosols, when incubated with healthy ones, can induce cleavage of CPP32 in a manner that is not concentration dependent.

This assay can now be used, both for drug testing, and for elucidation of the roles of potential upstream components in this pathway. One can deplete the cytosols of active CPP32, by immunoprecipitation with CPP32 antibodies, and look at potential protein candidates' ability to trigger this cleavage. Also, one can do *in vivo* crosslinking studies, looking at CPP32 binding proteins, thus determining proteins directly up or downstream of this apoptotic activator. One can also look at the role of reactive oxygen

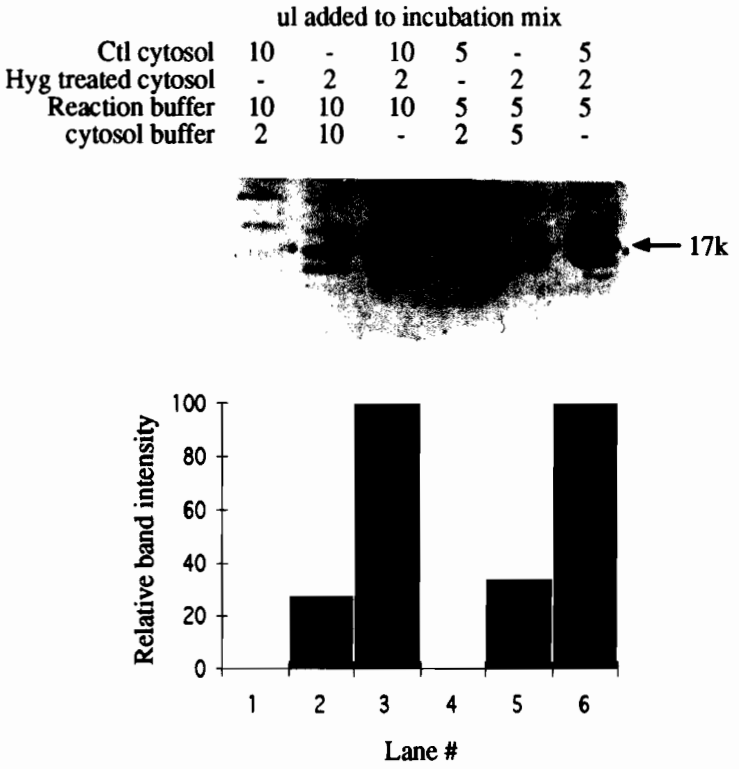

Figure 7: example of working in vitro assay.

species in apoptosis, by addition of free radicals or free radical sequesterers such as Vitamin C and E.

Another issue was the possibility of a quantifiable assay for looking at dying nuclei, devoid of the problems of statistical probabilities and subjectivity of choice of field-of view that were apparent with the previously used system of subjectively looking at small groups of nuclei under a microscope. A method extensively used for measuring death in whole cells (Hardin, et, al., 1992) was extended by myself for quantifiable viewing of death in nuclei - that of Fluorescence activated cell sorting (FACS) using a dye that was activated by a disruption of membrane integrity such as the one induced by the apoptotic process. Figure 8 shows a time-course of death in whole cells, sorted by FACS, after treatment (and induction of apoptosis) with hygromycin and staining with propidium iodide, one such dye. As the time-course progresses, one sees the increase in number of cells with activated dye, in the upper quadrant. Quantification of 10 000 cells can be done in a matter of seconds using this technique, and a time course death curve generated from FACS analysis closely correlates to one determined from trypan blue staining (Figure 9).

Extension of this system to the isolated nuclei is shown in figure 10, showing a time course of treatment with hygromycin, followed by isolation of the nuclei. A correlation to trypan blue was not possible, since all isolated nuclei would stain blue, regardless of healthiness - trypan blue can pass through the nuclear membrane once it is in the cell.

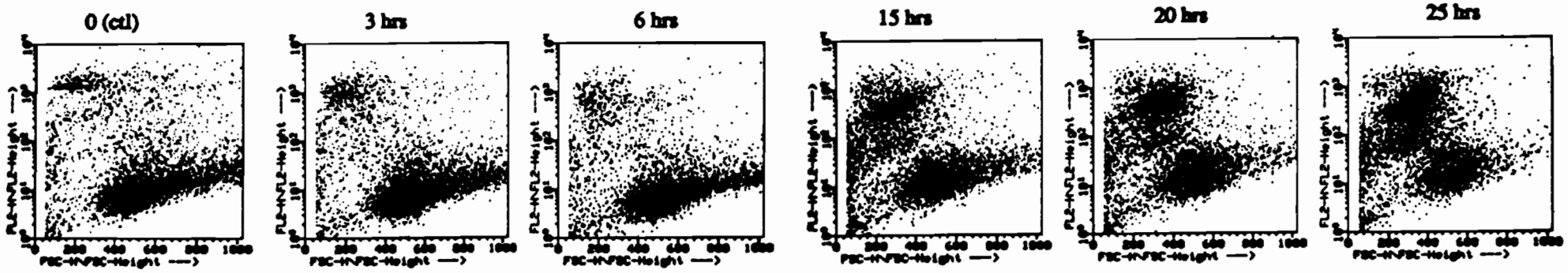

Figure 8 - Time course of hygromycin treatment - whole cells as viewed by FACS after treatment with propidium iodide.

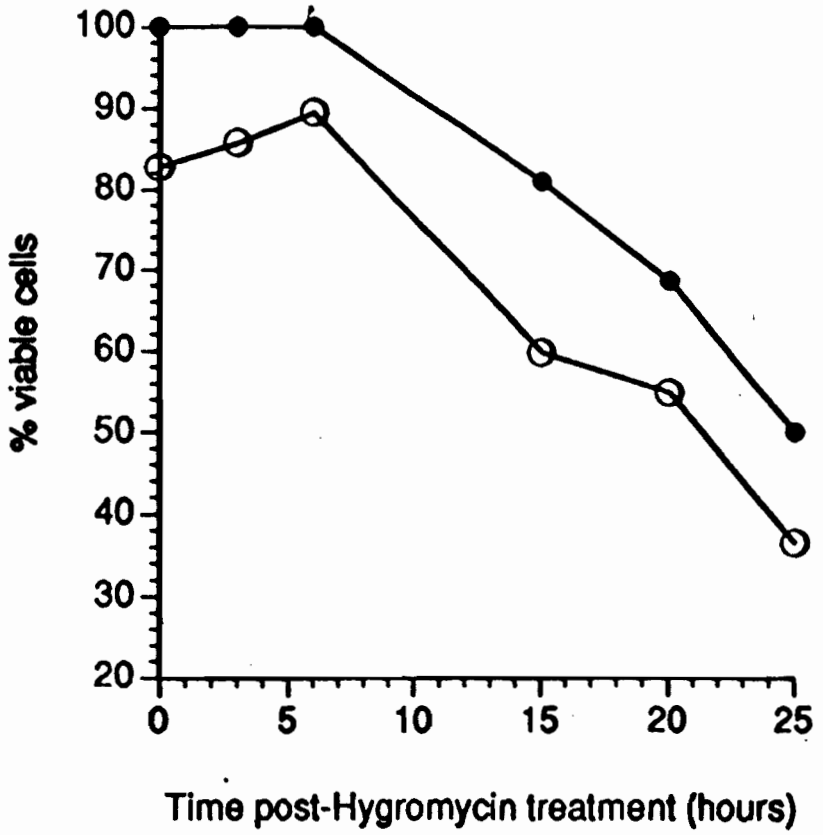

Figure 9 - Comparison of quantification of viability.
(●) Trypan blue exclusion (n=100-200).
(○) FACS after staining with propidium iodide (n=10 000).

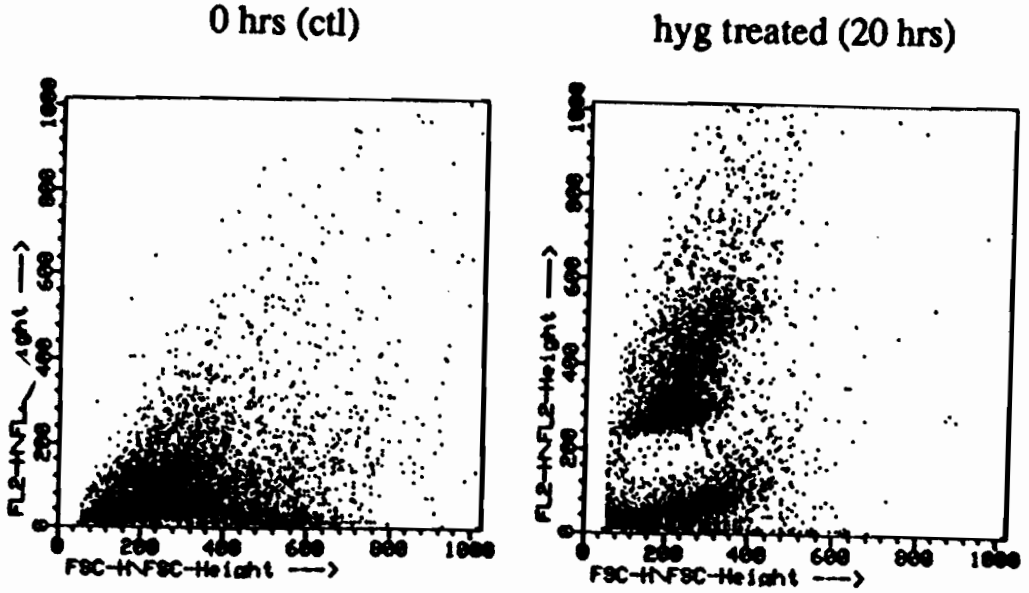

Figure 10 - Time course of hygromycin treatment - isolated nuclei as viewed by FACS after treatment with propidium iodide.

This quantifiable nuclear death assay can be used in further experiments, where it is necessary to directly look at nuclear morphology, rather than a biochemical event. This may happen when redundant systems are triggered, and PARP cleavage, or CPP32 activation, can no longer be correlated to the death of the cell. Although these apoptotic pathways have not yet been seen, their existence is quite likely, due to the extent of the ICE family members, and the reality of cellular redundancy.

Conclusion.

We have conclusively placed E1A whithin the apoptotic pathway that involves CPP32 activation and cleavage of PARP. We have also shown that these E1A-induced events can be blocked by Bcl-2 and E1B 19K; that these death repressors act upstream of this activation and cleavage. In addition, we have shown that E1A induction of PARP cleavage occurs independently of p53.

Finally, we have worked out an in vitro apoptotic system using human (KB) cell extracts, and worked out a quantitative method for analysis of death both biochemically, and in isolated nuclei.

These systems can be used to further elucidate the cell death pathway, hopefully with respect to the ROS system, and to work out new drugs and reagents for eventual clinical use in cancer therapy.

Appendix 1 - Materials and methods

Materials and Methods

Preparation of concentrated cytosolic extracts.

KB cells were grown up to 80% confluency on large 256x256 cm square cell culture plates (Nunc), scraped with a cell scraper (Falcon) and collected in their own media (10% Fetal Calf Serum (Gibco) in a-mem containing penicillin and streptomycin. Cells treated with hygromycin B were treated with1.5µl/ml (xx) hygromycin B for the indicated amounts of time, prior to scraping.

All steps after this point were done on ice, or in a cold room. Collected cells were spun down, and resuspended in Delbecco's PBS (Gibco). Cells were counted and resuspended at a concentration of 10^8 cells/ml in GIB buffer (46 mM sucrose; 1mM $MgCl_2$; 2mM Tris pH 7.5; 0.2% Triton X-100; 10µg/ml Pepstatin A; 10µg/ml Aprotinin; 20µg/ml Leupeptin; 1mM PMSF), and shaken on an eppendorf shaker (Eppendorf) for 1 minute. Cells were then spun at 12000g in an Eppendorf centrifuge (IEC) for 10 minutes. Supernatants were collected and frozen in liquid nitrogen until use.

Nuclear Isolations

KB cells were grown up to confluency on 256x256 cm plates. 10µM cytochalasin B was added to the cells for 30 minutes, prior to scraping. Cells were lifted off the plates using a cell scraper (Falcon), and spun down

at 1000 rpm and 4°C in a tabletop centrifuge (IEC). All steps following were done on ice. Cells were washed in Delbecco's PBS (Gibco) and counted in order to properly resuspend at a later step. Cells were washed in NIB (10mM Pipes (pH7.4); 10mM KCl; 2mM $MgCl_2$; 1mM dithiothrietol (DTT); 10mM Cytochalasin B; 1mM PMSF; 10μg/ml Pepstatin A; 10μg/ml Aprotinin; 20μg/ml Leupeptin), then resuspended in NIB and swollen on ice for 20 minutes. Swollen cells were then lysed in a ground glass homogenizer (50 strokes), then layered over 2x volume 30% sucrose in NIB and centrifuged at 3000 rpm (SS34 rotor, DuPont) for 4 minutes. Pellet was washed twice with RB (10mM Hepes (pH7.0); 40mM ß-glycerophosphate; 50mM NaCl; 2mM $MgCl_2$; 1mM DTT; 2mM ATP; 10mM Creatine Phosphate; 50μg/ml Creatine Kinase), and resuspended in RB to a volume of 10^8 nuclei/ml.

In Vitro Assay - CPP32 western

Various concentrations of cytosol, as indicated in figure 7, were added to RB and incubated for 2 hours, at 37°C. The entire incubation mix was then electrophoresed on a 12% polyacrylamide gel, and transferred to nitrocellulose. A 1:5000 dilution of anti-CPP32 antibody (D. Nicholson) was used, followed by 1:2000 anti-rabbit ECL secondary (Amersham). CPP32 bands were then viewed by autoradiography, scanned, and analyzed using NIH Image v. 1.57 and a Power Macintosh computer.

Immunofluorescence

Cells were suspended at a concentration of 1.5 x 106 cells/ml in PBS, and 1:1000 1M Propidium Iodide was added to them for 5 minutes just prior to counting.

For immunofluorescence of isolated nuclei, nuclei were isolated by the method described above ('Nuclear Isolations'), then washed and resuspended at a concentration of 1.5 x 106 nuclei/ml in PBS. 1:1000 1M Propidium Iodide was added for 5 minutes, just prior to counting.

Flow Cytometry (Counting).

Flow cytometry and Flow activated cell sorting was done using a FACScan (Beckinson Dickinson) series scanner, equipped with a 15mW, 488nm argon-ion lazer. For the propidium iodide excitation, a 578±13 nm band pass filter was used; for forward angle light scatter, a 530±30 nm band pass filter was used. Results were tabulated in graph form, with PI excitation (marked FL2), on the Y axis, and forward angle light scatter, or cell size (marked FLC) on the X axis. Scales are relative, and kept constant for every group of experiments.

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Original contributions to knowledge

1. The placing of E1A within the apoptotic pathway that involves CPP32 activation and cleavage of PARP.

2. That E1A-induced cleavage of PARP and processing of CPP32 can be blocked by Bcl-2 and Adenovirus E1B 19K.

3. That E1A induction of PARP cleavage occurs independently of p53.

4. That the FACS method of quantification of living cells using propidium iodide can be extended to isolated nuclei, and applied to an *in vitro* assay.

5. That CPP32 cleavage can be an effective and quantitative biochemical marker for use in an *in vitro* assay of cell death.