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Interactions between members of the Bcl-2 family, other protein partners and new survival factors

THESE

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CONTENTS

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II. ABBREVIATIONS

Ab	:	Antibody
Ac-DEVD-CHO	:	Acetyl-Asp-Glu-Val-Asp-aldehyde (caspase-3/-7 inhibitor)
Ac-YVAD-CHO	:	Acetyl-Tyr-Val-Ala-Asp-aldehyde (caspase-1 inhibitor)
AIDS	:	Acquired immunodeficiency syndrome
AIF	:	Apoptosis-inducing factor
Apaf-1	:	Apoptotic protease activating factor 1
Bad	:	Bcl-xL/Bcl-2-associated death promoter
Bak	:	Bcl-2 homologous antagonist/killer
Bax	:	Bcl-2-associated x protein
Bcl-2	:	B-cell lymphoma/leukemia-2
Bcl-xL, Bcl-xS	:	Bcl-2 related protein, L=long transcript, S=short transcript
BFA	:	Brefeldin A
BH1, BH2, BH3 and BH4	:	Bcl-2 homology domain 1, 2, 3 and 4
BIR	:	Baculovirus IAP repeat
Bok	:	Bcl-2 related ovarian killer
Boo	:	Bcl-2 ovary homologue
C. elegans	:	Caenorhabditis elegans
CAD	:	Caspase-activated deoxyribonuclease
CARD	:	Caspase activation and recruitment domain
Caspase	:	Cysteinyl aspartate-specific protease
ced-3, -4 and –9	:	Cell death abnormal 3, 4 and 9
CHAPS	:	3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate
CLARP/c-Flip$_L$/I-FLICE	:	Caspase-like apoptosis regulatory protein
CRADD	:	Caspase and Rip adapter with death domain
CrmA	:	Cowpox virus cytokine response modifier A
CTL	:	Cytotoxic T-Lymphocyte
DcR	:	Decoy receptor
DD	:	Death domain
DED	:	Death-effector domain
DEFCAP	:	Death effector filament-forming ced-4-like apoptosis protein
Diablo	:	Direct IAP binding protein with low isoelectric point (mouse Smac ortholog)
DFF-45	:	DNA fragmentation factor 45
DISC	:	Death-inducing signaling complex
DR	:	Death receptor
egl-1	:	Egg laying defective-1
ER	:	Endoplasmic reticulum
FADD	:	Fas-associating protein with death domain
FCS	:	Fetal calf serum
FLICE/MACH/Mch5	:	FADD-like ICE (caspase-8)
G418	:	Geneticin
GFP	:	Green fluorescent protein
GzmB	:	Granzyme B
GST	:	Glutathione S-transferase
HA	:	Hemagglutinin
IAP	:	Inhibitor-of-apoptosis protein
ICAD	:	Inhibitor of the caspase-activated DNase
ICE	:	Interleukin-1β-converting enzyme (caspase-1)

Ig	:	Immunoglobulin
IL	:	Interleukin
IP	:	Immunoprecipitation
JNK	:	Jun kinase
LPS	:	Lipopolysaccharide
MG132	:	Carbobenzoxyl-leucinyl-leucinyl-leucinal-H
NAC	:	Nucleotide-binding domain and CARD domain
NF-κB	:	Nuclear factor-κB
NGF	:	Nerve growth factor
NK	:	Natural killer
NP-40	:	Nonidet P-40
PARP	:	Poly(ADP-ribose) polymerase
PCD	:	Programmed cell death
PKC	:	Protein kinase C
PLAD	:	Pre-ligand assembly domain
PTP	:	Permeability transition pore
RAIDD	:	RIP-associated Ich-1/Ced-3 homologous protein with death domain
RIP	:	Receptor interacting protein
ROS	:	Radical oxygen species
Rpr	:	Reaper
RT	:	Room temperature
SDS	:	Sodium dodecylsulfate
Smac	:	Second mitochondria-derived activator of caspase
TM	:	Transmembrane domain
TNF	:	Tumor necrosis factor
TNFR1	:	Tumor necrosis factor receptor 1
TRADD	:	TNFR1 associated protein with death domain
TRAF1 and 2	:	TNF receptor associated factor 1 and 2
TRAIL	:	TNF-related apoptosis-inducing ligand
XIAP	:	X-chromosome-linked inhibitor of apoptosis protein
Z-DEVD-fmk	:	N-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone
Z-VAD-fmk	:	N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

III. ABSTRACT

Genetic and biochemical studies in *C. elegans* have shown that developmental programmed cell death requires a CED-4 mediated activation of the caspase CED-3. CED-9 acts as a death protector by directly interacting with and inhibiting the function of CED-4. CED-9 can be prevented from acting by binding to the death factor Egl-1. This apoptotic machinery seems to be conserved between worms and mammals, at least at its base. Several CED-3-like caspases have been identified which either activate each other in a proteolytic cascade or depend on an adapter-like CED-4 homologue for their activation. Members of the Bcl-2 family are plentiful in mammals and consist of both survival factors (Bcl-2, Bcl-xL), which are CED-9 homologues, and death factors (Bax, Bak), which are Egl-1 homologues, neutralizing each other through heterodimerization. However, as Bcl-2 and Bcl-xL do not simply act via Bax/Bak neutralization, they must somehow interact and work through mammalian CED-4 homologues if the death pathway is evolutionary conserved.

The first CED-4 homologue identified was Apaf-1. In contrast to death signals in *C. elegans*, Apaf-1 however depends for its caspase activation function (caspase-9) on a mitochondrial protein, cytochrome c, that is released into the cytosol upon apoptosis stimulation. Although it was previously published that Bcl-2 and Bcl-xL could interact with Apaf-1, many reports have shown that these proteins act upstream of Apaf-1 to block the release of cytochrome c. Thus, they probably interact with a CED-4 homologue that damages mitochondria directly or indirectly. In addition to this CED-4 interaction model, the homology of the crystal structure of Bcl-xL and Bax with pore-forming bacterial toxin suggested that Bcl-2 family members could act as ion or protein conducting channels or pores. While one could easily imagine such a mode of action for Bax and Bak, because their function on mitochondria is to release cytochrome c, it is more difficult to envisage a pore forming function for the anti-apoptotic activity of Bcl-2 and Bcl-xL.

The goal of this thesis was to get more insights into the interaction between Bcl-2 and Bax and whether these molecules could homodimerize and potentially form channels, to test whether Apaf-1 is indeed a protein target of Bcl-2 family members, to understand the mechanism by which Bax releases cytochrome c and to clone new survival factors. Firstly, we show that while Bax effectively di- or multimerizes, naturally occurring Bcl-2 does not readily form homodimers both *in vitro* and within cells even under conditions when Bcl-2 protects them from apoptosis. This indicates that Bcl-2 is probably not able to form a channel by itself. Secondly, we can not find any interaction between Apaf-1 and Bcl-2, Bcl-xL or Bax both *in vitro* and in intact cells. We therefore disfavor the notion that Apaf-1 forms a mammalian apoptosome with Bcl-2 or Bcl-xL even when they act as survival factors. Nevertheless, it is still possible that the interaction between Apaf-1 and the Bcl-2 family members is indirect or via another CED-4 homologue. Thirdly, we demonstrate that there is no other protein that associates stably in detectable amounts with Bcl-2, Bax or the heterodimer in normal and apoptotically stressed cells. Moreover, we reveal that the degree of protection against apoptosis does not correlate with the number of Bcl-2-Bax heterodimers but with the amount of Bcl-2 that is free of Bax indicating that Bcl-2 requires neither Bax nor major, stable interactions with other cellular proteins to exert its survival function. Fourthly, we were able to prove that Bcl-2 fails to prevent Bax-induced cytochrome c release in cells stably overexpressing both Bcl-2 and Bax. In addition, these cells show no signs of caspase activation and survive with significant amount of cytochrome c in the cytosol denoting that Bcl-2 can interfere with Bax killing downstream of and independently of cytochrome c release. Fifthly, we discover a crosstalk between the endoplasmic reticulum (ER) and mitochondria controlled by Bcl-2. Surprisingly, cytochrome c release induced by the drug brefeldin A (BFA) or tunicamycin is not only blocked by wild-type Bcl-2, but also by a Bcl-2 variant that is exclusively targeted to the ER. Finally, we isolate new putative survival factors from a functional assay where cells are rescued after transfecting a cDNA phagemid library from a human Burkitt lymphoma RNA source and exposing them to toxic doses of staurosporine.

IV. RESUME

Des études génétiques et biochimiques du *C. elegans* ont montré que durant le développement, la mort cellulaire programmée requiert une activation de la caspase CED-3 par l'intermédiaire de CED-4. CED-9 agit lui comme protecteur de mort en interagissant directement avec CED-4 et en inhibant sa fonction. L'action de CED-9 peut être empêchée par liaison au facteur de mort Egl-1. Cette machine apoptotique semble être conservée entre les vers et les mammifères, du moins à sa base. Plusieurs caspases, similaires à CED-3, ont été identifiées. Elles s'activent les unes les autres soit directement en une cascade proteolytique, soit par l'intermédiaire d'un adaptateur homologue à CED-4. Les membres de la famille de Bcl-2 sont abondants chez les mammifères et se composent de facteurs de survie (par exemple Bcl-2 et Bcl-xL) qui sont des homologues de CED-9 ainsi que des facteurs de mort (par exemple Bax et Bak) qui sont des homologues de Egl-1. Ces deux sous-familles se neutralisent les unes les autres par hétérodimérisation. Par contre, comme Bcl-2 et Bcl-xL n'agissent pas simplement en neutralisant Bax et Bak, ils doivent d'une façon ou d'une autre interagir et fonctionner à travers des homologues mammifères de CED-4 si le processus de mort est conservé durant l'évolution.
Le premier homologue de CED-4 à avoir été identifié, est Apaf-1. Cependant et contrairement aux signaux de mort dans le *C. elegans*, Apaf-1 dépend pour sa fonction d'activation des caspases (en l'occurrence caspase-9), d'une protéine mitochondriale, le cytochrome c, qui est libérée dans le cytosol par induction de l'apoptose. Bien que précédemment, il a été publié que Bcl-2 et Bcl-xL pouvaient interagir avec Apaf-1, beaucoup de comptes rendus ont montré que ces protéines agissent en fait en amont d'Apaf-1 afin de bloquer la libération du cytochrome c. Ils interagissent donc probablement avec un homologue de CED-4 qui endommage directement ou indirectement les mitochondries. En plus de ce modèle d'interaction de CED-4, la similitude des structures cristallines de Bcl-xL et de Bax avec la toxine bactérienne formant des pores, suggérait que les membres de la famille de Bcl-2 pouvaient agir comme des canaux ioniques ou protéiniques ou alors comme des pores. Un tel mode d'action serait facile à imaginer pour Bax et Bak étant donné que leur fonction sur les mitochondries est de libérer du cytochrome c, mais il est plus difficile d'envisager une fonction de formation de pores pour l'activité anti-apoptotique de Bcl-2 et de Bcl-xL.
Cette thèse avait comme objectif d'obtenir plus d'informations dans l'interaction entre Bcl-2 et Bax, de savoir si ces molécules peuvent homodimériser et potentiellement former des canaux, de tester si Apaf-1 est vraiment une protéine cible des membres de la famille de Bcl-2, de comprendre le mécanisme par lequel Bax libère le cytochrome c et finalement de cloner des nouveaux facteurs de survie. Premièrement, nous montrons que Bcl-2 ne forme pas volontiers des homodimères autant *in vitro* qu'à l'intérieur de la cellule, cela même sous des conditions où Bcl-2 la protège de l'apoptose, alors que Bax effectivement di- ou multimérise. Ceci indique que Bcl-2 n'est probablement pas capable de former un canal de lui-même. Deuxièmement, nous n'avons pu trouver aucune interaction entre Apaf-1 et Bcl-2, Bcl-xL ou Bax, *in vitro* et dans des cellules intactes. Par conséquent, nous sommes défavorables à la notion que Apaf-1 forme un apoptosome mammifère avec Bcl-2 ou Bcl-xL, même lorsqu'ils agissent comme des facteurs de survie. Néanmoins, il est encore possible que l'interaction entre Apaf-1 et les membres de la famille de Bcl-2 soit indirecte ou via un autre homologue de CED-4. Troisièmement, nous démontrons qu'il n'y a aucune autre protéine qui s'associe stablement et en quantités détectables avec Bcl-2, Bax ou leur hétérodimère dans des cellules normales et apoptotiquement stressées. De plus, nous révélons que le degré de protection contre l'apoptose ne correspond pas au nombre d'hétérodimères Bcl-2/Bax, mais à la quantité de Bcl-2 qui est libre de Bax. Cela indique que Bcl-2 ne requiert ni Bax, ni d'interaction majeure et stable avec d'autres protéines cellulaires pour exercer sa fonction de survie. Quatrièmement, nous sommes capables de prouver que Bcl-2 ne réussit pas à prévenir la libération du cytochorme c provoquée par Bax dans des cellules surexprimant stablement Bcl-2 et Bax. De plus, ces cellules ne montrent aucun signe d'activation des caspases et survivent avec des quantités importantes de cytochorme c dans le cytosol, dénotant ainsi que Bcl-2 peut interférer avec la mort induite par Bax en aval et indépendemment de la libération du cytochrome c. Cinquièmement, nous découvrons une connexion entre le réticulum endoplasmique et les mitochondries contrôlée par Bcl-2. De manière surprenante, la libération du cytochrome c induite par la drogue brefeldine A (BFA) ou tunicamycine n'est pas seulement bloquée par le Bcl-2 wild-type, mais aussi par une variante de Bcl-2 exclusivement présente

sur le réticulum endoplasmique. Finalement, nous isolons de possibles nouveaux facteurs de survie par une analyse fonctionnelle. Elle consiste à sauver des cellules qui ont été précédemment transfectées avec une librairie de phagemides de cDNA préparée à partir d'une source ARN de lymphomes Burkitt humains et exposées à des doses toxiques de staurosporine.

1. INTRODUCTION

1.1 The origin of cell death

When a cell in an organism dies due to a process encoded by that organism for the purpose of killing its own cells, that death can be considered to be a physiological process. The great majority of our cells are destined to die just by such a mechanism; relatively few die through injury or inability to sustain their own viability. That cell death occurs in a predictable "programmed" fashion in physiological circumstances was first recognized by Carl Vogt who saw dying cells in the neuronal system of developing toad embryos (Vogt, 1842). However, the very first cells described, those from cork and named "cells" by Hooke in 1665, were also corpses that had died physiologically. Developmental biologists were also quick to realize that cell death was involved in the process of metamorphosis, both in insects and mammals. For example, Lockshin coined the phrase "programmed cell death" in 1965 to describe cell death in insect metamorphosis (Lockshin and Williams, 1965), and a year later Tata showed loss of the tadpole's tail involved cell death that could be blocked by cycloheximide and therefore required expression of endogenous genes (Tata, 1966). When Kerr, Wyllie and Currie saw that the morphology of liver cells exposed to toxins, and lymphocytes treated with hormones, was the same as that described by the embryologist Glucksmann earlier in the century, they coined a new term, "apoptosis", for cell deaths with this distinct morphology-whether they be in response to physiologic or pathologic stimuli (Glucksmann, 1951; Kerr *et al.*, 1972; Vaux and Korsmeyer, 1999).
Although the first component of a cell death mechanism to be recognized was the mammalian gene Bcl-2 (Vaux *et al.*, 1988), the first evidence that a genetic program existed purely for physiological cell death came from studying development in *C. elegans* (Horvitz *et al.*, 1982; Ellis and Horvitz, 1986). The ability of human Bcl-2 to prevent programmed cell death in *C. elegans* showed that apoptosis in mammalian cells and programmed cell death in the nematode were the same highly conserved process (Vaux *et al.*, 1992). At about the same time, investigators in a seemingly unrelated area, inflammation, were engaged in the study of pro-interleukin(IL)-1β processing, in the hope that identification of the responsible protease would provide a starting point for the development of new therapeutic agents. This work led to the discovery of a novel protease, IL-1β converting enzyme (ICE), now known as caspase-1 (Ceretti *et al.*, 1992; Thornberry *et al.*, 1992). The connection between caspases and apoptosis was made late in 1993 when Yuan and her colleagues reported that caspase-1 is related to a *C. elegans* death gene, CED-3 (Yuan *et al.*, 1993). This work not only reinforced the prevailing view that molecular mechanism of cell death were highly conserved, but established proteases, caspases in particular, as key mediators of this process (Thornberry, 1999).

1.2 Types and morphologies of cell death

Based on morphological and biochemical analyses (Table 1), cell death can occur via two quite distinct mechanisms : necrosis, also called accidental or pathological cell death, and apoptosis.

Necrosis	Apoptosis
Morphological features	**Morphological features**
- Loss of membrane integrity	- Membrane blebbing, but no loss of integrity
- Flocculation of chromatin	- Aggregation of chromatin at the nuclear membrane
- Swelling of the cell and lysis	- Cellular condensation (cell shrinkage)
- No vesicle formation, complete lysis	- Formation of membrane bound vesicles (apoptotic bodies)
- Disintegration (swelling) of organelles	- No disintegration of organelle; organelles remain intact
Biochemical features	**Biochemical features**
- Loss of regulation of ion homeostasis	- Tightly regulated process involving activation and enzymatic step
- No energy requirement (passive process, also occurs at 4°C)	- Energy (ATP)-dependent (active process, does not occur at 4°C)
- Random digestion of DNA (smear of DNA after an agarose gel electrophoresis)	- Non-random mono- and oligonucleosomal lenght DNA fragmentation (ladder pattern after an agarose gel electrophoresis)
- Postlytic DNA fragmentation (=late event of death)	- Prelytic DNA fragmentation (=early event of cell death)
Physiological features	**Physiological features**
- Death of cell groups	- Death of single, individual cells
- Evoked by non-physiological disturbances	- Induced by physiological stimuli
- Phagocytosis by macrophages	- Phagocytosis by adjacent cells or macrophages
- Significant inflammatory response	- No inflammatory response

Table 1 : Differential features and significance of necrosis and apoptosis.

While necrosis occurs when cells are exposed to an extreme variance from physiological conditions, like complement attack, severe hypoxia, hyperthermia, lytic viral infection, exposure to toxins or respiratory poisons, leading to the rupture of the cell and an inflammation (Trump *et al.*, 1981 ; Searle *et al.*, 1982), apoptosis acts in a single cell and in response to defined stimuli. It proceeds via a genetically encoded cell suicide machinery that sometimes even requires RNA and protein synthesis. Therefore it is often called programmed cell death or PCD (Tata, 1966 ; Lockshin, 1969 ; Oppenheim *et al.*, 1990).

During necrosis, the cell swells, its mitochondria dilate, other organelles dissolve and the plasma membrane ruptures, releasing cytoplasmic material; this often elicits an inflammatory response. By contrast, during apoptosis, the cytoplasm shrinks and the chromatin condenses, but the organelles retain their integrity. The plasma membrane blebs and exposes phosphatidylserine on its outer surface, which is normally retained in the inner leaflet, but it does not rupture, preventing the release of cellular compounds into the extra-cellular medium. *In vitro*, apoptotic cells ultimately fragment into membrane-enclosed vesicles (apoptotic bodies), which swell and lyse resulting in the so-called “secondary necrosis” phase (Mills *et al.*, 1999; Desagher and Martinou, 2000), whereas, *in vivo*, they are recognized and removed by either phagocytes or adjacent cells, thereby avoiding inappropriate inflammation. Biochemical hallmarks of apoptosis also include the activation of endonucleases, DNA degradation into oligonucleosomal fragments and the activation of a family of cysteine proteases called caspases (Desagher and Martinou, 2000).

Nevertheless, there is increasing evidence that classical apoptosis and necrosis represent only the extreme ends of a wide range of possible morphological and biochemical deaths. The two classical types of demise can occur simultaneously in tissues or cell cultures exposed to the same stimulus and often, the intensity of the same initial insult decides the prevalence of either apoptosis or necrosis. For example, pre-emptying human T-cells of ATP switches the

type of demise caused by two classic apoptotic triggers (staurosporine and CD95 stimulation) from apoptosis to necrosis (Nicotera *et al.*, 1999).
Moreover, for several years, a major discussion point was definition of the type of cell death : apoptosis, programmed, active, physiological, necrotic. Elucidation of some of the molecular pathways has resolved some of these issues, but has indicated that there is sufficient variation that one needs either to take a larger view of the meaning of the word "apoptosis" or to consider apoptosis to be one of a family of physiological cell deaths. For instance, in the last 10 years, caspases have been identified as major players in apoptosis, and have even been proposed as the defining element of apoptosis, but we also now recognize that the certainly powerful caspase pathway is not necessary to kill cells. More recently, evidence has accumulated that non-caspases, including cathepsins, calpains, granzymes and the proteasome complex, also have roles in mediating and promoting cell death (Borner and Monney, 1999; Johnson, 2000). In addition, in many instances cells in which caspases are blocked or knocked out will die, even though they do not acquire apoptotic morphology. They look more like cells with elaborately developed autophagy, a form of death that is encountered, more commonly than acknowledged, in large post-mitotic epithelial and other cells, including insect organs and mammary epithelium in which lysosomal and perhaps other proteases are activated (Lockshin *et al.*, 2000). Morphologically, autophagic cell death includes degradation of Golgi apparatus, polyribosomes, and endoplasmic reticulum, which precedes nuclear destruction. Intermediate and microfilaments are largely preserved; presumably the cytoskeleton is required for autophagocytosis (Bursch *et al.*, 2000).

1.3 Cell death and disease

Programmed cell death plays a critical role in controlling the number of cells in development and throughout an organism's life by the removal of cells at the appropriate time. It is an important biological process for the elimination of unwanted cells such as those with potentially harmful genomic mutations, autoreactive lymphocytes, or virally infected cells. Alterations of this normal process can result in the disruption of the delicate balance between cell proliferation and cell death and can lead to a variety of diseases (Thompson, 1995). For example, in many forms of cancer, key pro-apoptotic proteins are mutated or anti-apoptotic proteins are upregulated, leading to the accumulation of cells and the inability to respond to harmful mutations, DNA damage, or chemotherapeutic agents. Since effective chemotherapy depends on the induction of programmed cell death, cancers with defects in the cell death signaling pathways are particularly difficult to treat.
PCD is also important for eliminating autoreactive T cells after an immune response. When this normal process is disrupted through mutations of the proteins that trigger apoptosis (e.g. Fas ligand or the Fas receptor), an autoimmune lymphoproliferative syndrome (ALPS) can result, with complications such as hypersplenism, autoimmune hemolytic anemia, thrombocytopenia, and neutropenia (Strauss *et al.*, 1999).
Inappropriate apoptosis also contributes to several neurologic disorders. In Alzheimer's, Parkinson's and Huntington's disease, specific neurons prematurely commit suicide, which can lead to irreversible memory loss, uncontrolled muscular movements and depression (Fesik, 2000).
While the relationship of apoptosis to disease has been postulated and argued in many settings, on one topic there is a building consensus. In diseases as diverse as AIDS, myocardial infarction (MI), hyperoxia in the lung epithelium, immunological irregularities, and autoimmune disease, there is substantial apoptosis, often including cells that appear not to be seriously damaged but may be bystanders activating their cell death pathways in response

to local events (Finkel *et al.*, 1995; Finkel and Casella, 1998; Garaci *et al.*, 1999; Gougeon and Montagnier, 1999). In some situations such as MI, massive apoptosis may result in incomplete clearance of apoptotic cells and consequent inflammation. There is growing evidence that the primary antigens of autoimmne disease result from immunization against apoptotic blebs. In all pathological situations, we often know the outline of the origin of the pathology, but we need to understand it with far greater subtlety: the means by which a cell is forced into apoptosis, following what appears to be longterm, chronic stress; by misreading or misdelivery of transcellular signals by other cells (death of bystander cells); by a self-destructive turn of recruitment of phagocytic attack when a chronic infection allows T-cells to be recruited to destroy apoptotic blebs (in autoimmune disease); or the subtle changes in development or cancer in which, without abrogating the apoptotic response, patterns in which cell death occurs are shifted or the sensitivity of cells to normal controls is altered (Lockshin *et al.*, 2000).

1.4 Molecular biology of apoptosis

1.4.1 The basic apoptosis machinery

The past few years have seen significant advances in our understanding of both the machinery that executes apoptosis and the mechanisms by which this potentially catastrophic process is controlled. Much of our knowledge stems from studies of the developmentally invariant nematode *C. elegans*. During its ontogeny, 1090 cells are born, of which 131 die by suicide. Genetic analysis has identified four principal genes that are directly involved in the regulation and execution of each cell death : the products of the ced-3, ced-4 and egl-1 genes promote apoptosis, whereas the product of the ced-9 gene inhibits apoptosis (Meier and Evan, 1998). ced-3 encodes a caspase which is a cysteine protease that cleaves certain proteins after specific aspartic acid residues ; it exists as a zymogen which is activated through self-cleavage (Salvesen and Dixit, 1997 ; Thornberry and Lazebnik, 1998). The ATPase CED-4 binds to the inactive pro-form of CED-3 and facilitates autoactivation of the latter by oligomerization, whereas CED-9 interferes with this process by interacting with CED-4 (Figure 1). EGL-1 sequesters CED-9 and allows death to proceed in a CED-4/CED-3-dependent manner (Conradt and Horvitz, 1998). In living cells, CED-9 binds CED-4 on mitochondria, while in dying cells CED-4 is released from CED-9 and translocates to the nuclear membrane (Chen *et al.*, 2000).

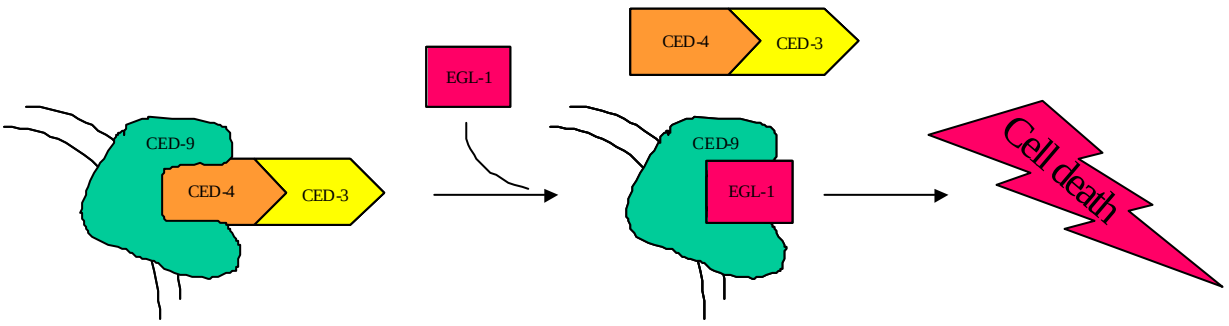

Figure 1 : Apoptosis in *C. elegans*

Vertebrates have evolved entire gene families that resemble *C. elegans* cell death genes. Mammalian caspases are similar to CED-3 (Salvesen and Dixit, 1997; Thornberry and Lazebnik, 1998). Apaf-1, Nod-1/CARD-4, Nod-2, DEFCAP and NAC are the only

mammalian CED-4 homologue known so far. The products of the mammalian Bcl-2 gene family are related to CED-9 but include two subgroups of proteins that either inhibit (anti-apoptotic) or promote (pro-apoptotic) apoptosis. These pro-apoptotic proteins might be the mammalian equivalent of EGL-1. But as expected, due to the higher degree of complexity of mammals, the mechanism of apoptosis is more elaborate and degenerate. For convenience, apoptosis can be divided into separate phases (Figure 2). The "initiation" phase, during which cells receive signals that may result in the activation of the death program. The "commitment"/"amplification" phase, the point after which death signals become irreversible and where multiple caspases are recruited to cooperate in the destruction of the cell. Finally, the "demolition" phase, where a panoply of active caspases dismantle cellular structures, either directly, or via activation of other enzymes such as CAD/DFF45 (Slee *et al.*, 1999a).

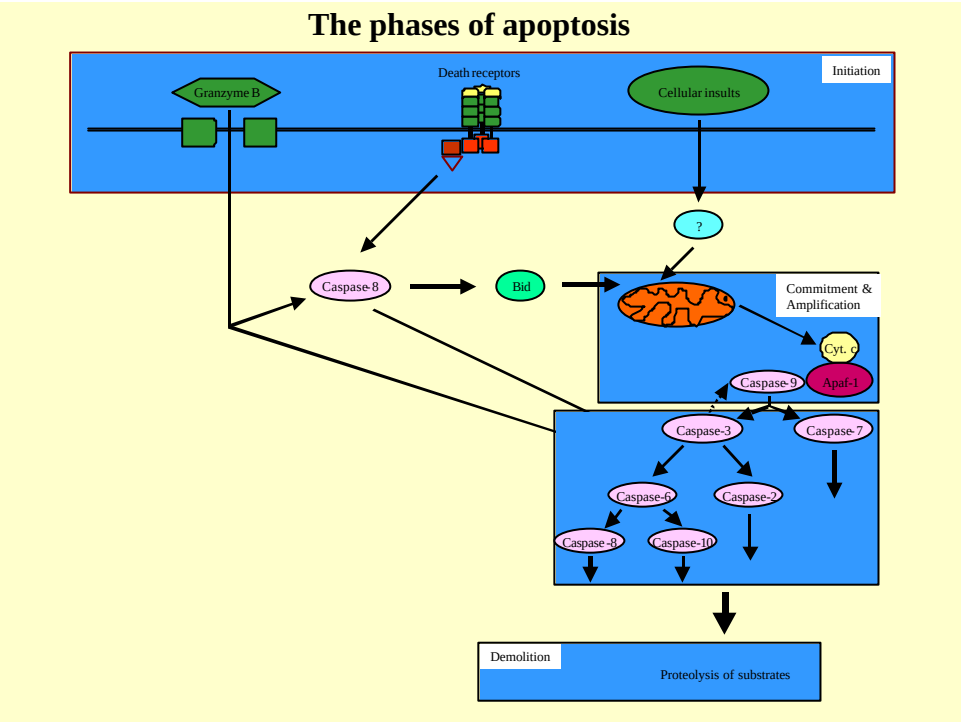

Figure 2 : Schematic representation of the routes to caspase activation within apoptosis.

1.4.2 The initiation phase

Initiators of the apoptosis machinery can be broadly grouped into three different categories : (a) death receptors, (b) the contents of cytotoxic T and NK cell granules, and (c) stimuli that provoke generalized cellular damage.

(a) Death receptor-initiated cell death signals

Death receptors belong to the tumor necrosis factor receptor (TNF-R) gene superfamily, which is defined by one to five similar, cysteine-rich extracellular domains (Smith *et al.*, 1994; Gruss and Dower, 1995). The death receptors contain in addition a homologous cytoplasmic sequence termed the "death domain" (DD) (Tartaglia *et al.*, 1993; Nagata, 1997). Death domains typically enable death receptors to engage the cell's apoptotic machinery, called the "extrinsic" pathway, but in some instances they mediate functions that are distinct

from or even counteract apoptosis. Some molecules that transmit signals from death receptors contain death domains themselves.
The best characterized death receptors are CD95 (also called Fas or Apo1) and TNFR1 (also called p55 or CD120a) (Smith *et al.*, 1994; Gruss and Dower, 1995; Nagata, 1997). Additional death receptors are death receptor 3 (DR3; also called Apo3, WSL-1, TRAMP, or LARD) (Chinnaiyan *et al.*, 1996a; Kitson *et al.*, 1996; Marsters *et al.*, 1996a; Bodmer *et al.*, 1997; Screaton *et al.*, 1997a), DR4 (also called TRAIL-R1) (Pan *et al.*, 1997a), DR5 (also called Apo2, TRAIL-R2, TRICK 2, or KILLER) (Chaudhary *et al.*, 1997; McFarlane *et al.*, 1997; Pan *et al.*, 1997b; Schneider *et al.*, 1997; Screaton *et al.*, 1997b; Sheridan *et al.*, 1997; Walczak *et al.*, 1997; Wu *et al.*, 1997), decoy receptor 1 (DcR1; also called TRID, TRAIL-R3, or LIT) (Degli-Esposti *et al.*, 1997a; McFarlane *et al.*, 1997; Pan *et al.*, 1997b; Schneider *et al.*, 1997; Sheridan *et al.*, 1997; Mongkolsapaya *et al.*, 1998), DcR2 (also called TRAIL-R4 or TRUNDD) (Degli-Esposti *et al.*, 1997b; Marsters *et al.*, 1997; Pan *et al.*, 1998b) and DcR3 (Pitti *et al.*, 1998). The p75 nerve growth factor (NGF) receptor also contains a death domain (Liepinsh *et al.*, 1997). The ligands that activate these receptors, with the exception of NGF, are structurally related molecules that belong to the TNF gene superfamily (Smith *et al.*, 1994; Gruss and Dower, 1995). CD95 ligand (CD95L) binds to CD95 and DcR3; TNF and lymphotoxin α bind to TNFR1; Apo3 ligand (Apo3L, also called TWEAK) (Chicheportiche *et al.*, 1997; Marsters *et al.*, 1998) binds to DR3 (Marsters *et al.*, 1998); and Apo2 ligand (Apo2L, also called TRAIL) (Wiley *et al.*, 1995; Marsters *et al.*, 1996b ; Pitti *et al.*, 1996;) binds to DR4 (Pan *et al.*, 1997a), DR5 (Chaudhary *et al.*, 1997; McFarlane *et al.*, 1997; Pan *et al.*, 1997b; Schneider *et al.*, 1997; Screaton *et al.*, 1997b; Sheridan *et al.*, 1997; Walczak *et al.*, 1997; Wu *et al.*, 1997;), DcR1 (Degli-Esposti *et al.*, 1997a; McFarlane *et al.*, 1997; Pan *et al.*, 1997b; Schneider *et al.*, 1997; Sheridan *et al.*, 1997; Mongkolsapaya *et al.*, 1998) and DcR2 (Degli-Esposti *et al.*, 1997b; Marsters *et al.*, 1997; Pan *et al.*, 1998b).

Signaling by CD95

CD95 is a widely expressed glycosylated cell-surface molecule of relative molecular mass ~45'000-52'000 (335 amino-acid residues). It is a type I transmembrane receptor, but alternative splicing can result in a soluble form (Cascino *et al.*, 1995). CD95 expression can be boosted by cytokines such as interferon-γ and TNF but also by the activation of lymphocytes (Klas *et al.*, 1993; Leithäuser *et al.*, 1993). CD95L is a TNF-related type II transmembrane molecule (Krammer, 1999).
CD95 and CD95L play an important role mainly in four types of physiologic apoptosis : (1) negative selection of pre-T lymphocytes that fail to rearrange their T-cell receptors (TCR) genes and CD95-positive B-cells killing by T-cells (Krammer, 2000); (2) peripheral deletion of activated mature T cells at the end of an immune response; (3) killing of targets such as virus-infected cells or cancer cells by cytotoxic T cells and by natural killer cells; and (4) killing of inflammatory cells at "immune-privileged" sites such as the testis and the eye. They are implicated also in pathological suppression of immune surveillance, namely, elimination of tumor-reactive immune cells by certain tumors that constitutively express CD95L. Furthermore, CD95L can be cleaved from the membrane by a metalloprotease (Mariani *et al.*, 1995; Yagita *et al.*, 1995; Tanaka *et al.*, 1996). This cleavage of membrane-bound FasL to a soluble form (sFasL) does not affect its ability to bind to Fas but drastically decreases its cytotoxic activity. Conversely, cross-linking epitope-tagged sFasL with anti-tag antibodies to mimic membrane-bound ligand results in a 1000-fold increase in cytotoxicity (Schneider and Tschopp, 2000).
Like other TNF family members, CD95L is a homotrimeric molecule. The crystal structure of lymphotoxin α in complex with TNFR1 suggests by analogy that each CD95L trimer binds

three CD95 molecules (Smith *et al.*, 1994; Gruss and Dower, 1995; Nagata, 1997; Ashkenazi and Dixit, 1998) (Figure 3). This oligomerization is required for transduction of the apoptotic signal. A complex of proteins associates with activated CD95 (Kischkel *et al.*, 1995; Peter *et al.*, 1998). This death-inducing signaling complex (DISC) forms within seconds of receptor engagement. First, the adaptor FADD (Fas-associated death domain protein, also known as Mort1) binds via its own death domain to the death domain in CD95. FADD also carries a so-called death-effector domain (DED), and, again by homologous interaction, recruits the DED-containing pro-caspase-8 (also known as FLICE, MACH or Mch5) or pro-caspase-10 (also known as Mch4) into the DISC (Krammer, 2000). The death effector domain is a specific example of a more global homophilic interaction domain termed CARD (caspase recruitment domain), which is found in several caspases, like caspase-2, -8, -9 and –10 (Hofmann *et al.*, 1997). Upon recruitment by FADD, caspase-8 (or –10) oligomerization drives its activation through self-cleavage (Muzio *et al.*, 1998). Next, active caspase-8 (or –10) is released from the DISC into the cytoplasm and cleaves various proteins in the cell including pro-caspase-3 and Bid, which results in their activation and completion of the cell death program. Various other proteins have been described to bind to activated CD95 and the DISC, such as them found in a certain class of herpes virus. These proteins contain two DEDs and inhibits the recruitment and activation of caspase-8, formerly known as FLICE ; hence their name v-FLIPs (for viral FLICE-inhibitory proteins). In transfected cells, v-FLIP inhibited the apoptosis induced by several apoptosis-inducing receptors (CD95, TNF-R1, TRAMP/DR3 and TRAIL-R1/DR4), indicating that these receptors use similar signaling pathways (Bertin *et al.*, 1997; Hu *et al.*, 1997; Thome *et al.*, 1997). Two human homologues of v-FLIP have been identified by several groups at the same time and are known under a variety of names. Although it is widely assumed that the cellular FLIPs (c-FLIP) block apoptosis as well, the data are ambiguous and c-FLIP might be pro-apoptotic or anti-apoptotic depending on the cellular context. Recent results with cells from c-FLIP-deficient mice support the role of c-FLIP as an anti-apoptotic molecule (Yeh *et al.*, 2000).
Studies with FADD gene knockout mice (Yeh *et al.*, 1998; Zhang *et al.*, 1998) and with transgenic mice expressing a dominant negative mutant of FADD (FADD-DN) in T cells (Newton *et al.*, 1998; Zornig *et al.*, 1998) establish that FADD is essential for apoptosis induction by CD95. Surprisingly, these mice display reduced proliferation of mature T cells in response to antigenic stimulation; moreover, FADD deletion causes embryonic lethality (Newton *et al.*, 1998; Yeh *et al.*, 1998; Zhang *et al.*, 1998; Zornig *et al.*, 1998). However, another sort of receptor, called decoy receptor 3 (DcR3) is able to inhibit apoptosis by blocking the CD95-FADD pathway. DcR3 has four cysteine-rich domains and is a secreted soluble protein which is able to bind to CD95L with an affinity equal to that of Fas, suggesting that DcR3 acts as a decoy receptor that competes with CD95 for binding to CD95 ligand (Pitti *et al.*, 1998).

Recently, with the use of fluorescence resonance energy transfer, another model of CD95 signaling has been worked out. Extracellular pre-ligand binding assembly domains (PLADs) were described for CD95 and TNF-R, which are supposed to aggregate the receptors before ligand binding. To prevent the premature signaling of pre-associated receptors, which is a dangerous situation, intracellular receptor-associated apoptosis blockers were postulated (Chan *et al.*, 2000; Siegel *et al.*, 2000). On the basis of the PLAD model, it is not entirely clear how ligand binding interferes with PLAD association and leads to receptor association, which initiates apoptosis. More structural work is needed to resolve these issues. It is also unclear whether the DISC model and the PLAD model complement each other to describe initial signaling events *in vivo* (Krammer 2000).

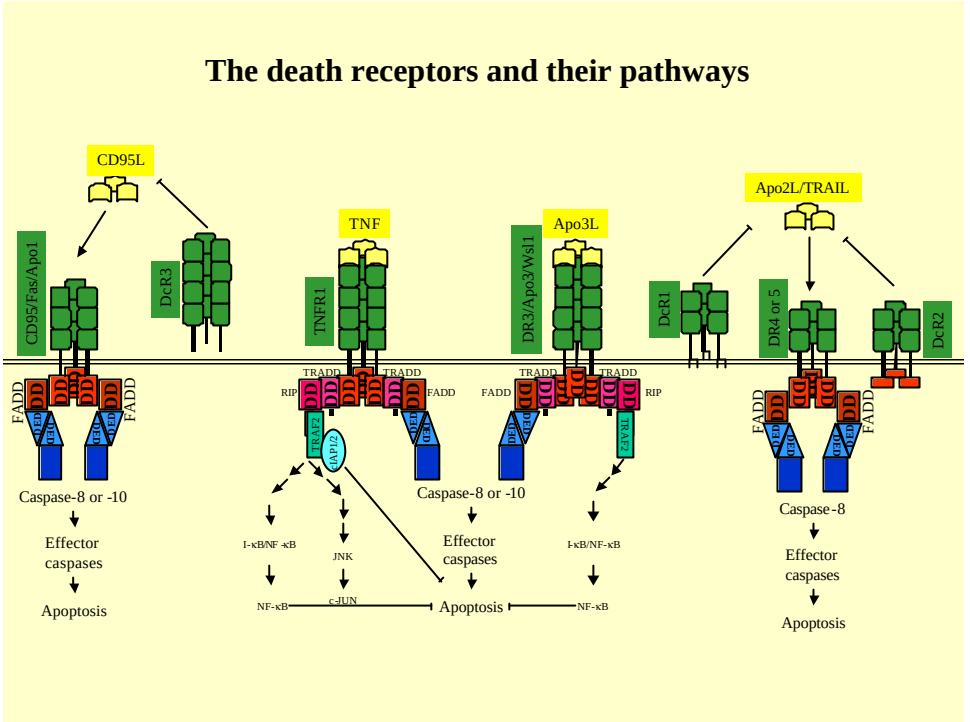

Figure 3 : The different death receptors with their respective ligands and adapters.

Signaling by other death receptors

Signaling of apoptosis by other members of the death receptor subfamily seems to follow the same basic rules and is initiated by the same sequential steps (Bodmer *et al.*, 2000; Kischkel *et al.*, 2000; Sprick *et al.*, 2000) : a) ligand binding, b) receptor trimerization and DISC formation, c) attraction of the adapter molecule FADD into the DISC, d) association of pro-caspase-8 (or –10), and e) autocatalytic cleavage of the pro-caspase and the formation of active caspase-8 (or –10). Active casapse-8 then serves as the initiator caspase, activating other further downstream executioner caspases that cleave cellular death substrates, resulting in the morphological and biochemical catastrophe termed apoptosis (Krammer, 2000).

TNFR1

TNF is produced mainly by activated macrophages and T cells in response to infection. By engaging TNFR1, TNF activates the transcription factor NF-κB and AP-1, leading to induction of pro-inflammatory and immunomodulatory genes (Tartaglia and Goeddel, 1992). In some cell types, TNF also induces apoptosis through TNFR1. Unlike CD95L, however, TNF rarely triggers apoptosis unless protein synthesis is blocked, which suggests the pre-existence of cellular factors that can suppress the apoptotic stimulus generated by TNF. Expression of these suppressive proteins probably is controlled through NF-κB and JNK/AP-1, as inhibition of either pathway sensitizes cells to apoptosis induction by TNF (Beg and Baltimore, 1996; Van Antwerp *et al.*, 1996; Wang *et al.*, 1996; Roulston *et al.*, 1998).
TNF trimerizes TNFR1 upon binding (Smith *et al.*, 1994; Gruss and Dower, 1995), inducing association of the receptors' death domains (Figure 3). Subsequently, an adapter termed TRADD (TNFR-associated death domain) (Hsu *et al.*, 1995) binds through its own death domain to the clustered receptor death domains. TRADD functions as a platform adapter that recruits several signaling molecules to the activated receptor : TNFR-associated factor-2

(TRAF-2) (Rothe *et al.*, 1995; Hsu *et al.*, 1996a) and receptor-interacting protein (RIP) (Hsu *et al.*, 1996b; Ting *et al.*, 1996) stimulate kinase pathways leading to activation of NF-κB and of JNK/AP-1, whereas FADD couples the TNFR1-TRADD complex to activation of caspase-8 (or –10), thereby initiating apoptosis (Chinnaiyan *et al.*, 1996b; Hsu *et al.*, 1996a; Varfolomeev *et al.*, 1996). TRAF-2 also binds to cIAP1 and cIAP2 (cellular inhibitor of apoptosis-1 and –2) (Shu *et al.*, 1996), which belong to a family of mammalian and viral proteins with anti-apoptotic activity.
Cells from FADD knockout mice are resistant to TNF-induced apoptosis, demonstrating an obligatory role of FADD in this response (Yeh *et al.*, 1998; Zhang *et al.*, 1998). Besides FADD, TNFR1 can engage an adapter called RAIDD or CRADD (Ahmad *et al.*, 1997; Duan and Dixit, 1997). RAIDD binds through a death domain to the death domain of RIP and through a CARD motif to a similar sequence in the death effector caspase-2, thereby inducing apoptosis (Ashkenazi and Dixit, 1998).

DR3

DR3 shows close sequence similarity to TNFR1 (Chinnaiyan *et al.*, 1996a; Kitson *et al.*, 1996; Marsters *et al.*, 1996a; Bodmer *et al.*, 1997; Screaton *et al.*, 1997a). Upon overexpression, DR3 triggers responses that resemble those of TNFR1, namely, NF-κB activation and apoptosis. Like TNFR1, DR3 activates NF-κB through TRADD, TRAF2, and RIP and apoptosis through TRADD, FADD and caspase-8 or –10 (Figure 3). DR3 binds to Apo3L, which is related most closely to TNF (Marsters *et al.*, 1998). Apo3L activates NF-κB through TRADD, TRAF2, RIP and triggers apoptosis through TRADD, FADD and caspase-8 (or –10), consistent with signaling through DR3. Thus, with respect to the regulation of NF-κB and apoptosis, Apo3L closely resembles TNF. There are notable differences, however, in the expression of these ligands and receptors. TNF expression occurs mainly in activated macrophages and lymphocytes (Tartaglia and Goeddel, 1992), whereas Apo3L messenger RNA is expressed constitutively in many tissues (Chicheportiche *et al.*, 1997; Marsters *et al.*, 1998). Conversely, TNFR1 is expressed ubiquitously (Tartaglia and Goeddel, 1992), whereas DR3 transcripts are present mainly in spleen, thymus, and peripheral blood and are induced by activation in T cells (Chinnaiyan *et al.*, 1996a; Kitson *et al.*, 1996; Marsters *et al.*, 1996a; Bodmer *et al.*, 1997; Screaton *et al.*, 1997a). Hence, despite overlapping signaling mechanisms, Apo3L-DR3 and TNF-TNFR1 interactions probably have distinct biological roles (Ashkenazi and Dixit, 1998).

DR4, DR5 and modulation by decoy receptors

A TNF family member that shows the most similarity to CD95L was identified independently by two groups who named it TRAIL or Apo2L (Wiley *et al.*, 1995; Pitti *et al.*, 1996). Similar to CD95L, Apo2L triggers rapid apoptosis in many tumor cell lines (Wiley *et al.*, 1995; Marsters *et al.*, 1996b; Pitti *et al.*, 1996; Mariani *et al.*, 1997; Mariani and Krammer, 1998). Unlike expression of CD95L, which is restricted mainly to activated T cells and NK cells, and to immune-privileged sites (Nagata, 1997), Apo2L messenger RNA expression is constitutive in many tissues (Wiley *et al.*, 1995; Pitti *et al.*, 1996); however, like CD95L, Apo2L transcription is elevated upon stimulation in peripheral blood T cells (Screaton *et al.*, 1997b; Jeramias *et al.*, 1998; Martinez-Lorenzo *et al.*, 1998). A subset of mature T cells acquires sensitivity to Apo2L-induced apoptosis after stimulation by interleukin-2, suggesting that Apo2L may play some role in peripheral T cell deletion (Marsters *et al.*, 1996b; Martinez-Lorenzo *et al.*, 1998). In addition, T cells from human immunodeficiency virus-infected

individuals show increased sensitivity to Apo2L, implicating this ligand in the killing of virus-infected cells (Jeramias *et al.*, 1998).
Apoptosis induction by TRAIL requires caspase activity (Marsters *et al.*, 1996b; Mariani *et al.*, 1997; Mariani and Krammer, 1998; Martinez-Lorenzo *et al.*, 1998). Overexpression of DR4 or DR5, which bind to Apo2L, triggers apoptosis via FADD and caspase-8 (Kischkel *et al.*, 2000) (Figure 3). Cells from FADD-deficient mice, which are resistant to apoptosis induction by CD95, TNFR1, and DR3, show also a jamming of TRAIL-induced apoptosis (Suliman *et al.*, 2001).
Like the Apo2L mRNA, DR4 and DR5 transcripts are expressed in several tissues, suggesting that there may be mechanisms that protect cells from apoptosis induction by Apo2L. One type of protection is based on a unique set of decoy receptors (DcR), which compete with DR4 and DR5 for binding to Apo2L (Golstein, 1997) (Figure 3). DcR1 (also called TRID, TRAIL-R3, or LIT) is a glycosyl phophatidylinositol (GPI)-anchored cell surface protein that resembles DR4 and DR5, but lacks a cytoplasmic tail. DcR1 binds to Apo2L, and its transfection into Apo2L-sensitive cells substantially reduces responsiveness to the ligand (Pan *et al.*, 1997b; Sheridan *et al.*, 1997; Mongkolsapaya *et al.*, 1998). Treatment of DcR1-bearing cells with a phospholipase that cleaves the GPI anchor results in marked sensitization to Apo2L-induced apoptosis (Sheridan *et al.*, 1997). Thus, DcR1 appears to function as a decoy that prevents Apo2L from binding to its death receptors. DcR2 (also called TRAIL-R4 or TRUNDD) is another receptor that resembles DR4 and DR5, but it has a substantially truncated cytoplasmic death domain. DcR2 transfection inhibits apoptosis induction by Apo2L; deletion of the DcR2 cytoplasmic region does not abrogate the inhibitory activity (Marsters *et al.*, 1997), indicating that this receptor acts as a decoy that competes with DR4 and DR5 for binding to Apo2L. Overexpression of DcR2 seems to activate NF-κB. Osteoprotegerin (OPG) is a secreted, soluble receptor for the more distantly TNF homologue TRANCE/RANKL/OPGL. OPG is also able to bind to Apo2L/TRAIL and inhibits apoptosis induction by Apo2L suggesting that it is a decoy receptor for this latter ligand (Emery *et al.*, 1998).

More recently, a new member of the tumor necrosis factor (TNF) receptors family, termed death receptor-6 (DR6) was discovered. Like other death receptors, DR6 is a type I transmembrane receptor, possesses four extracellular cysteine-rich motifs and a cytoplasmic death domain. It is expressed in most human tissues and abundant transcript was detected in heart, brain, placenta, pancreas, thymus, lymph node and several non-lymphoid cancer cell lines. DR6 interacts with TRADD, which has previously been shown to associate with TNFR1. Furthermore, ectopic expression of DR6 in mammalian cells induces apoptosis and activation of both NF-kappaB and JNK. However, the ligand of DR6 is still unknown (Pan *et al.*, 1998c).

(b) Cytotoxic lymphocyte-initiated death signals

Cytotoxic lymphocytes (T and NK cells) contain granules that can be discharged onto the surface of target cells, delivering what has been called “the kiss of death” (Figure 2). These granules contain, amongst other things, granzyme B (also called fragmentin-2), a serine protease which cleaves after Asp residues, and a pore-forming protein called perforin (or cytolysin) that is likely to permit entry of the other granule components into the target cell. This immediately suggests a mechanism for cytotoxic lymphocyte-initiated apoptosis where granzyme B enters the cell, with the help of perforin, and triggers the caspase cascade by directly cleaving and activating caspases (Darmon *et al.*, 1995; Martin *et al.*, 1996). Nevertheless, granzyme B was recently demonstrated to enter cells in a perforin-independent manner, thus predicting the existence of a cell surface receptor, which is the cation-

independent mannose 6-phosphate/insulin-like growth factor receptor (CI-MPR) (Motyka *et al.*, 2000).
While it has been demonstrated that granzyme B (GzmB) can cleave most of the caspases *in vitro*, it appears that in cells its preferred target is caspase-8 (Medema *et al.*, 1997) or caspase-3 (Yang *et al.*, 1998a), which then proceeds to activate downstream caspases. Nonetheless, the absolute requirement of caspase activation for GzmB-induced apoptosis is controversial. Another report demonstrated that GzmB can initiate apoptosis in the absence of caspase-3 activity by directly cleaving DFF45/ICAD to liberate activated DFF40/CAD. DFF45/ICAD cleavage occurs less efficiently in cells that lack caspase-3 activity, suggesting that the caspases normally amplify the GzmB death signal. DFF45/ICAD-deficient mouse embryo fibroblasts are partially resistant to GzmB-induced death, demonstrating the biological importance of DFF45/ICAD for GzmB-mediated apoptosis (Thomas *et al.*, 2000). Moreover, more recently, a group demonstrated that mitochondrial cytochrome c release is the primary mode of GzmB-induced apoptosis and that Bcl-2 is a potent inhibitor of this pivotal event. Caspase activation is not required for cytochrome c release, an activity that correlates with cleavage and activation of Bid, which they have found to be cleaved more readily by granzyme B than either caspase-3 or caspase-8. Bcl-2 blocks the rapid destruction of targets by granzyme B by blocking mitochondrial involvement in the process (Pinkoski *et al.*, 2001).

(c) Generalized cellular damage-initiated death signals

All other stimuli that can provoke apoptosis have been grouped into this category, mainly because it is still far from clear how these stimuli engage the caspases components of the death machinery. This group includes diverse apoptosis-promoting stimuli such as chemotherapeutic or cytotoxic drugs (for example staurosporine), gamma- and UV-radiation, DNA damaging agents, heat shock, survival factor deprivation (for example NGF) and other cellular stresses like changes in redox state, energy metabolism or ionic concentrations (Ca^{2+}, K^{+}). The signals that these stimuli evoke within the cell are disparate, but the death pathways (also called the "intrinsic" pathway) engaged by the vast bulk of these stresses seem to converge on the mitochondria (Kluck *et al.*, 1997). Furthermore, the anti-apoptotic Bcl-2 family members (Bcl-2 and Bcl-xL) protect in the majority of these cases, again implicating the mitochondrial pathway as an important conduit for death signals triggered by stimuli within this group (Figure 2).

1.4.3 The commitment/amplification phase

Mitochondria are the well-known "powerhouses" of the cell, producing ATP to drive the various physiological functions and to maintain life itself. But they are also intimately involved in cell demise. The first sign that mitochondria play an active role in apoptosis came in 1994 with the observation that induction of nuclear apoptotic changes (chromatin condensation, nuclear fragmentation) in extracts of *Xenopus* eggs required the presence of mitochondria (Newmeyer *et al.*, 1994). The stage was thus set for a period of rapid advance, leading to radically new insights into mitochondrial function (Crompton, 2000) such as the finding that, in apoptosis triggered by many stimuli, mitochondria play a pivotal role in coordinating caspases activation through the release of cytochrome c (Desagher and Martinou, 2000).
Mitochondria are organized into several distinct spaces, based on reconstructions of mitochondria imaged by electron tomography (Perkins *et al.*, 1997; Manella *et al.*, 1998). The matrix is filled with the enzymes of the tricarboxylic acid cycle. Projecting through the matrix

space are hollow flattened or tubular structures, the cristae. The crista membranes are continuous with the rest of the inner mitochondrial membrane, and contain the electron transfer complexes. The intermembrane space can be functionally separated into two compartments, the cristal lumen and the intermembrane space that lies between the inner membrane and the outer membrane. It is estimated that 85-97% of the cytochrome c resides in the crystal lumen, with the remainder in the intermembrane space (Bernardi *et al.*, 1999; Scorrano *et al.*, 1999). Cytochrome c is electrostatically associated with cytochrome c reductase and cytochrome oxidase, and is also associated with cardiolipin, which is asymmetrically distributed across the lipid bilayer of the inner membrane. Also present in the intermembrane are pro-caspases-2, -3 and –9, apoptosis-inducing factor (AIF) (Mancini *et al.*, 1998; Susin *et al.*, 1999a), and Smac/DIABLO (Du *et al.*, 2000; Verhagen *et al.*, 2000). The F_0F_1-ATPase decorates the matrix side of the crista membrane. The inner membrane/crista membrane is highly resistant to proton leakage, allowing the buildup of a significant membrane potential (about –220 mV). The accumulated protons on the outer face of the inner membrane (in the lumen of the cristae) leak back down their electrochemical gradient through the F_0F_1-ATPase to drive ATP synthesis. Cardiolipin is an anionic phospholipid that is only found in the mitochondrial inner membrane and is believed to contribute to the proton-impermeable nature of the inner membrane. The outer mitochondrial membrane is generally considered to be permeable to small molecules (< 1000 Da) because of the presence of a very abundant protein, the voltage-dependent anion channel (VDAC). VDAC comprises as much as 20% of the protein of the outer membrane and is ordinarily open. The outer membrane is dimpled with contact points where the outer and inner membranes are closely juxtaposed. Contact points may represent two different biochemical entities: the protein translocation machinery made up of the translocase outer and inner membrane complexes (reviewed in Herrmann *et al.*, 2000), and the components of the nucleotide transport system, including VDAC, the adenine nucleotide translocase (ANT) and creatine kinase. These latter components are also constituents of the permeability transition pore (PTP), whose opening is often believed to herald mitochondrial destruction and certain cell death. The PTP can be reconstituted with VDAC, ANT, and cyclophilin D (reviewed in Crompton, 1999). The association of cyclophilin D with the PTP is important, as it underlies the ability of cyclosporin A (ligand of cyclophilin D and inhibitor of this latter) to prevent pore opening and mitochondrial dysfunction with cyclosporin A (Andreeva *et al.*, 1995). Additional proteins that may co-purify with the PTP include hexokinase, the peripheral benzodiazepine receptor, creatine kinase and the Bcl-2 family member Bax (Marzo *et al.*, 1998a; Gottlieb, 2000).

Mitochondria and the Bcl-2 family proteins

Currently, at least 15 Bcl-2 family proteins have been identified in mammals and several others in viruses (Chao and Korsmeyer, 1998; Gross *et al.*, 1999). Bcl-2 family members can be grouped into three categories (Figure 4):

1. Anti-apoptotic members such as Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1 (Bfl-1) and Boo, all of which exert anti-cell death activity and share sequence homology, particularly within four regions, Bcl-2 homology (BH) 1 through BH4, although some members lack an apparent BH4 domains.
2. Pro-apoptotic members such as Bax, Bak, Bad, Bok (Mtd), Diva, which share sequence homology in BH1, BH2 and BH3 but not in BH4, although significant homology at BH4 has been noticed in some members.

3. "BH3-only proteins", the pro-apoptotic proteins which include Bik, Bid, Bim, Hrk (DP5), Blk, Bnip3 and Bnip3L, and share sequence homology only in BH3.

In addition, many of the Bcl-2 proteins contain a hydrophobic C-terminal tail that is responsible for localizing the proteins predominantly to the mitochondrial outer membrane, but also to the endoplasmic reticulum (ER) and nuclear membranes (Desagher and Martinou, 2000; Fesik, 2000).
One of the unique features of Bcl-2 family proteins is heterodimerization between anti-apoptotic and pro-apoptotic proteins, which is considered to inhibit the biological activity of their partners (Oltvai *et al.*, 1993; Yang *et al.*, 1995). In fact, the relative levels of these proteins in a cell determine whether a cell will live or die (Yin *et al.*, 1994). Mutagenesis established that the BH1, BH2, and BH3 domains strongly influence homo- and heterodimerization (Yin *et al.*, 1994; Chittenden *et al.*, 1995; Kelekar and Thompson, 1998), and the three-dimensional structure of Bcl-xL provided the explanation. Coalescence of the α helices in its BH1, BH2 and BH3 regions creates an elongated hydrophobic cleft, to which a BH3 amphipathic α helix can bind (Sattler *et al.*, 1997). BH3-cleft coupling, reminiscent of ligand-receptor engagement, may account for all dimerization within the family. In addition to the BH1 and BH2, the BH4 domain is required for anti-apoptotic activity (Huang *et al.*, 1998). In contrast, BH3 is essential and, itself, sufficient for pro-apoptotic activity (Chittenden *et al.*, 1995).

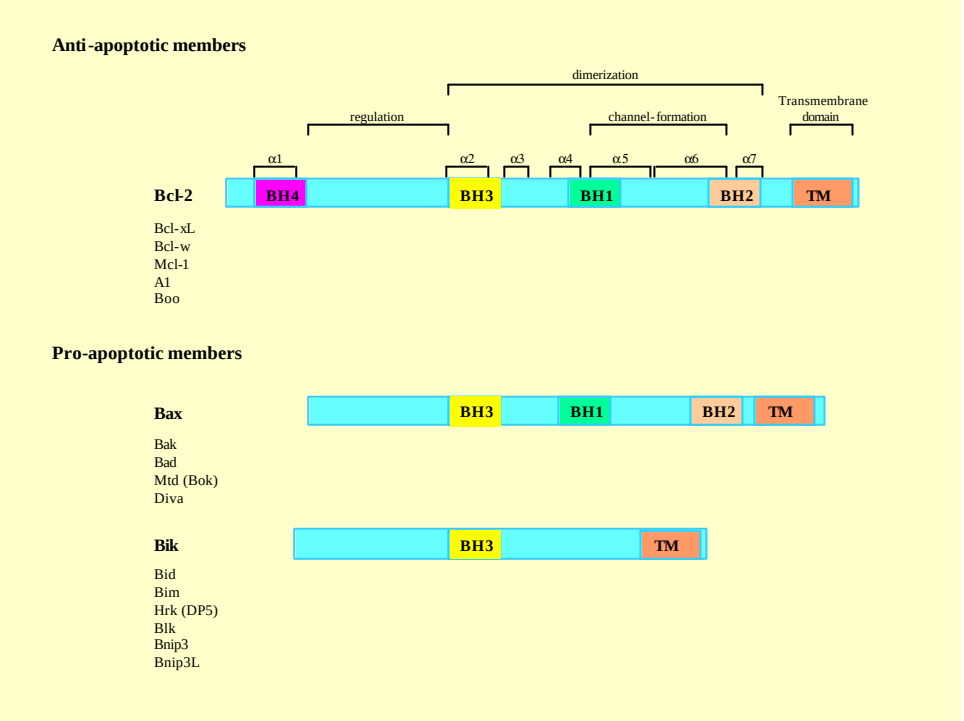

Figure 4 : Schematic drawing of Bcl-2 and related proteins.
BH1 to BH4 are conserved sequence motifs. Several functional domains of Bcl-2 are shown (see text). α1-α7 indicate helices identified in Bcl-xL, in which a core of two hydrophobic helices α5 and α6 is surrounded by five amphipathic helices. The region between α1 and α2 is called a flexible, non-conserved loop which appears to have a negative regulatory role in the signaling of apoptosis. A membrane-anchoring domain is not carried by all members of the family (Tsujimoto and Shimizu, 2000).

The principal mechanism by which Bcl-2 family proteins regulate apoptosis is probably by controlling the release of apoptogenic factors from mitochondria, such as cytochrome c. Overexpression of the anti-apoptotic proteins Bcl-2 or Bcl-xL in many cell types subjected to various cytotoxic treatments prevents cytochrome c release from mitochondria, caspase activation and cell death (Kluck *et al.*, 1997; Vander Heiden *et al.*, 1997; Yang *et al.*, 1997). Moreover, the use of cell-free systems indicates that exogenous Bcl-2 must be associated with mitochondria to prevent cytochrome c release (Kluck *et al.*, 1997). Conversely, ectopic expression of the death promoter Bax triggers cytochrome c release from mitochondria in the absence of any death signal (Eskes *et al.*, 1998; Jürgensmeyer *et al.*, 1998; Pastorino *et al.*, 1998; Rossé *et al.*, 1998; Finucane *et al.*, 1999). This loss of cytochrome c is associated with caspase activation and the induction of apoptosis. The direct addition of Bax to isolated mitochondria also induces cytochrome c release (Eskes *et al.*, 1998; Jürgensmeyer *et al.*, 1998; Narita *et al.*, 1998; Finucane *et al.*, 1999; Kluck *et al.*, 1999; Pastorino *et al.*, 1999). These effects can be blocked by overexpression of Bcl-xL in intact cells or addition of recombinant Bcl-xL to isolated mitochondria (Jürgensmeyer *et al.*, 1998; Narita *et al.*, 1998; Finucane *et al.*, 1999). However, caspase inhibitors do not affect Bax-induced cytochrome c leakage but they do effectively block caspase activation and delay apoptosis (Eskes *et al.*, 1998; Jürgensmeyer *et al.*, 1998; Rossé *et al.*, 1998; Finucane *et al.*, 1999). These data thus suggest that Bax can directly induce the loss of cytochrome c and indicate that caspases do not participate in this event (Desagher and Martinou, 2000).

Although Bcl-2 appears to be exclusively membrane bound, particularly in mitochondria and endoplasmic reticulum/nuclear membranes, other related proteins (e.g. Bid, Bad and Bim) are cytosolic but translocate to mitochondria during apoptosis (Desagher *et al.*, 1999; Downward, 1999; Puthalakath *et al.*, 1999). These proteins play a major role in transducing signals from the cytosol to mitochondria, where they bind to and regulate the activity of the Bcl-2 family members that control the release of cytochrome c. Translocation of these proteins is triggered by specific posttranslational modifications such as dephosphorylation by the Ca^{2+}-sensitive phosphatase calcineurin or the protein phosphatase 1α following a growth-factor deprivation (in the case of Bad, Downward, 1999) or by the death receptor and cytotoxic T/NK cell granule-initiated pathways which provoke the caspase-dependent route to mitochondrial damage via the cleavage of caspase-8 (in the case of Bid, Figure 2, Budihardjo *et al.*, 1999). Similarly, under normal conditions, Bax appears to be predominantly cytosolic and, in some cells, in peripheral association with mitochondria (Desagher and Martinou, 2000).

Following a death receptor-independent stimuli via as yet unidentified factors (Figure 2), several changes affect Bax that lead to its activation. First, Bax translocates from cytosol to mitochondria (Hsu *et al.*, 1997; Gross *et al.*, 1998) unless Bax is already loosely attached to the organelle (Goping *et al.*, 1998; Desagher *et al.*, 1999). Then, the conformation of Bax changes, unmasking its N-terminus (Goping *et al.*, 1998; Desagher *et al.*, 1999). This is accompanied by an apparent oligomerization, which was detected by cross-linking experiments (Gross *et al.*, 1998; Eskes *et al.*, 2000). Finally, Bax inserts into the outer membrane of mitochondria and becomes an integral membrane protein (Goping *et al.*, 1998; Eskes *et al.*, 2000). This is rapidly followed by cytochrome c release from mitochondria. Interestingly, it has been shown that enforced dimerization of Bax is sufficient to induce Bax translocation, mitochondrial dysfunction and apoptosis, which indicates that the mitochondrial localization of Bax and its oligomerization play a crucial role in triggering apoptosis (Gross *et al.*, 1998; Desagher and Martinou, 2000).

These changes in Bax can be prevented by Bcl-2 and Bcl-xL (Gross *et al.*, 1998; Desagher *et al.*, 1999), and they can be reproduced in isolated mitochondria by treatment with recombinant full-length or truncated Bid (Desagher *et al.*, 1999; Eskes *et al.*, 2000). Both Bcl-xL and Bid seem to exert their influence by interacting directly with Bax (Desagher *et al.*,

1999; Eskes *et al.*, 2000). Taken together, these data suggest that Bax or a Bax-like protein act as kind of on-off switch regulating cytochrome c release, and that Bax or a Bax-like protein is the focus of many apoptosis-regulating pathways (Desagher and Martinou, 2000).
Finally, most effects of the Bcl-2 family proteins that have been described seem to be conditional on their mitochondrial localization. This does not exclude the possibility that Bcl-2-related proteins could influence apoptosis independently of mitochondria. Consistent with this thesis, some Bcl-2 mutants that are not addressed to the mitochondrial membrane retain significant ability to inhibit apoptosis (Oltvai *et al.*, 1993). Similarly, apoptosis in different cell lines induced by microinjection of cytochrome c is strongly reduced by Bcl-2 overexpression (Zhivotovsky *et al.*, 1998). In some circumstances, Bcl-2 has also been shown to inhibit Bax-induced caspase activation and cell death without blocking cytochrome c release (Rossé *et al.*, 1998). Taken together, these observations suggest that anti-apoptotic proteins can also act downstream of cytochrome c release (Desagher and Martinou, 2000).
Recently, Goldstein *et al.* (2000) studied the kinetics of cytochrome c release from mitochondria using cytochrome c tagged with green fluorescent protein (GFP) and found that, during apoptosis induced by a variety of stimuli, all of the cytochrome c-GFP was released from all mitochondria of the cell within 5 min. They also reported that the release was temperature-independent. Although these observations were made using an artificial protein, they argue strongly against the involvement of enzymatic transport systems. However, what exactly are the mechanisms that underlie cytochrome c release ? Two prevailing theories have been postulated: the non-specific rupture of the outer mitochondrial membrane and the formation of cytochrome c-conducting channels (Desagher and Martinou, 2000).

The non-specific outer mitochondrial membrane rupture

According to the first theory, water and solutes enter the matrix during apoptosis, causing swelling of the mitochondria (Figure 5a,b). Because the inner membrane, with its numerous cristae, has a considerably larger surface area than the outer membrane, expansion of the inner membrane upon matrix swelling can break the outer membrane, and such rupture has been observed in different apoptotic systems (Vander Heiden *et al.*, 1997). This would be expected to trigger the release and irreversible dilution in the cytosol of the whole content of the intermembrane space, because it is no longer constrained by the outer membrane. However, the content of the matrix is retained in mitochondria because the inner membrane remains intact even though it expands (Desagher and Martinou, 2000).
Two models can account for matrix swelling. The first model implicates the initial hyperpolarization of the inner membrane that precedes cytochrome c release in some systems (Figure 5a) (Vander Heiden *et al.*, 1997; Samali *et al.*, 1999). According to Vander Heiden *et al.* (1999a), this hyperpolarization might result from the inability to exchange mitochondrial ATP for cytosolic ADP during apoptosis. This antiport is normally mediated by the voltage-dependent anion channel (VDAC, or mitochondrial porin) and the adenylate translocator (ANT). Impairment of ATP-ADP exchange by VDAC closure should inhibit F_1F_0-ATPase activity, resulting in an inhibition of H^+ re-entry to the matrix, and should thereby contribute to the hyperpolarization of the inner mitochondrial membrane. Such an increase of the mitochondrial transmembrane potential ($\Delta\psi_m$) is predicted to promote an osmotic matrix swelling (Vander Heiden *et al.*, 1999b). However, these hypothetical mechanisms conflict with a second model that implicates a mitochondrial megachannel called the “permeability transition pore” or PTP (Figure 5b) (Desagher and Martinou, 2000).
The PTP is a high-conductance unselective channel whose opening can be triggered by several physiological effectors such as Ca^{2+}, reduced concentrations of adenine nucleotides, inorganic phosphate, reactive oxygen species (ROS), changes in pH or low $\Delta\psi_m$ (Crompton,

1999) and possibly Bax (Marzo *et al.*, 1998a). PTP opening causes a sudden increase in the permeability of the inner mitochondrial membrane to molecules of mass < 1.5 kDa. This event, the "permeability transition", results in the immediate dissipation of the proton-dependent $\Delta\psi_m$ and chemical equilibration between the cytoplasm and mitochondrial matrix (Crompton, 1999). This causes a progressive osmotic swelling of the matrix owing to its high solute concentration and can ultimately lead to disruption of the outer membrane (Desagher and Martinou, 2000).

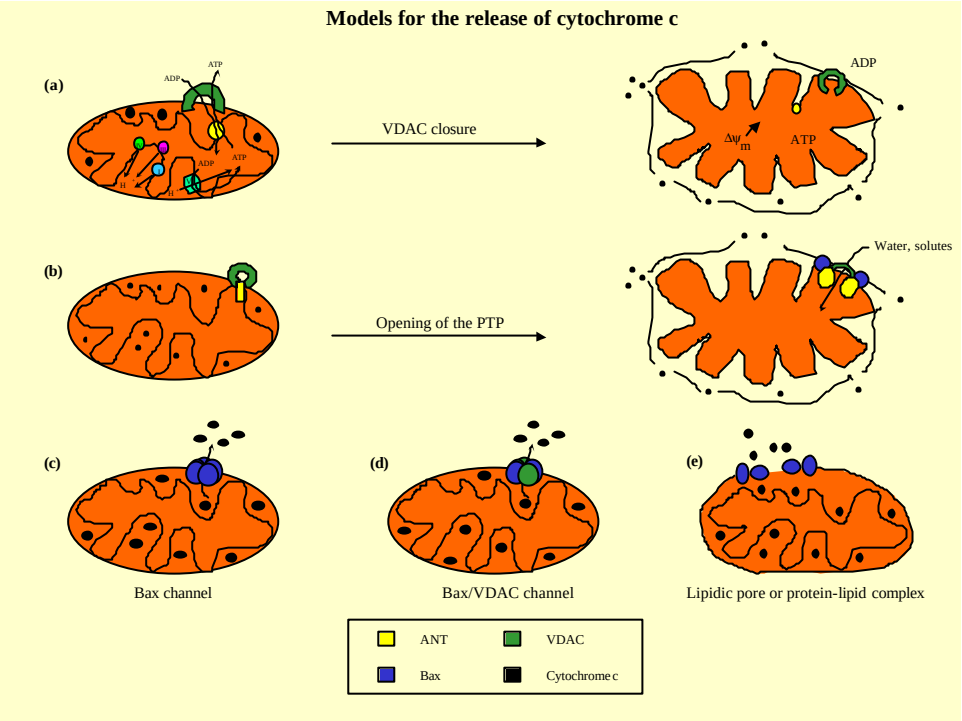

Figure 5 : Models for the cytochrome c release from mitochondria during apoptosis. (a,b) The outer mitochondrial membrane ruptures as a result of swelling of the mitochondrial matrix, allowing cytochrome c and other proteins to escape from the intermembrane space. Model (a) involves closure of the voltage-dependent anion channel (VDAC) and impairment of ATP-ADP exchange. Under normal conditions (left), the protons that accumulate in the intermembrane space as a result of electron transport re-enter the mitochondrial matrix through F_1F_0-ATPase (complex V of the respiratory chain). By inhibiting F_1F_0-ATPase, impairment of ATP-ADP exchange results in the accumulation of protons in the intermembrane space and an increase in the mitochondrial transmembrane potential ($\Delta\psi_m$), which is predicted to promote osmotic swelling (right). Model (b) suggests that the permeability-transition pore (PTP) opens and that the inner membrane permeability increases, causing mitochondrial–matrix swelling. Bax might induce this pore to open by binding to the adenine-nucleotide translocator (ANT) at the contact sites between the inner and outer membranes or after translocation to the inner membrane. (c-e) A large channel forms in the outer mitochondrial membrane, allowing cytochrome c release, but mitochondria are not damaged. This channel could be formed by Bax only (c), Bax in association with VDAC (d) or could be a lipidic channel or protein-lipid complex (e) formed after Bax insertion into the outer mitochondrial membrane (Desagher and Martinou, 2000).

The rupture of the outer mitochondrial membrane is, of course, an attractive hypothesis because it could explain the release of many proteins from the intermembrane space such as

cytochrome c, Smac/DIABLO, some caspases, AIF (57 kDa), Hsp60, Hsp10, adenylate kinase (25.2 kDa) and sulfite oxidase (104 kDa) (Kluck *et al.*, 1999; Samali *et al.*, 1999; Susin *et al.*, 1999b). Moreover, members of the Bcl-2 family can regulate opening of the PTP (Marzo *et al.*, 1998b). Bcl-2 can prevent the PTP-mediated mitochondrial depolarization in intact cells and isolated mitochondria (Kroemer *et al.*, 1997a; Shimizu *et al.*, 1998), but Bax can provoke $\Delta\psi_m$ loss, matrix swelling and cytochrome c release in some cell types and isolated mitochondria in a cyclosporin A-inhibitable fashion (Marzo *et al.*, 1998; Narita *et al.*, 1998; Pastorino *et al.*, 1998; Pastorino *et al.*, 1999). Bax could trigger PTP opening through binding to ANT, as suggested by co-immunoprecipitation and yeast two-hybrid studies (Marzo *et al.*, 1998). However, such a physical rupture has rarely been described and many data suggest that it is more likely to be a consequence than a cause of cytochrome c release. Moreover it has been shown that recombinant Bid and Bax cause complete cytochrome c loss from isolated mitochondria *in vitro*, but preserve the ultrastructure and protein import function of mitochondria, which depend on inner membrane polarization (Von Ahsen *et al.*, 2000). This argues for the existence of other mechanisms responsible for the initial cytochrome c leakage (Desagher and Martinou, 2000).

The formation of cytochrome c-conducting channels

According to the second theory, channels are formed that are large enough for the passage of soluble proteins (Figure 5c-e). One clue to how Bcl-2 family proteins exert their mitochondrial activity has come from the three-dimensional structure of Bcl-xL (Muchmore *et al.*, 1996). Bcl-xL consists of two central, predominantly hydrophobic, α helices (α5 and α6, Figure 4) surrounded by five amphipathic helices (Muchmore *et al.*, 1996). A similar structure can be predicted for other Bcl-2 family members such as Bcl-2 and Bax, which have a high degree of sequence homology with Bcl-xL (Schendel *et al.*, 1998). Surprisingly, however, the structure of Bid is also similar to that of Bcl-xL, even though the amino acid sequence identity between Bid and Bcl-xL is limited to the 16-residue BH3 domain (Chou *et al.*, 1999; McDonnell *et al.*, 1999; Desagher and Martinou, 2000).
Importantly, the structure of Bcl-xL and Bid resembles that of the pore-forming domains of diphteria toxin and some bacterial colicins (Muchmore *et al.*, 1996), which can form channels for ions and proteins. Bcl-xL, Bcl-2, Bax (Schendel *et al.*, 1998) and the truncated form of Bid (Schendel *et al.*, 1999) have been shown to form functional ion channels in synthetic lipid vesicles and planar lipid bilayers. In a similar way to bacterial toxin pores, these channels have multiple conductance states, are pH and voltage-sensitive, and show a low ion selectivity. The intrinsic channel properties of pro- and anti-apoptotic proteins differ significantly, which might account for their opposite influences on cytochrome c release (Schlesinger *et al.*, 1997). More interestingly, Bcl-2 is able to prevent Bax-channel formation in liposomes (Antonsson *et al.*, 1997; Desagher and Martinou, 2000).
These data, taken together with the fact that the addition of Bax or Bak directly to mitochondria triggers cytochrome c release, suggest that these pro-apoptotic members of the Bcl-2 family could form mitochondrial channels. Could these channels be responsible for the outer mitochondrial membrane permeabilization that occurs during apoptosis ? Bcl-2-related proteins have only two helices long enough to span a membrane bilayer, which is insufficient to form an aqueous channel. However, Bax can oligomerize under some conditions, allowing it to form very high-conductance channels in liposomes and to trigger cytochrome c release from mitochondria (Antonsson *et al.*, 2000). Oligomerization, a process so far described only for Bax, might represent the mechanism by which pro-apoptotic proteins form megachannels (Figure 5c).

Alternatively, it has been found that Bax and the truncated form of Bid (tBid), but not Bcl-xL, can decrease the stability of planar phospholipid bilayers, suggesting that pro-apoptotic proteins might act directly by destabilizing the outer mitochondrial membrane (Basanez *et al.*, 1999; Kudla *et al.*, 2000). By reducing the linear tension of the membrane, Bax and tBid could promote the formation of a lipidic pore or a protein-lipid complex large enough to allow intermembrane proteins to diffuse into the cytosol (Figure 5e) (Desagher and Martinou, 2000).

A third possibility is that Bax cooperates with VDAC to form a large cytochrome c-conducting channel (Figure 5d). When VDAC is reconstituted in liposomes, Bak and Bax promote opening of the channel, whereas Bcl-xL facilitates its closure. Furthermore, monitoring the movement of fluorescein-labelled cytochrome c indicates that Bax and Bak allow cytochrome c to pass through VDAC, possibly by inducing a conformational change or by cooperating with VDAC to form a megapore. However, none of Bax, Bak or VDAC is able by itself to form channels permeable to cytochrome c in liposomes (Shimizu *et al.*, 1999).

In summary, the channel theory offers the advantage of maintaining functional mitochondria during the time necessary for the activation of caspases, because the latter process requires ATP. Nevertheless, we have thus far no evidence that Bcl-2 family proteins do form channels *in vivo*. Whether the lumen diameter of these pores would be sufficient to allow the passage of cytochrome c and other proteins is even less certain (Desagher and Martinou, 2000).

Regulation of mitochondrial homeostasis by anti-apoptotic Bcl-2 family proteins

As already described, mitochondria play a vital role in the cell, providing most of the cell's energy and participating in the Ca^{2+}, redox and pH homeostasis. This means that a major mitochondrial dysfunction is likely to cause cell death. For instance, disruption of electron transport might be responsible for the increased production of reactive oxygen species (ROS) and the cytoplasmic acidification that are observed early during apoptosis. In such circumstances, the electrons that escape in large amounts from the respiratory chain can reduce molecular oxygen to produce superoxide anions, and the reduced ATP synthesis promotes the accumulation of lactate by stimulating glycolysis (Desagher and Martinou, 2000).

Consistent with the idea that mitochondrial status can influence the life-death decision, anti-apoptotic Bcl-2 proteins have also been implicated in the protection of mitochondrial integrity. One of the first anti-apoptotic functions attributed to Bcl-2 was the reduction of ROS levels (Vander Heiden *et al.*, 1999b). Since then, Bcl-2 has been shown to stabilize $\Delta\psi_m$, to affect mitochondrial proton influx and to modulate mitochondrial Ca^{2+} homeostasis (Kroemer *et al.*, 1997a; Shimizu *et al.*, 1998; Zhu *et al.*, 1999); similarly, Bcl-xL has been implicated in the regulation of mitochondrial metabolism and matrix volume (Vander Heiden *et al.*, 1997; Vander Heiden *et al.*, 1999a). Some of these effects can be ascribed to the blockade of cytochrome c release by Bcl-2 and Bcl-xL because the depletion of cytochrome c from mitochondria can impair electron transport and be responsible for some types of mitochondrial dysfunction (Desagher and Martinou, 2000).

Alternatively, the control of cytochrome c release by anti-apoptotic proteins might be the indirect result of their effects on mitochondrial homeostasis. For example, Bcl-2 can maintain $\Delta\psi_m$ in response to various agents such as Ca^{2+} and ROS by enhancing proton efflux (Shimizu *et al.*, 1998). By offsetting ion imbalances that would otherwise result in a lowered $\Delta\psi_m$ and an increased probability of PTP opening, Bcl-2 could prevent cytochrome c redistribution. Similarly, Bcl-xL appears to inhibit cytochrome c release by maintaining mitochondrial ADP-ATP exchange and by preventing mitochondrial swelling (Vander Heiden *et al.*, 1997; Vander

Heiden *et al.*, 1999a). Therefore, Bcl-2 and Bcl-xL seem to regulate an aspect of mitochondrial physiology more general than the simple redistribution of cytochrome c (Desagher and Martinou, 2000).

The release of mitochondrial apoptogenic factors activates downstream events

With a few exceptions (e.g. Fas or TNF-triggered apoptosis in certain cells), mitochondria represent an essential component of many apoptotic pathways by releasing cytochrome c and pro-caspases into the cytosol. Once released, cytochrome c interacts with the adaptor protein Apaf-1 (Figures 2 and 6) (Green and Reed, 1998). Apaf-1 contains at least three functional domains: (i) an N-terminal caspase-activation recruitment domain (CARD), which binds the pro-domain of caspase-9; (ii) a CED-4 domain required for Apaf-1 self-oligomerization; and (iii) a series of C-terminal WD-40 repeats thought to mediate protein-protein interactions (Zou *et al.*, 1997). Apaf-1, when bound to cytochrome c, apparently hydrolyzes dATP/ATP and undergoes oligomerization via its CED-4 domains (Hu *et al.*, 1998a, 1999; Srinivasula *et al.*, 1998). Simultaneously, the CARD domain recruits and facilitates processing of pro-caspase-9 (Li *et al.*, 1997; Srinivasula *et al.*, 1998; Qin *et al.*, 1999). This complex of cytochrome c, Apaf-1 and caspase-9 is commonly referred to as the apoptosome. Reconstitution experiments using purified recombinant proteins indicate that the apoptosome is ~1.4 kDa in size (Saleh *et al.*, 1999; Zou *et al.*, 1999), whereas in native cell lysates, Apaf-1 oligomerizes into an ~700 kDa complex and, in addition to processed caspase-9, contains fully processed caspases-3 and –7 (Cain *et al.*, 1999, 2000). Thus, the initial processing of downstream caspases by caspase-9 and their subsequent autocatalytic processing appears to take place within the apoptosome. The specific mechanisms that govern these processes remain unclear (Bratton *et al.*, 2001).

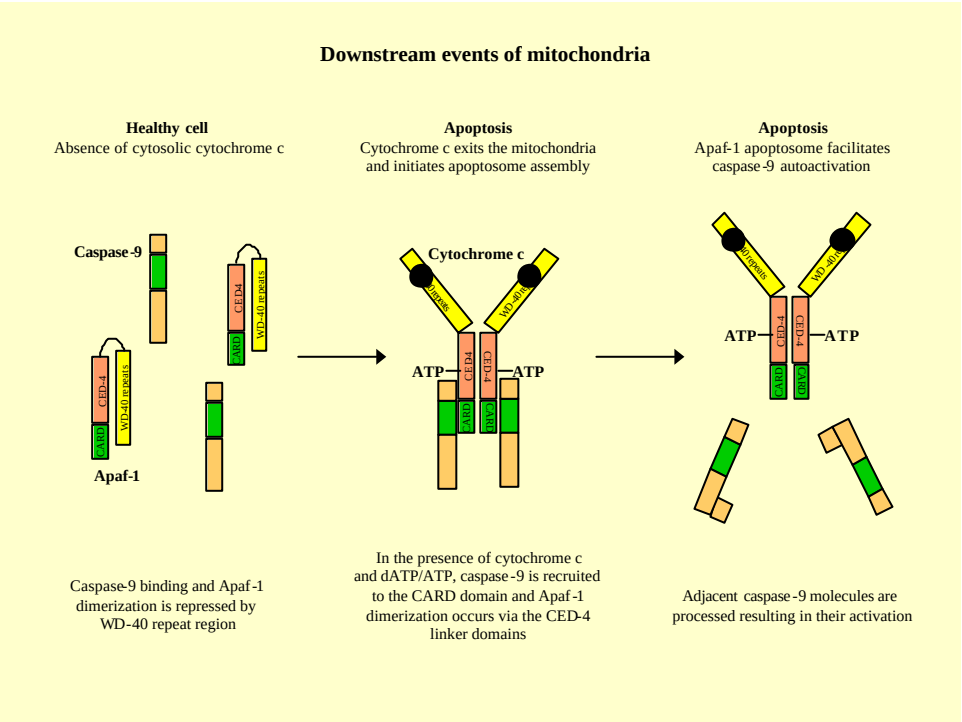

Figure 6 : Apaf-1 promotes caspase-9 activation by oligomerization.
Cytochrome c and dATP/ATP regulate the ability of Apaf-1 to recruit caspase-9 and form multimers.

The physiological importance of cytochrome c release and the subsequent activation of caspase-9 has been shown by Apaf-1 or caspase-9 knockout mice, which die perinatally with brain overgrowth and reduced apoptosis in the central nervous system (Vaux *et al.*, 1999). More recently, these observations have been confirmed by the generation of cytochrome c-deficient mice (Susin *et al.*, 1999b). These mice die earlier during embryonic development, but this is presumably due to defective mitochondrial oxidative phosphorylation. Nevertheless, cells derived from the cytochrome c knockout embryos (as well as cells lacking Apaf-1 or caspase-9) are resistant to a wide range of apoptotic stimuli, even though they undergo apoptosis normally after Fas or TNF-receptor activation (Susin *et al.*, 1999b; Vaux *et al.*, 1999; Desagher and Martinou, 2000). Moreover, it has been proposed that Bcl-xL might interact with Apaf-1 and prevent it from activating caspase-9 (Hu *et al.*, 1998b; Pan *et al.*, 1998a). However, this assumption is very controversial and requires further investigation.

In addition of and together with cytochrome c, other proteins, such as apoptosis-inducing factor (AIF), are released during apoptosis. Cloning of the full-length cDNAs corresponding to mouse AIF (612 amino acids) and human AIF (613 a.a.) (Susin *et al.*, 1999b) revealed that AIF is strongly conserved between the two mammalian species (92% a.a. identity in the whole protein). The mouse AIF cDNA codes for a protein which is organized in three domains : (1) an amino-terminal mitochondrial localization sequence (MLS) of 100 amino acids; (2) a spacer sequence of 27 amino acids; and (3) a carboxy-terminal 485 amino acid oxidoreductase domain. AIF is encoded by one single gene located on the X chromosome, and ubiquitously expressed, both in normal tissues and in a variety of cancer cell lines. The AIF precursor is synthesized in the cytosol and is imported into mitochondria. The mature protein, a flavoprotein (prothestic group: flavine adenine dinucleotide) with significant homology to plant ascorbate reductase and bacterial NADH (nicotinamide adenine dinucleotide, reduced form) oxidase, is normally confined to the mitochondrial intermembrane space. When released, AIF translocates through the outer mitochondrial membrane to the cytosol and to the nucleus. If microinjected into the cytoplasm of normal cells, recombinant AIF suffices to cause three hallmarks of apoptosis, namely (1) the dissipation of the mitochondrial transmembrane potential and the release of cytochrome c, (2) the condensation of nuclear chromatin, (3) and the exposure of phosphatidylserine on the plasma membrane surface (Susin *et al.*, 1999b; Ferri *et al.*, 2000; Vieira and Kroemer, 2000). These alterations are rapid (30-120 min.) and are not prevented by addition of the general caspase inhibitor Z-VAD-fmk (Susin *et al.*, 1999b; Ferri *et al.*, 2000). Moreover, they are not affected by overexpression of the anti-apoptotic protein Bcl-2 (Susin *et al.*, 1999b). Similar *in vivo* effects have been obtained by transfection-enforced overexpression of a truncated AIF-GFP construct lacking the N-terminal MLS (AIF-GFPΔ1-100) and that is misdirected to the extramitochondrial compartment (Vieira and Kroemer, 2000). These data confirm that ectopic (extramitochondrial) AIF is a caspase-independent effector of apoptosis that acts beyond the Bcl-2-controlled checkpoint of cell death (Daugas *et al.*, 2000a).
The apoptogenic effects of AIF, as revealed in intact cells, have been recapitulated in cell-free systems. Addition of recombinant AIF to purified nuclei causes chromatin condensation (Susin *et al.*, 1999b). This is accompanied by a large-scale DNA fragmentation, but not by oligonucleosomal DNA fragmentation. In intact cells, Z-VAD-fmk prevents oligonucleosomal DNA degradation, yet frequently has no effect on the large-scale fragmentation pattern (Daugas *et al.*, 2000b). These results are compatible with the hypothesis that AIF is (one of) the mediator(s) responsible for large-scale chromatin degradation. But how AIF mediates this apoptogenic function remains entirely elusive. Thus, the functional relationship between AIF and other factors involved in chromatin condensation and degradation (caspases, CAD, acinus, cyclophilin etc.) remains unknown (Zamzami and

Kroemer, 1999). Moreover, the AIF-interacting protein(s) in the cytosol (which are required for its membrane permeabilizing effects) and the nuclear AIF target(s) have not yet been identified. Based on our knowledge of other mitochondrial proteins and their pro-apoptotic effects, it may be speculated that endogenous AIF inhibitors exist. Such inhibitors would prevent accidental cell death induction by partial AIF release and set the threshold at which AIF levels suffice to drive the full cell death program (Daugas *et al.*, 2000a).

In conclusion, similar to cytochrome c, AIF is a phylogenetically old, bifunctional protein with an electron acceptor/donor (oxidoreductase) function and a second apoptogenic function. In contrast to cytochrome c, however, AIF acts in a caspase-independent fashion (Daugas *et al.*, 2000a). Moreover, very recently, Joza *et al.* provide genetic evidence that the AIF-dependent, caspase-independent PCD pathway is crucial for cell death following growth factor deprivation and early mammalian development (Joza *et al.*, 2001). In fact, they show that AIF inactivation abolishes all signs of cell death in early morphogenesis, including the mitochondrial release of cytochrome c. In addition, they show that AIF is a rate-limiting factor of embryonic stem cell death induced by serum withdrawal (independently of caspases inhibition), indicating a stimulus-dependent contribution of AIF to the apoptotic cascade. Therefore, they postulate the coexistence of two separate pathways linking the mitochondria to apoptosis, one that requires AIF (which is thought to predate to the caspase pathway) and the other that relies on caspase activation.

Activation of caspases

Upon activation of caspase-8/–10 by death receptors or caspase-9 by Apaf-1, these caspases can then propagate the death signal by activating other caspases (see Figure 2). The study of this phase of the death program has been greatly facilitated by the observation that cytochrome c (in conjunction with dATP/ATP) can trigger apoptosis in cell-free extracts, an unusual property that is not displayed by the vast majority of agents that can promote apoptosis in intact cells (Liu *et al.*, 1996a; Li *et al.*, 1997; Slee *et al.*, 1999b). Studies using cell-free systems have revealed that cytochrome c activates caspase-9 (via Apaf-1) and that caspase-9 then initiates a series of other caspase activation events that represent a *bona fide* caspase cascade, since removal, mutation of certain members of the cascade or the use of small pharmacological inhibitors will slow down or even prevent apoptosis (Earnshaw *et al.*, 1999; Slee *et al.*, 1999b).

Most of the morphological changes that were observed by Kerr *et al.* are caused by a set of cysteine proteases that are activated specifically in apoptotic cells and can be thought of as the central executioners of the apoptotic pathway. These death proteases are homologous to each other, and are part of a large protein family known as the caspases (Alnemri *et al.*, 1996). Caspases are highly conserved through evolution, and can be found from humans all the way down to insects, nematodes, and hydra (Budihardjo *et al.*, 1999; Cikala *et al.*, 1999; Earnshaw *et al.*, 1999). Over a dozen caspases (currently 14 are known) have been identified in humans; about two-thirds of these have been suggested to function in apoptosis (Thornberry and Lazebnik, 1998; Earnshaw *et al.*, 1999; Hengartner, 2000). The others appear to play a primary role in cytokine maturation and inflammation.

Given the great importance of caspases in the apoptotic process, it is reasonable to propose that a proper understanding of apoptosis will require us to understand how caspases are activated.

As is true of most proteases, caspases are synthesized as enzymatically inert zymogens. These zymogens are composed of three domains: an N-terminal pro-domain, a large subunit with the catalytic site "QA**C**XG" (also called the p20 domain) and a small subunit (also called the p10 domain) (Figure 7a), which are found in the mature enzyme. Therefore, caspases can be

divided into two types: those with large pro-domains (like caspase-2, -8, -9, and -10) that function upstream as initiators of the death cascade and those with a small pro-domain (like caspase-3, -6 and –7) that act downstream as effectors (Fesik, 2000). In all cases examined so far, the mature enzyme is a heterotetramer containing two p20/p10 heterodimers and two active sites (Figure 7b) (Earnshaw *et al.*, 1999). Although, much has been made about the fact that active caspases are dimers containing two active sites, there is no obvious structural reason why this should be so, and it seems quite possible that caspases could exist as active monomers under the right conditions (Hengartner, 2000).

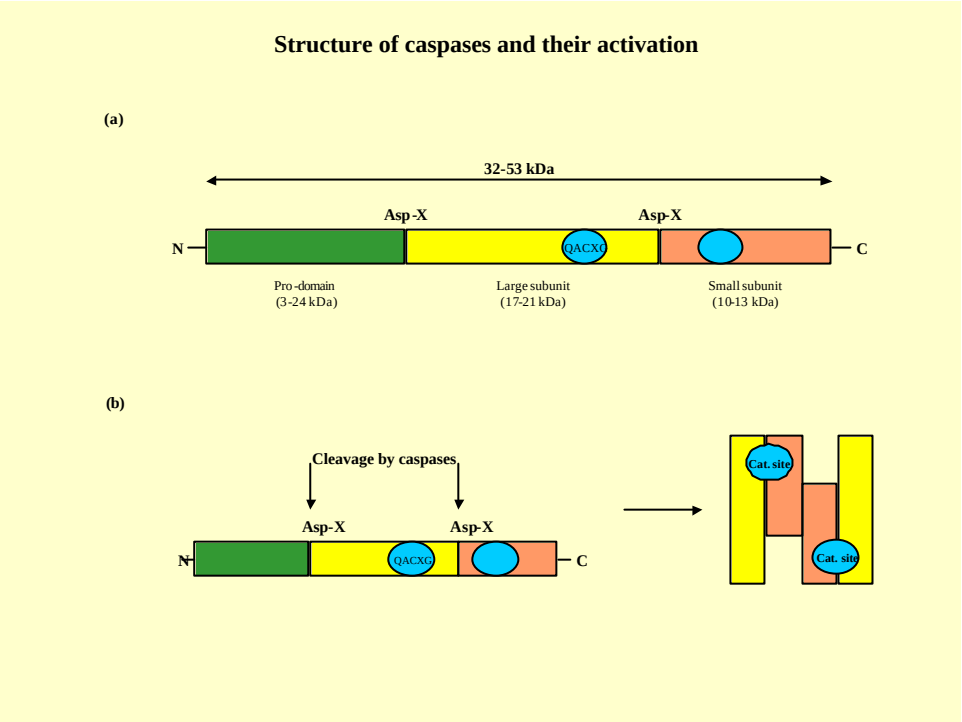

Figure 7 : Schematic representation of caspase structure and their activation.

Three general mechanisms of caspases activation have been described so far: (1) processing by an upstream caspase, (2) induced proximity, and (3) association with a regulatory subunit.

(1) Processing by an upstream caspase

Most caspases are activated by proteolytic cleavage of the zymogen between the p20 and p10 domains, and usually also between the pro-domain and the p20 domain. Interestingly, all these cleavage sites occur at Asp-X sites- candidate caspase substrate sites- suggesting the possibility of autocatalytic activation (Figure 7b) (Thornberry *et al.*, 1997). Indeed, the simplest way to activate a pro-caspase is to expose it to another, previously activated caspase molecule. This “caspase cascade” strategy of caspase activation is used extensively by cells for the activation of the three short pro-domain caspases, caspase-3, -6 and –7. These three downstream effector caspases are considered the workhorses of the caspase family, and are usually more abundant and active than their long pro-domains cousins (Hengartner, 2000).

The caspase cascade is a useful method to amplify and integrate pro-apoptotic signals, but it cannot explain how the first, most upstream caspase gets activated. At least two other approaches are used to get the ball rolling (Hengartner, 2000).

(2) Induced proximity

Caspase-8 and –10 are the key initiator caspases in the death-receptor pathway. Upon ligand binding, death receptors such as CD95 (Apo-1/Fas) aggregate and form membrane-bound signaling complexes (Figures 2 and 3). These complexes then recruit, through adapter proteins, several molecules of pro-caspase-8 or –10, resulting in a high local concentration of zymogens. The induced proximity model posits that under these crowded conditions, the low intrinsic protease activity of pro-caspase-8 or –10 (Muzio *et al.*, 1998) is sufficient to allow the various pro-enzyme molecules to mutually cleave and activate each other. A similar mechanism of action has been proposed to mediate the activation of several other caspases, including caspase-2 and the nematode caspase CED-3 (Yang *et al.*, 1998b).
Although forced crowding of zymogens clearly is sufficient in many cases to activate caspases (Salvesen and Dixit, 1999), it is a rather crude a way to control the fate of a cell. Whereas the basic concept is probably correct, additional levels of regulation surely must exist *in vivo* to modulate the process (Hengartner, 2000).

(3) Association with a regulatory subunit

The most complex activation mechanism described so far is the one used by caspase-9. Unlike other caspases, proteolytic processing of pro-caspase-9 has only a minor effect of the enzyme's catalytic activity (Rodriguez and Lazebnik, 1999; Stennicke *et al.*, 1999). Rather, the key requirement for caspase-9 activation is its association with a dedicated protein co-factor, Apaf-1 (Hengartner, 2000). Apaf-1 was identified through a biochemical approach as one of two proteins that are required for caspase-9 activation (the other being cytochrome c) (Li *et al.*, 1997; Zou *et al.*, 1997). Initially believed to be required only transiently, for caspase-9 activation, the Apaf-1/caspase-9 complex is now thought to actually represent the true active form of caspase-9 (Rodriguez and Lazebnik, 1999). Thus, we must view Apaf-1 not simply as a caspase-9 activator, but rather as an essential regulatory subunit of a caspase-9 holoenzyme (Cain *et al.*, 1999; Beere *et al.*, 2000; Cain *et al.*, 2000).
In addition, all caspases have a distinct substrate specificity that requires cleavage after an aspartic acid at the P_1 position N-terminal to the cleavage site. The preference of amino acids further to the N-terminus of peptide substrates at the P_2-P_4 positions differs among caspases family members and defines their substrates specificity (Thornberry *et al.*, 1997). The initiator caspases such as caspase-8 and caspase-9 prefer (V,L)EXD-containing substrates like those found in the cleavage sites used to process caspase-zymogens into active enzymes. In contrast, the effector caspases such as caspase-3 and caspase-7 cleave DEXD-containing substrates such as those found in structural proteins that are cleaved during apoptosis, resulting in the typical morphological changes in the cell (Fesik, 2000).
In summary, effector caspases are usually activated proteolytically by an upstream caspase, whereas initiator caspases are activated through regulated protein-protein interactions. In fact, each of the long pro-domain caspases contains in its pro-domain a protein-protein interaction module, which allows it to bind to and associate with its upstream regulators. Caspase-8 and –10 contain a death-effector domain (DED), whereas caspase-2 and –9 contain a caspase activation and recruitment domain (CARD). These two domains share little sequence identity, but fold into very similar three dimensional structures, consisting of six anti-parallel α-helices arranged in a Greek key configuration (Hofmann, 1999). The same fold is also found in the

death domain, a third protein interaction module present in several upstream regulators of apoptosis, such as CD95 and the adapter molecule FADD (Huang *et al.*, 1996). The structure of the death domain, DED and CARD perfectly suits their function. The anti-parallel helices bundle into a tight core, leaving exposed large surfaces onto which evolution has carved extended protein-protein interaction domains. The particular face of the module that is used for interaction varies greatly from one protein to the next (Huang *et al.*, 1996; Eberstadt *et al.*, 1998; Zhou *et al.*, 1999). Work so far suggests that the death adaptor modules usually mediate intra-family interactions that is, death domain/death domain (DD/DD), DED/DED and CARD/CARD. However, structural analyses show that there is enough surface area left on DDs, DEDs and CARDs to also interact with other proteins. Indeed, death adaptor modules might well act as integration platforms, binding to several different proteins, which could modulate their dimerization and hence caspase activation (Hengartner, 2000).

Ordering caspase activation in apoptosis

As we know how caspases are activated and which are their possible targets, it is reasonable to try to establish the activation ordering of the different caspases from the initiator caspases to the final effector caspases.

As already described, following stimuli implicating either death receptors or mitochondria, initiator caspase such as caspase-8, -10 or –9 can be processed, inducing the activation of downstream caspases. In fact, when caspase-8 or –10 is activated efficiently, it can directly activate caspase-3, thus bypassing an unnecessary mitochondrial step. In contrast, when only a small amount of caspase-8 is activated after a death-receptor-induced stimulus, mitochondria in these cells may function as amplifiers via the BID cleavage, release of cytochrome c, and caspase-9 activation (Scaffidi *et al.*, 1998).

The caspase activation events driven by caspase-9 appear to be the simultaneous activation of caspase-3 and –7 (Pan *et al.*, 1998d; Srinivasula *et al.*, 1998; Slee *et al.*, 1999b). Caspase-3, then drives the activation of caspase-2 and –6, followed by the activation of caspase-8 and –10 (Slee *et al.*, 1999b). In the absence of caspase-3, caspase-7 and –9 are still activated but activation of other caspases downstream of this point (Figure 2) is arrested (Slee *et al.*, 1999b). Removal of caspase-6 blocked the activation of caspase-8 and –10 in this context, suggesting that these activation events are, somewhat surprisingly, driven by caspase-6 (Slee *et al.*, 1999b). The activation of long pro-domain caspases late in this cascade may be unexpected, but these observations are bolstered by similar observations in other systems. In addition, it seems quite plausible that caspases that are apical (initiator) caspases in the context of certain pro-apoptotic stimuli may participate in an amplification role in other contexts. This phase of the death program may serve as an amplification step that activates the full complement of caspases that are required to dismantle the cell in the appropriate manner (Slee *et al.*, 1999a).

These two emerging pathways (death receptors- and mitochondria-induced) represent linear and simplified schemes that ignore an extraordinary complex network of interactions involving the caspases themselves as well as other regulatory molecules. For example, caspase-3 can activate pro-caspase-9 (Srinivasula *et al.*, 1996). Likewise, caspase-6 can activate pro-caspase-3 (Liu *et al.*, 1996b; Slee *et al.*, 1999b). Furthermore, pro-caspase-8 has also been shown to be a substrate for caspase-3 (Sun *et al.*, 1999). Finally, although BID is most likely to be activated by even low concentrations of caspase-8, it can also be cleaved by higher concentrations of caspase-3 (Li *et al.*, 1998a).

It is important to note that not all of the caspases that are activated during this phase may be necessary for the cell to die *per se*. However, they are likely to be required for the cell to adopt the typical apoptotic phenotype. For example, cells deficient in caspase-3 (either from

CASP-3 null mice, or due to a frame-shift mutation, i.e. MCF-7 cells) clearly die in response to many apoptotic stimuli (Kuida, *et al.*, 1996; Jänicke *et al.*, 1998a; Jänicke *et al.*, 1998b). However, caspase-3-deficient cells undergo an aberrant form of apoptosis (restricted blebbing, delayed DNA fragmentation), suggesting that the downstream amplification events and non-caspase cleavage events that are mediated by caspase-3 cannot be carried out by caspases activated earlier in the pathway. In a similar but more dramatic way, if all caspases activity is blocked using broad spectrum caspase inhibitors (like Z-VAD-fmk), cells that progress beyond the mitochondrial commitment point typically exhibit features of necrosis rather than apoptosis (McCarthy *et al.*, 1997; Amarante-Mendes *et al.*, 1998).

Regulation of caspases

Once activated, the caspases cleave a small subset of the proteins in the cell, and it is the cumulative effect of these cleavage events that accounts for the physical characteristics of apoptosis (Nicholson, 1999). By definition, then, the caspases are potentially dangerous molecules, and an elaborate system of checks and balances exists to regulate these harbingers of cellular mortality (Nicholson, 2001).
Caspases can be controlled in two ways: the processing and activation of a caspase can be regulated by molecules such as Bcl-2 family members and inhibitor-of-apoptosis proteins (IAPs), or active caspases can be controlled by a variety of inhibitors that directly interact with the protease.

Molecules as regulators

While the regulation of caspases by Bcl-2 family members, which sequestering caspase-activating adaptor proteins, has been largely discussed, we would like to present a more recently finding. Srinivasula *et al.* (2001) reveal another regulatory mechanism by showing that a key catalyst in the apoptotic pathway, caspase-9, uses a peptide from the end of one of its own subunits as bait to attract a molecule from the IAP family. In doing so, caspase-9 remains catalytically dormant even though the initial steps in committing it to its active state have taken place. This mechanism may work to eliminate the untoward effects that small amounts of activated caspase-9 would have on a cell that is not fully committed to apoptotic death (Nicholson, 2001).
Once launched, the caspase-9/caspase-3 apoptotic pathway does not necessarily proceed unopposed: it is regulated further by proteins of the IAP family. These proteins were initially discovered in baculoviruses where they were found to inhibit apoptosis in host cells during viral infection (Crook *et al.*, 1993). Subsequently, IAPs have been found in other viruses, yeast, flies, worms, and mammals (Uren *et al.*, 1998). They contain one to three baculovirus IAP repeat (BIR) domains which are composed of about 70 amino acids and have a characteristic signature sequence ($CX_2CX_{16}HX_6C$). Some of the IAPs also contain a C-terminal ring finger. Several functions have been attributed to the IAPs. In Drosophila, IAPs have been shown to interact with the pro-apoptotic proteins REAPER, HID, and GRIM (Vucic *et al.*, 1998; Goyal *et al.*, 2000). The mammalian IAPs, cIAP-1 (MIHB) and cIAP-2 (MIHC), bind to TNF-receptor associated factors (TRAFs) –1 and –2, while survivin (Ambrosini *et al.*, 1997) has been implicated in the cell cycle (Li *et al.*, 1998b). Some IAPs have also been shown to potently inhibit caspases. For example, human X-linked IAP (XIAP) inhibits caspase-3 and caspase-7 (Deveraux *et al.*, 1997) and also inhibits caspase-9 (Deveraux *et al.*, 1999a). The portion of XIAP responsible for inhibiting caspase-3 was found to contain the BIR2 domain (Takahashi *et al.*, 1998) whereas the BIR3 domain of XIAP was shown to inhibit caspase-9 (Deveraux *et al.*, 1999a; Fesik, 2000; Sun *et al.*, 2000). So the

presence of XIAP slows down and often (although not always) blocks the apoptotic pathway (Nicholson, 2001).

The caspase-inhibiting effects of XIAP are antagonized by another apoptosis-promoting mitochondrial protein, called Smac/DIABLO (Du *et al.*, 2000; Verhagen *et al.*, 2000). This recently found protein promotes cytochrome c/Apaf-1-dependent caspase activation. Like cytochrome c, Smac/DIABLO is normally located in mitochondria and released, together with cytochrome c, into the cytosol when cells undergo apoptosis. The amino terminus of Smac/DIABLO binds to a surface groove in the BIR3 domain of XIAP, activating this latter for a ubiquitin/proteasome degradation and preventing the inhibition of caspases and of apoptosis (Chai *et al.*, 2000; Liu *et al.*, 2000; Wu *et al.*, 2000). The ability of caspase-9 and caspase-3 to commit a cell to apoptotic death appears to depend on the dynamics and stoichiometry of XIAP and its antagonist, Smac/DIABLO. Other regulatory polypeptides, such as XIAP-associated factor-1 (Liston *et al.*, 2001), may also contribute to these dynamics (Nicholson, 2001).

There is no doubt some leakage in this finely tuned regulatory system. But the consequence of even low levels of an unregulated initiator caspase could be devastating to a cell. So it appears that caspase-9 has a back-up strategy: it actually recruits its own inhibitor. Srinivasula *et al.* (2001) show that the amino terminus of the small subunit of mature caspase-9 contains a four-amino-acid peptide that is remarkably similar to the key amino terminus of mammalian Smac/DIABLO and the apoptosis-promoting fruitfly proteins Reaper, Hid and Grim. However, unlike the peptide in Smac/DIABLO, the peptide in mature caspase-9 does not block the caspase-inhibiting effects of XIAP. Instead, this peptide helps XIAP to bind and inhibit caspase-9. The relatively dormant caspase-9 pro-enzyme (in which this peptide is masked) does not bind XIAP. But once caspase-9 takes on its small subunit is liberated, it uses the exposed peptide as bait to recruit XIAP (Nicholson, 2001).

But does this make sense in a cell that may need to fully activate its "mitochondrial" apoptosis pathway? As it turns out, all else holds true. Smac/DIABLO competes with the small subunit of mature caspase-9 for XIAP, and can tear XIAP away. The following scenario may thus play out (Figure 8). During the normal stresses of cellular existence within a multicellular organism, small amounts of caspase-9 may become activated by self-catalytic or other mechanisms. This rogue protease is inherently dangerous, so the newly exposed amino terminus of its small subunit binds to the BIR3 domain of XIAP; the BIR3 domain in turn inhibits caspase-9. However, under certain circumstances in which cell death should take place, signals emanating from mitochondria help to reverse or overcome this process. In other words, Smac/DIABLO competes for binding to XIAP and relieves the inhibition of mature caspase-9, while cytochrome c stimulates Apaf-1-mediated activation of the broader pool of caspase-9 pro-enzymes (Nicholson, 2001).

Although it was not demonstrated by Srinivasula *et al.* (2001), the XIAP bound to the small subunit of caspase-9 may also be liberated by further proteolytic clipping of the small subunit. Nicholson *et al.* show that caspase-3 cleaves this subunit at a position downstream of the XIAP-binding peptide, releasing the peptide and, presumably, the bound XIAP too. In support of the idea that XIAP remains bound to this peptide even when it is cleaved from the small subunit, Srinivasula *et al.* showed that a synthetic form of the peptide antagonizes the interaction between XIAP and caspase-9, in much the same way that equivalent Smac/DIABLO peptides can block the interaction between XIAP and Smac/DIABLO (Chai *et al.*, 2000; Liu *et al.*, 2000; Nicholson, 2001).

In addition to binding active caspase-9 and –3, XIAP also associates with oligomerized Apaf-1. Thus, by associating with the apoptosome, XIAP appears not only to influence the activation of caspase-3 by caspase-9, but also to inhibit the release of active caspase-3 from the complex (Bratton *et al.*, 2001).

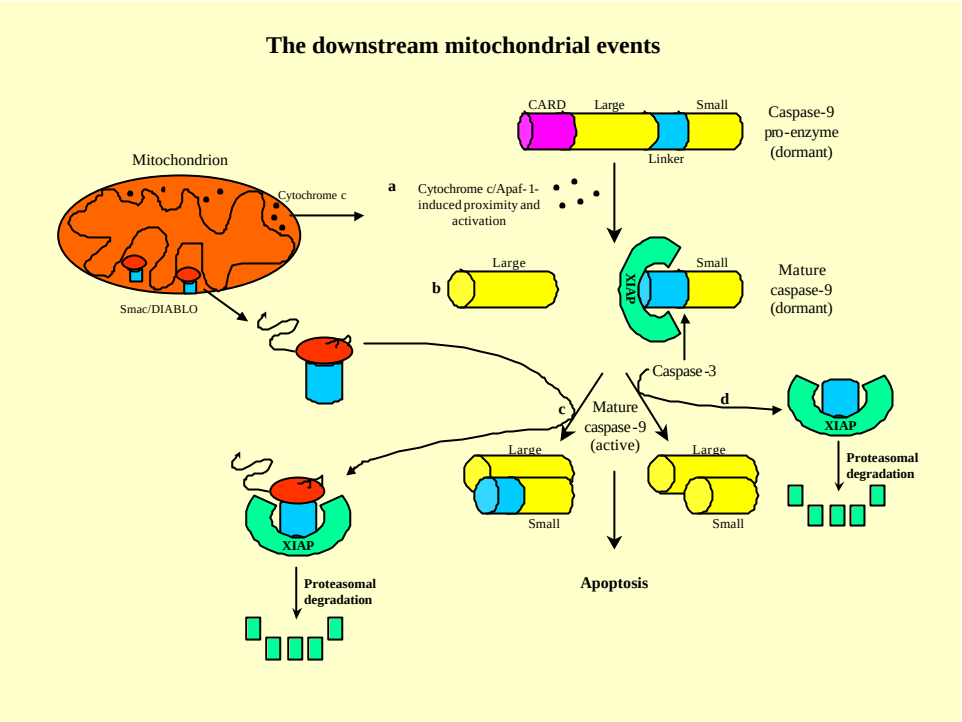

Figure 8 : The apoptosis pathway that includes caspase-9 and its regulators.
(a) In response to death stimuli (not shown), a molecule called Apaf-1, together with cytochrome c, which is released from mitochondria, induces the aggregation and processing of caspase-9 pro-enzymes. The processing includes the removal of the "CARD" domain, and separation of the large subunit from the linker peptide that connects it to the small subunit. This exposes the linker peptide. (b) One end of the linker peptide mimics the XIAP-binding peptide of Smac/DIABLO, allowing XIAP (an inhibitor of apoptosis) to bind to mature caspase-9. This keeps caspase-9 in a catalytically dormant state. (c)-(d) This suppression of caspase-9 might be counteracted by two mechanisms: competition for XIAP by the apoptosis-promoting protein Smac/DIABLO and a proteasomal degradation of XIAP (c), or caspase-3-mediated removal of the XIAP-binding peptide from caspase-9 and also a proteasomal degradation of XIAP (d) (Nicholson, 2001).

Inhibitors as regulators

This type of caspases regulators can be separated into two different classes : the viral/cellular inhibitors and the synthetic inhibitors.

<u>Viral and cellular inhibitors:</u>

Viruses must co-opt a cell's synthetic machinery in order to reproduce. Therefore a most effective way of preventing viral replication is for an infected cell to kill itself. However, just as apoptosis is used to defend against viruses, viruses carry cell death inhibitors to block this response of their host cells. Several viral cell death inhibitors act by binding to activated caspases. The first caspase inhibitor to be identified was the Cowpox virus product Cytokine Response Modifier A (CrmA), which was found to inhibit mainly caspase-1 and caspase-8. In this way, CrmA can prevent apoptosis triggered by TNF receptor family members (Los *et al.*, 1995; Tewari and Dixit, 1995; Varfolomeev *et al.*, 1998). Structurally, CrmA belongs to the serine protease inhibitor (serpin) group, but unlike other serpins, it inhibits caspases, which are cysteine proteases (Ray *et al.*, 1992; Komiyama *et al.*, 1994). However, like conventional

serpins, CrmA acts as a pseudo-substrate that binds to the active proteases. The pseudo-substrate region of CrmA has the residue LVAD (designated P_4-P_1). A second, important viral inhibitor is p35. p35 is a baculoviral protein that can block the defensive apoptotic response of insect cells to viral infection (Clem *et al.*, 1991). As well as being able to inhibit several caspases, p35 can inhibit *C. elegans* CED-3 and mammalian caspase-1, -3, -6, -7, -8 and –10, but does not inhibit non-caspase cysteine proteases or serine proteases important for the caspase-independent apoptosis. In addition, p35 is able to inhibit caspases *in vivo* (Ekert *et al.*, 1999). Cellular inhibitors for caspases are members of the IAP family as discussed above.

Synthetic inhibitors:

As inappropriate apoptosis has been implicated in many diseases, there has been a tremendous effort to develop caspase inhibitors for pharmacological use. Such synthetic caspase inhibitors are of course also useful for analysis of caspase activity in experimental models. Therefore, a number of specific or general caspase inhibitors have been developed based upon the substrate cleavage sites of the caspases. These peptides act as pseudo-substrates for active caspases and are competitive inhibitors. The chemical mechanism of action of the synthetic inhibitors is determined by the chemical groups which the peptides are linked. Linking the appropriate peptide to fluoro- or chloro-methyl ketones (-FMK, -CMK respectively) groups produces irreversible, competitive inhibitors. Peptides linked to aldehyde groups (-CHO) (or nitriles or ketones) act as reversible inhibitors. In addition, the -FMK adducts are much more permeable than the aldehyde based inhibitors, which enter cells poorly, and do not inhibit any caspases in intact cells at concentrations less than 1 μM. Thus, the best known caspase inhibitors are Z-VAD-FMK, which inhibits all known caspases except perhaps the caspase-2, Ac-YVAD-CHO, which inhibits mainly caspase-1, and Z-DEVD-FMK or Ac-DEVD-CHO, which inhibits caspase-1, -3, -6, -7, -8, -9 and –10 (Ekert *et al.*, 1999).

1.4.4 The demolition phase

When the full complement of caspases that are necessary for the proper execution of the death program have become activated, the final demolition phase can begin. The distinction between the commitment/amplification and demolition phase is obviously an artificial one, since it is presumed that substrates will become cleaved as soon as the caspases responsible for particular cleavage events are activated. However, it should be noted that whereas many of the caspases have a cytosolic localization, many of their known substrates are contained within the nucleus or other compartments. Thus, there is likely to be some delay between activation of a caspase and the subsequent proteolytic attack of substrate(s) (Slee *et al.*, 1999a).

Activation of caspases does not result in the wholesale degradation of cellular proteins. Rather, caspases selectively cleave a restricted set of target proteins, usually at one, or at most a few positions in the primary sequence (always after an aspartate residue). In most cases, caspase-mediated "protein surgery" results in inactivation of the target protein. But caspases can also activate proteins, either directly, by cleaving off a negative regulatory domain, or indirectly, by inactivating a regulatory subunit (Hengartner, 2000).

Several important caspase substrates have been identified in recent years. One of the more exciting discoveries has been the elucidation of the mechanism of activation of the nuclease responsible for the famous nucleosomal ladder. First described by Wyllie (1980), this nuclease cuts the genomic DNA between nucleosomes, to generate DNA fragments with lengths corresponding to multiple integers of approximately 180 base pairs. The presence of

this DNA ladder has been used extensively as a marker for apoptotic cell death (Hengartner, 2000).

In an elegant series of experiments, the group of Wang and Nagata showed that the DNA ladder nuclease (now known as caspase-activated DNase, or CAD) pre-exists in living cells as an inactive complex with an inhibitory subunit, dubbed ICAD (Nagata, 2000). Activation of CAD occurs by means of caspase-3-mediated cleavage of the inhibitory subunit, resulting in the release and activation of the catalytic subunit (Liu *et al.*, 1997; Enari *et al.*, 1998; Sakahira *et al.*, 1998).

Caspase-mediated cleavage of specific substrates also explains several of the other characteristic features of apoptosis. For example, cleavage of the nuclear lamins is required for nuclear shrinking and budding (Rao *et al.*, 1996; Buendia *et al.*, 1999). Loss of overall cell shape is probably caused by the cleavage of cytoskeletal proteins such as fodrin and gelsolin (Kothakota *et al.*, 1997). Finally, caspase-mediated cleavage of PAK2, a member of the p21-activated kinase family, seems to mediate the active blebbing observed in apoptotic cells. Interestingly, in this last case, caspase cleavage occurs between the negative regulatory subunit and the catalytic subunit, and results in a constitutive activation of PAK2 (Rudel and Bokoch, 1997; Hengartner, 2000).

Close to 100 additional caspase substrates have been reported over the years, and there will certainly be many more (Earnshaw *et al.*, 1999; Nicholson, 1999). Why are there so many substrates ? Perhaps apoptosis is just more complicated that we currently believe. Indeed, several of the key apoptotic sub-programs, such as cell shrinking and the emission of pro-engulfment signals, are still poorly understood. Alternatively, it is possible that many of the described caspase substrates are not relevant substrates, but simply “innocent bystanders” that get caught in the action. According to this line of reasoning, there might be little selection against the presence of fortuitous caspase cleavage sites on irrelevant proteins, as the cell is about to stop functioning anyway. Further experimentation will allow this issue to be resolved (Hengartner, 2000).

2. RESULTS

2.1 ARTICLE : Bcl-2 is a monomeric protein: prevention of homodimerization by structural constraints

Conus, S., Kaufmann, T., Fellay, I., Otter, I., Rossé, T. and Borner, C. (2000) *Embo* **19**(7), 1534-1544.

Bcl-2 and related cytoplasmic proteins are key regulators of apoptosis, the cell suicide program critical for development, tissue homeostasis, and protection against pathogens (Adams and Corry, 1998). Therefore, it has become widely accepted that the sensitivity of a cell for apoptosis is determined by the kind and degree of physical interaction between anti- and pro-apoptotic members of the Bcl-2 family (rheostat model). While Bax-Bax homodimers seem to favor apoptosis, Bcl-2-Bcl-2 homodimers are supposed to confer death protection. It was reported that Bax-Bax homodimers form *in vitro* as well as in intact cells and that these homodimers are required for the apoptotic action of Bax in most situations (Simonen *et al.*, 1997; Zha and Reed, 1997; Kelekar and Thompson, 1998). Moreover, mutagenesis analyses and stable or transient co-overexpression studies have validated the concept that Bcl-2-Bax heterodimers neutralize the pro-apoptotic activity of Bax and the anti-apoptotic activity of Bcl-2 (Zha *et al.*, 1996). However, it is unknown whether Bcl-2 has to homodimerize to protect cells from apoptosis as proposed by the rheostat model.
We decided to challenge this hypothesis because it is crucial to know how Bcl-2 really acts inside cells. We showed by co-immunoprecipitation and FPLC purification of recombinant proteins that whereas Bax readily forms homodimers and heterodimers with Bcl-2 that can be disrupted by an interacting BH3-Bax peptide, we did not find any evidence for Bcl-2 homodimer formation even under conditions when Bcl-2 protects cells from apoptosis. Moreover, we showed by immunofluorescence (an analysis without detergent, because the immunoprecipitation technique, which uses detergent to solubilize membrane proteins, is controversial to study interactions between proteins) that membrane-bound Bcl-2 is incapable of dislocating soluble Bcl-2. However, when we changed the structure of Bcl-2 by mutations or deletions in the BH1 and the BH2 domains, the mutated Bcl-2 is now capable to form homodimers with wild-type Bcl-2. This indicated that the naturally occurring Bcl-2 acts as a monomer to protect cells from apoptosis, a notion that is in agreement with the published monomeric structure of the Bcl-2 homologue Bcl-xL (Muchmore *et al.*, 1996). But our findings were inconsistent with previously published data indicating that Bcl-2 interacts with another Bcl-2 molecule in a head-to-tail fashion in the yeast two-hybrid system (Hanada *et al.*, 1995) and in co-immunoprecipitates (Yin *et al.*, 1994). A possible explanation of this discrepancy may be that in such previous studies tagged (GST, HA, LexA, etc.) versions of Bcl-2 were used where the native structure of Bcl-2 may have been disrupted.
Taken together, our results showed that Bcl-2 stays monomeric inside cells to protect cells from apoptosis and is therefore probably not capable of forming a channel as suggested by Schendel *et al.* (1998) with recombinant Bcl-2 on artificial membranes.

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Bcl-2 is a monomeric protein: prevention of homodimerization by structural constraints

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The pro-apoptotic activity of the Bcl-2 family member Bax has been shown to be facilitated by homodimerization. However, it is unknown whether Bcl-2 or Bcl-x_L have to homodimerize to protect cells from apoptosis. Here we show by co-immunoprecipitation and FPLC analyses that while Bax multimerizes and forms heterodimers with Bcl-2, there is no evidence for Bcl-2 homodimerization, even in conditions under which Bcl-2 protects cells from apoptosis. Immunofluorescence studies confirmed that Bax can attract active, soluble Bcl-2 to mitochondrial membranes, but that nuclear/ER membrane-bound Bcl-2 was incapable of dislocating soluble Bcl-2. The failure of Bcl-2 to homodimerize is due to structural constraints as versions of Bcl-2 deleted or mutated in the BH1 and BH2 domains effectively dimerized with wild-type Bcl-2 and were dislocated by Bcl-2 inside cells. These data indicate that naturally occurring Bcl-2 does not expose protein domains that mediate homodimerization and therefore most likely acts as a monomer to protect cells from apoptosis.

Keywords: apoptosis/Bax/Bcl-2/BH3 domain/dimerization

Introduction

Apoptosis is a tightly controlled, physiological mechanism to eliminate superfluous and damaged cells from a multicellular organism. At the center stage of this process are caspases, a family of cysteine-dependent, aspartate-directed proteases, which dismantle cells by cleaving survival factors, cytoskeletal and nuclear scaffold proteins and components of the replication, transcription, splicing or repair machineries (Earnshaw *et al*., 1999). Inhibition of caspases and hence interference with apoptosis occurs either directly through a pseudosubstrate strategy (p35, IAPs) (Clem *et al*., 1996) or indirectly by sequestering and inactivating caspase-activating adaptor proteins (Thome *et al*., 1997). Some members of the Bcl-2 family have emerged as prototypes for the latter mechanism (Reed, 1997).

Bcl-2 family members can be grouped into anti-apoptotic survival factors such as Bcl-2 or Bcl-x_L, which block caspase activation, and pro-apoptotic killer proteins such as Bax and Bak, which facilitate apoptosis via caspase-dependent and -independent mechanisms (Reed, 1997). Sequence analysis has not provided any clue as to how these proteins may work. However, they share one to four α-helical Bcl-2 homology domains, called the BH domains, whose subtle differences in primary sequence and spatial arrangements may determine pro- and anti-apoptotic functions (Muchmore *et al*., 1996; Aritomi *et al*., 1997).

The anti-apoptotic proteins Bcl-2 and Bcl-x_L possess four BH domains (BH1–4), which are all required for death protection (Adams and Cory, 1998). Based on the crystal structure of Bcl-x_L, these domains cooperate to form a hydrophobic face on one side of the molecule to which numerous proteins, including caspase activators, may bind (Muchmore *et al*., 1996; Aritomi *et al*., 1997). An additional, amphipathic C-terminus allows the proteins to be tail-anchored to intracellular membranes (mainly mitochondria and nuclear/ER) (Nguyen *et al*., 1993; Lithgow *et al*., 1994) and thus to sequester apoptosis regulatory molecules from the cytoplasm to specific membrane sites. This 'docking effect' may, however, not be the only activity of Bcl-2 or Bcl-x_L (Reed, 1997). Structurally, Bcl-x_L resembles the bacterial, pore-forming toxins colicin or diphtheria toxin, especially in an α-helical region that mediates the membrane insertion of the toxins (Muchmore *et al*., 1996). This region corresponds to BH1 and part of the BH2 domains of Bcl-2 and Bcl-x_L, and is called the α5/α6 region. As recombinant Bcl-2 and Bcl-x_L can form ion channels on artificial membranes, it was proposed that, like the bacterial toxins, these proteins use their α5/α6 region for membrane insertion and/or channel formation (Schendel *et al*., 1998).

Apart from lacking an N-terminal BH4 domain, the pro-apoptotic family members Bax and Bak contain BH3, BH1 and BH2 domains that are highly homologous and similarly spaced as in Bcl-2 or Bcl-x_L (Adams and Cory, 1998). Studies on the structure of the pro-apoptotic family member Bid (Chou *et al*., 1999; McDonnell *et al*., 1999) as well as molecular modeling of Bax (Aritomi *et al*., 1997; Schendel *et al*., 1998) suggest that the overall structure of Bax or Bak may be very similar to that of Bcl-x_L. In addition, like Bcl-x_L and Bcl-2, Bax can form ion channels on artificial membranes, and this also depends on the α5/α6 membrane spanning region encompassing the BH1/BH2 domains (Schendel *et al*., 1998). However, a major difference between Bax/Bak and Bcl-2/Bcl-x_L is that the former undergo conformational changes inside cells. While Bcl-2 and Bcl-x_L are mostly membrane bound, Bax and Bak are often found in the cytoplasm of normal cells. In response to apoptotic stimuli, the pro-apoptotic

proteins change their conformation, expose an N-terminal epitope and translocate to mitochondria where they perform their cytotoxic action (Wolter *et al*., 1997; Goping *et al*., 1998; Griffiths *et al*., 1999). Part of this action involves the release of cytochrome *c* into the cytoplasm (Jürgensmeier *et al*., 1998; Rossé *et al*., 1998) where it binds to the adapter protein Apaf-1 to activate caspases-9 and -3 (Green and Reed, 1998). Bax may itself form a channel through which cytochrome *c* can pass or regulate an outer mitochondrial channel, such as the voltage-dependent anion channel (VDAC), to make it more permeable for cytochrome *c* (Shimizu *et al*., 1999).

In addition to the α5/α6 membrane spanning regions, Bax and Bak depend on the BH3 domain for cytotoxicity (Kelekar and Thompson, 1998). Two modes of action have been proposed for this domain. First, it can interact with Bcl-2 and Bcl-x_L and inhibit their death protective activities (Chittenden *et al*., 1995; Zha *et al*., 1996; Holinger *et al*., 1999). Structural analysis revealed that the BH3 domain of Bak nestles into a shallow groove in the hydrophobic face of Bcl-x_L because it changes from a random coil to an α-helical structure and rotates outward to expose its hydrophobic surface (Sattler *et al*., 1997). Secondly, the BH3 domain is used for Bax or Bak homodimerizations (Simonen *et al*., 1997; Zha and Reed, 1997; Kelekar and Thompson, 1998). While there is still some disagreement whether such homodimerizations are required for pro-apoptotic activities (Simonian *et al*., 1996), enforced dimerization of Bax was shown to facilitate mitochondrial translocation, mitochondrial dysfunction and apoptosis induction (Gross *et al*., 1998). Moreover, purified Bax can form heptamers in solution (Lewis *et al*., 1998).

It has remained even more controversial whether Bcl-2 and Bcl-x_L require homodi- or multimerization for their survival activity. This is an important issue if one wants to know whether these proteins form channels within cells. Although the crystal structure of soluble, anchorless Bcl-x_L is a monomer (Muchmore *et al*., 1996), it could still form dimers after conformational changes and/or insertion into membranes. Bcl-2 was shown to interact with another Bcl-2 molecule in a head-to-tail fashion in the yeast two-hybrid system (Hanada *et al*., 1995) and in co-immunoprecipitates (Yin *et al*., 1994). However, these analyses were often done with tagged [glutathione *S*-transferase (GST), LexA, hemagglutinin (HA), etc.] versions of Bcl-2 where the native structure of Bcl-2 may have been disrupted. Here we show that while Bax dimerizes in a BH3-dependent manner, Bcl-2 does not readily form homodimers both *in vitro* and within cells. This is not because the BH3 domain cannot participate at dimerization reactions, but because this domain is not accessible and/or recruitable in the wild-type Bcl-2 molecule.

Results

Bax homodimerizes and heterodimerizes with Bcl-2 in a BH3-dependent manner

We previously showed homodimerization between exogenous and endogenous Bax and heterodimerization between exogenous Bax and Bcl-2 in anti-Bax immunoprecipitates from rat 6 (R6) embryo fibroblasts stably co-overexpressing Bax and Bcl-2 (R6-Bcl-2/Bax#9) (Otter *et al*., 1998). As these interactions are thought to be mediated via the BH3 domain of Bax, we performed competition experiments using a 19 amino acid Bax–BH3 peptide either in its wild-type (^{56}TKKLSECLKRIG**D**EL-DSNM^{74}) or mutant (D68A) configuration. When mixed at a concentration of 100 μM into a [^{35}S]methionine/cysteine-labeled R6-Bcl-2/Bax#9 cell lysate, the wild-type, but not the mutant Bax–BH3 peptide prevented the co-immunoprecipitation of endogenous rat Bax (rBax) with exogenous human Bax (hBax) (Figure 1A). The same peptide also interfered with the co-immunoprecipitation

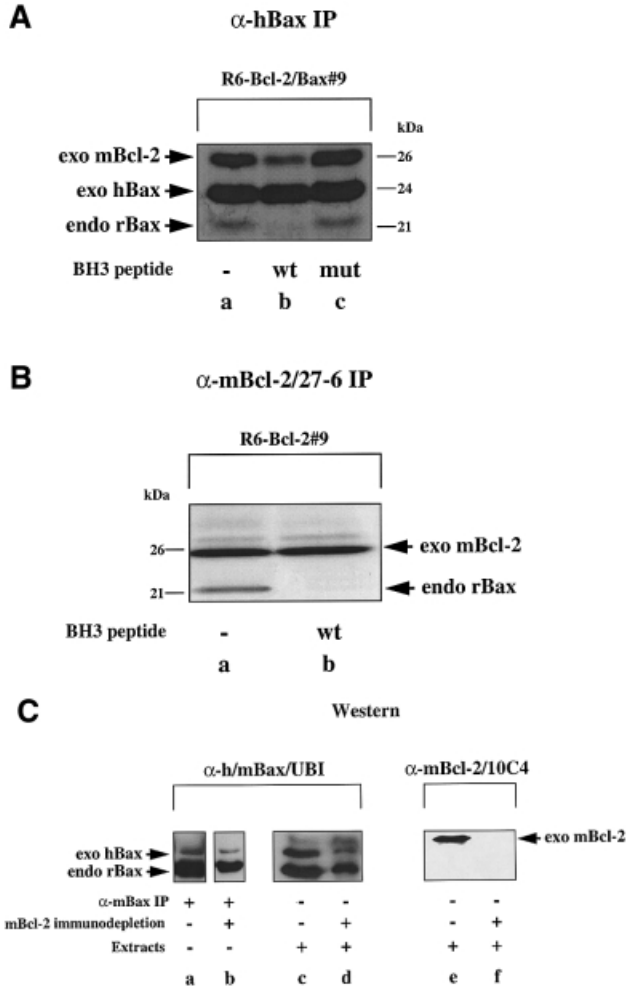

Fig. 1. BH3-dependent interaction between Bax and Bax/Bcl-2 in R6 cell extracts. (**A**) α-hBax immunoprecipitates (IP) from radiolabeled R6 cell extracts co-overexpressing hBax and mBcl-2 (R6-Bcl-2/Bax#9), performed in the absence (lane a) or presence of a wild-type (lane b) or a mutant (D68A) (lane c) Bax–BH3 peptide. The location of the immunoprecipitated overexpressed hBax (exo hBax) [24 kDa; see Otter *et al*., (1998)], the co-precipitating endogenous rBax (endo rBax) (21 kDa) and overexpressed mBcl-2 (exo mBcl-2) (26 kDa) are indicated. (**B**) α-mBcl-2/27-6 immunoprecipitates from R6 cell extracts overexpressing mBcl-2 (R6-Bcl-2#9), performed in the absence (lane a) or presence of the wild-type Bax–BH3 peptide (lane b). (**C**) α-h/mBax/UBI Western blot of α-mBax immunoprecipitates from R6-Bcl-2/Bax#9 cell extracts before (lane a) and after (lane b) three rounds of mBcl-2 immunodepletion using the α-mBcl-2/10C4 antibody. We also show α-h/mBax/UBI and α-mBcl-2/10C4 Western blots of the cell extracts before (lanes c and e) and after (lanes d and f) mBcl-2 immunodepletion to prove the total disappearance of Bcl-2 (compare lanes e and f) and the partial disappearance of exo hBax and endo rBax (compare lanes c and d).

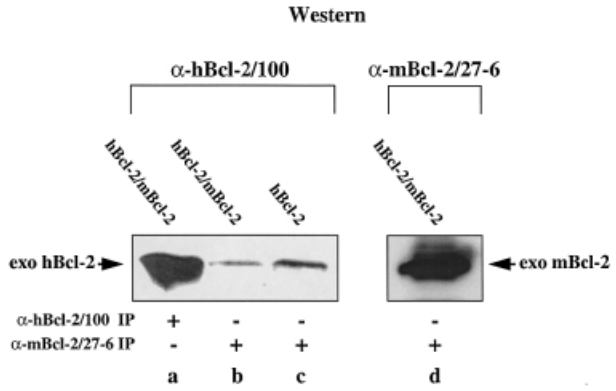

Fig. 2. No interaction between Bcl-2 molecules in HEK cell extracts. α-hBcl-2/100 Western blot of unlabeled α-hBcl-2/100 (lane a) and α-mBcl-2/27-6 (lanes b and c) immunoprecipitates from extracts of HEK cells transfected with hBcl-2 alone or co-transfected with hBcl-2 and mBcl-2. Lane d shows an α-mBcl-2/27-6 Western blot of an α-mBcl-2/27-6 immunoprecipitate from extracts of co-transfected cells. Note that there is not more hBcl-2 in lane b than in lane c, indicating that hBcl-2 does not co-precipitate with mBcl-2.

of endogenous rat Bax with exogenous mouse Bcl-2 (mBcl-2) from an R6-Bcl-2#9 cell lysate (Figure 1B). To rule out the possibility that Bax formed homodimers via Bcl-2, we immunodepleted the R6-Bcl-2/Bax#9 cell extract of Bcl-2 prior to anti-Bax immunoprecipitations. Although this procedure also diminished the levels of Bax (due to its binding to Bcl-2) (Figure 1C, lanes c and d), we could still detect the co-immunoprecipitation of exogenous hBax with endogenous rBax (lane b) in the absence of any detectable Bcl-2 (lane f). Thus, Bax can form either Bcl-2-independent homodimers or heterodimers with Bcl-2 in a BH3-dependent manner.

No evidence for Bcl-2 homodimers in anti-Bcl-2 and anti-Flag immunoprecipitates

Next, we wanted to know whether Bcl-2 formed homodimers under the same extraction/immunoprecipitation conditions. For that purpose we transiently co-overexpressed hBcl-2 and mBcl-2 in HEK cells. Following an immunoprecipitation with a Bcl-2 antibody against one species, the immunoprecipitate was immunoblotted and probed with a Bcl-2 antibody against the other species. As shown in Figure 2, the mouse Bcl-2-specific antibody α-mBcl-2/27-6 immunoprecipitated high amounts of mBcl-2 from an HEK-hBcl-2/mBcl-2 cell extract (lane d). This antibody slightly crossreacted with hBcl-2 as seen in an α-mBcl-2/27-6 immunoprecipitate from HEK-hBcl-2 lysates (Figure 2, lane c). The amount of crossreactive hBcl-2 was, however, far below that immunoprecipitated with the human Bcl-2-specific antibody α-hBcl-2/100 (Figure 2, compare lanes a and c). Moreover, an α-mBcl-2/27-6 immunoprecipitate from an HEK-hBcl-2/mBcl-2 lysate did not contain more hBcl-2 than an α-mBcl-2/27-6 immunoprecipitate from an HEK-hBcl-2 lysate (Figure 2, compare lanes b and c), indicating that the two Bcl-2 species did not interact.

To confirm the absence of Bcl-2 dimers *in vitro*, we investigated the interaction of mBcl-2 with an N-terminally Flag-tagged version of mBcl-2 (Flag–mBcl-2) in lysates from R6 cells stably overexpressing the two proteins individually (R6-Bcl-2#9, R6-Flag–Bcl-2mix) or in combination (R6-Bcl-2/Flag–Bcl-2#7). Flag–mBcl-2 was as efficient in protecting cells from apoptosis as mBcl-2 (Otter *et al.*, 1998; also see Supplementary material available in *The EMBO Journal* Online). Thus, if dimer formation was crucial for Bcl-2 function it should also occur between Flag–mBcl-2 and mBcl-2. When we compared α-Flag immunoprecipitates

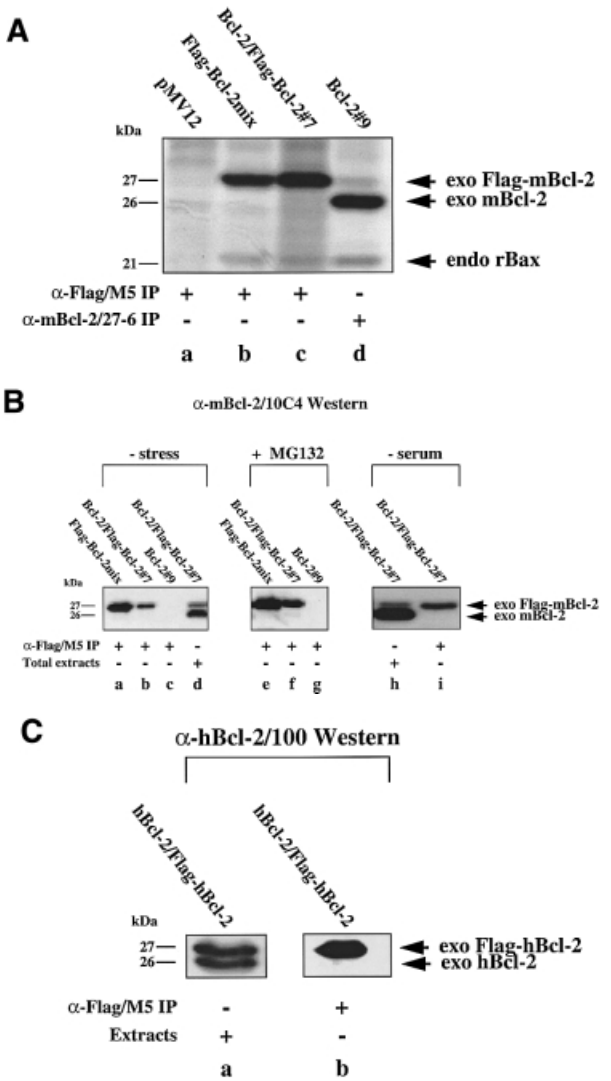

Fig. 3. No interaction between Bcl-2 and Flag-tagged Bcl-2 in R6 extracts. (**A**) α-Flag/M5 (lanes a–c) or α-mBcl-2/27-6 (lane d) immunoprecipitates from radiolabeled R6 cell extracts harboring either the transfer vector (pMV12) (lane a), Flag–mBcl-2 (R6-Flag–Bcl-2mix) (lane b), mBcl-2 (R6-Bcl-2#9) (lane d) or both (R6-Bcl-2/Flag–Bcl-2#7) (lane c). Note the absence of a band at 26 kDa in lane c, indicating that 26 kDa mBcl-2 does not co-precipitate with 27 kDa Flag–mBcl-2. (**B**) α-mBcl-2/10C4 Western blot of α-Flag/M5 immunoprecipitates from unlabeled extracts of R6 cells overexpressing Flag–mBcl-2 (R6-Flag–Bcl-2mix) (lanes a and e) or mBcl-2 (R6-Bcl-2#9) (lanes c and g) or both (R6-Bcl-2/Flag–Bcl-2#7) (lanes b, f and i) before (– stress) and after treatment with 1 μM MG132 (+ MG132) or depletion of serum for 48 h (– serum). Total extracts from R6-Bcl-2/Flag–Bcl-2#7 cells in the presence or absence of serum are shown in lanes d and h, respectively. Note that neither lane b nor lanes f and i contain co-precipitating 26 kDa exo mBcl-2 although it is clearly present in the total extracts (lanes d and h). (**C**) α-hBcl-2/100 Western blot of an extract (lane a) or an α-Flag/M5 immunoprecipitate of this extract (lane b) prepared from HEK cells co-transfected with Flag–hBcl-2 and hBcl-2. Note that the human forms of Bcl-2 also do not interact.

from extracts of [^{35}S]methionine/cysteine-labeled R6-Bcl-2/Flag–Bcl-2#7 and R6-Flag–Bcl-2mix cells, we found in both cases a 27 kDa protein corresponding to Flag–mBcl-2 as well as a 21 kDa protein corresponding to co-precipitated endogenous rBax (Figure 3A, lanes b and c). However, there was no additional 26 kDa mBcl-2 that co-immuno-precipitated from the R6-Bcl-2/Flag–Bcl-2#7 cell extract (Figure 3A, lane c), indicating that Flag–mBcl-2 and mBcl-2 did not interact.

To increase the sensitivity of immunodetection, we probed the α-Flag immunoprecipitates from unlabeled R6-Bcl-2/Flag–Bcl-2#7 and R6-Flag–Bcl-2mix lysates on an α-mBcl-2/10C4 Western blot. In addition, we considered the possibility that Bcl-2 homodimers would only form when the cells were exposed to apoptotic stimuli, i.e. when Flag–mBcl-2 and mBcl-2 acted as survival factors. For that purpose, we treated the cells with 1 μM MG132 or removed the serum for 48 h. Under these conditions most of the R6 cells underwent apoptosis while the Bcl-2 overexpressing counterparts were effectively protected (Otter *et al*., 1998; our unpublished data). α-Flag immunoprecipitates from both unstressed and stressed R6-Bcl-2/Flag–Bcl-2#7 lysates still contained only the 27 kDa Flag–mBcl-2 and no co-precipitated 26 kDa mBcl-2 when tested on an α-mBcl-2/10C4 Western blot (Figure 3B, lanes b, f and i). This was not due to the degradation or lack of expression of the 26 kDa mBcl-2 as this protein was clearly immunodetected in a total extract of unstressed (Figure 3B, lane d) or stressed (lane h) R6-Bcl-2/Flag–Bcl-2#7 cells. In addition, the lack of Bcl-2 homodimerization was not species specific since the human forms of Flag–Bcl-2 and Bcl-2 also did not interact in α-Flag immunoprecipitates (Figure 3C, lane b). Taken together, our data show that even under apoptotic stress conditions when Bcl-2 effectively acts as a survival factor, it does not form homodimers.

Purified recombinant Bax multimerizes, while Bcl-2 stays monomeric

We embarked on yet another approach to study di- or multimer formation of Bcl-2 and Bax *in vitro*. Both proteins were generated as GST fusions in bacteria, attached to a glutathione–Sepharose column and cleaved on the column by thrombin to release the recombinant mBcl-2 or hBax proteins. Since we worked with the full-length molecules, detergent had to be included at all steps of the purification. However, to minimize micelle formations on the FPLC column, elutions of mBcl-2 and hBax from the glutathione column were performed in the presence of 0.05% *N*,*N*-dimethyldodecylamine-*N*-oxide (LDAO) instead of 1% Triton X-100. After passing through an FPLC Superose 12 sizing column and comparing with the elution of standard proteins (see figure legends), the two recombinant proteins exhibited a strikingly different elution profile. While hBax eluted in several peaks that were multiples of ~21 kDa (Figure 4A), mBcl-2 eluted in a single peak of ~26 kDa (Figure 4B). These peaks all contained the respective hBax or mBcl-2 proteins as tested by Western blotting of the eluted fractions (our unpublished data). Moreover, when Flag–mBcl-2 was affinity purified from an R6-Flag–Bcl-2mix extract and applied to the Superose column, it also eluted as a single peak at ~27 kDa

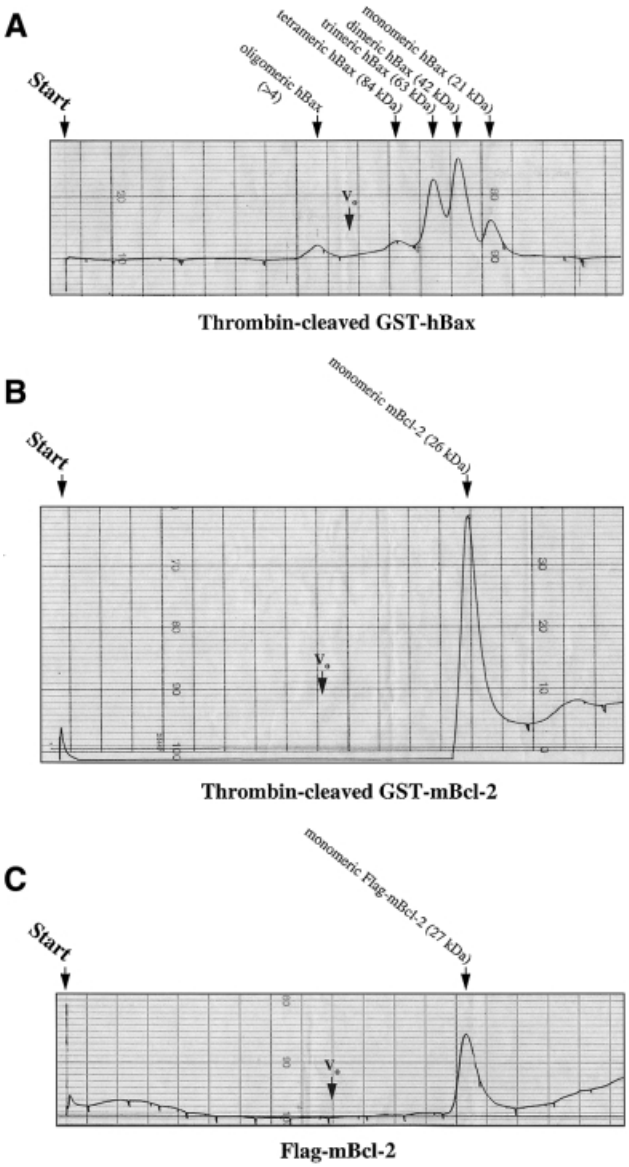

Fig. 4. Recombinant Bax, but not Bcl-2 or Flag–Bcl-2, forms multimers *in vitro*. On-line 280 nm measurements of glutathione affinity-purified, thrombin-cleaved GST–hBax (**A**), GST–mBcl-2 (**B**) or α-Flag/M2-purified Flag–mBcl-2 (**C**) eluting from an FPLC Superose 12 column. The time of sample application (Start) and the void volume of the column (V_o) are indicated. While hBax elutes in multiple peaks corresponding to the molecular masses of its multimers (21, 42, 63, 84 kDa and higher), mBcl-2 and Flag–mBcl-2 are recovered in one single peak each (monomeric 26 or 27 kDa forms, respectively). The molecular masses were calculated according to the elutions of the standard proteins carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa) and β-galactosidase (115 kDa).

(Figure 4C). These data show that Bax, but not Bcl-2, can multimerize *in vitro*.

Bax, but not Bcl-2, dislocates a soluble form of Bcl-2 inside cells

It has been reported that detergent can either promote or disrupt Bcl-2 family protein interactions (Hsu and Youle, 1997; Otter *et al*., 1998). We therefore needed to confirm our Bcl-2–Bax interaction studies in an experimental

system that does not require detergent extraction. We chose an immunofluorescence analysis on fixed, permeabilized R6 cells and investigated how Bax and Bcl-2 molecules recruit each other to different subcellular compartments. Our previous data showed that while Bax was mainly associated with mitochondria, a high portion of Bcl-2 was bound to the nuclear envelope and the associated ER membrane (Borner *et al*., 1994a; Rossé *et al*., 1998). Upon co-overexpression, Bax could attract Bcl-2 to the mitochondrial membrane, indicating that the two proteins interacted inside cells (Otter *et al*., 1998; Rossé *et al*., 1998); however, it was difficult to monitor Bcl-2–Bcl-2 interactions as mBcl-2, hBcl-2 and Flag–mBcl-2 all localized to the same nuclear/ER site (our unpublished data). We therefore took advantage of a Bcl-2 mutant that was devoid of its C-terminal membrane anchor and thus localized to the cytoplasm (mBcl-2ΔTM). This mutant was previously shown to retain its function to protect neurons from NGF-deprived apoptosis (Borner *et al*., 1994a). We first validated our strategy by testing whether Bax could attract mBcl-2ΔTM similarly to the way in which it attracted wild-type mBcl-2. As expected, while wild-type Bcl-2 was detected on nuclear/ER membranes, mBcl-2ΔTM exhibited a diffuse cytoplasmic and intranuclear staining after transfection into R6 cells (Figure 5A). The reason for a presumed nuclear staining of mBcl-2ΔTM is unknown but the lack of its membrane anchor and its low molecular mass (24 kDa) may allow a free passage into the nucleus. In contrast, transfected hBax showed a punctated, mitochondrial pattern as previously reported (Rossé *et al*., 1998), and triggered nuclear fragmentation (Figure 5A). Upon co-transfection with Bax, mBcl-2ΔTM lost most of its cytoplasmic/nuclear association, became co-localized with Bax in punctated mitochondria and inhibited Bax-induced nuclear fragmentation (Figure 5A). Thus, cytosolic mBcl-2ΔTM behaved like wild-type membrane-bound Bcl-2 in that it was attracted by Bax to mitochondria and inhibited the pro-apoptotic activity of the latter. This set the stage to investigate whether mBcl-2ΔTM interacted similarly with wild-type Bcl-2. To distinguish between the two proteins, we co-transfected R6 cells with mBcl-2ΔTM and a human form of wild-type Bcl-2 and used species-specific antibodies for immunofluorescence. As shown in Figure 5B, while hBcl-2 was mainly detected on the nuclear envelope/ER network, mBcl-2ΔTM remained diffuse, cytoplasmic and intranuclear after co-transfection into R6 cells. This was also the case when the cells were exposed to the apoptotic stimulus MG132 (our unpublished data). Thus, unlike Bax, hBcl-2 cannot significantly recruit mBcl-2ΔTM to the ER/nuclear membrane, suggesting that Bcl-2 homodimers do not readily form within cells.

Bcl-2 lacking BH1/BH2 domains dimerizes with wild-type Bcl-2 in a BH3-dependent manner

Our data so far indicate that despite a high sequence homology, Bcl-2 and Bax may be structurally different. An attractive possibility is that Bax can form di- or multimers because its BH3 domain is exposed or at least easily recruitable. In Bcl-2 this domain would be hidden inside the molecule. Indeed, based on the crystal structure of Bcl-x_L, the BH3 domain of Bcl-2 is in close contact with the BH1/BH2 domains (Muchmore *et al*., 1996; Aritomi *et al*., 1997). Thus, by breaking this intramolecular interaction, we may obtain a Bcl-2 molecule that now has its BH3 domain exposed for dimerizations. We therefore generated two Bcl-2 deletion mutants: an mBcl-2 lacking BH1/BH2 (mBcl-2ΔBH1/2) and an mBcl-2 lacking BH1/BH2 and BH3 (mBcl-2ΔBH1/2/3). The purpose of the latter mutant was to test whether an eventual interaction between mBcl-2ΔBH1/2 and Bcl-2 was BH3 mediated. Both mBcl-2ΔBH1/2 and mBcl-2ΔBH1/2/3 were stable proteins of 19 and 17 kDa, respectively, which could be highly expressed in HEK, R6 and other cell types (Figure 6, lanes g–j; our unpublished data). To see whether mBcl-2BHΔ1/2, but not mBcl-2ΔBH1/2/3, was capable of dimerizing with hBcl-2, we first performed the above described Western blot/immunoprecipitation analysis on HEK cells transiently transfected with hBcl-2, each of the mutants, or in combinations. As shown in Figure 6, the α-hBcl-2/100 antibody detected similar amounts of 26 kDa hBcl-2 in HEK-hBcl-2 (lane a), HEK-hBcl-2/mBcl-2ΔBH1/2 (lane c) and HEK-hBcl-2/mBcl-2ΔBH1/2/3 lysates (lane e). Also, the α-mBcl-2/10C4 antibody detected similar amounts of 19 kDa mBcl-2ΔBH1/2 or 17 kDa mBcl-2ΔBH1/2/3 in HEK-mBcl-2ΔBH1/2 (lane h), HEK-mBcl-2ΔBH1/2/3 (lane j), HEK-hBcl-2/mBcl2ΔBH1/2 (lane g) and HEK-hBcl-2/mBcl-2ΔBH1/2/3 lysates (lane i). This showed that the respective proteins were similarly expressed in single and double transfectants. As previously seen with α-mBcl-2/27-6 (Figure 2, lane c), the α-mBcl-2/10C4 antibody slightly crossreacted with hBcl-2 and immunoprecipitated a low amount of the protein from an HEK-hBcl-2 extract (Figure 6, lane b). The amount of hBcl-2 was, however, 5–10 times higher in an α-mBcl-2/10C4 immmunoprecipitate from HEK-hBcl-2/mBcl-2ΔBH1/2 cells (Figure 6, lane d). This was not the case when the immunoprecipitation was performed on an HEK-hBcl-2/mBcl-2ΔBH1/2/3 extract (Figure 6, lane f), indicating that hBcl-2 and mBcl-2ΔBH1/2 interacted in a BH3-dependent fashion.

Bcl-2 dislocates mitochondrial Bcl-2ΔBH1/2 to the ER/nuclear membrane

To substantiate our *in vitro* findings, we used immunofluorescence to see whether Bcl-2 could dislocate mBcl-2ΔBH1/2, but not mBcl-2ΔBH1/2/3, inside cells. Strikingly, when mBcl-2ΔBH1/2 was transiently expressed in R6 cells, it displayed a 'spaghetti-like' immunodetection pattern that co-localized with the mitochondrial marker Tom20 (Figure 7A). This was detected with both the α-mBcl-2/10C4 (Figure 7A) and α-mBcl-2/27-6 (our unpublished data) antibodies. In contrast, as shown in Figure 5B, hBcl-2 was mainly associated with ER/nuclear membrane structures (Figure 7B). Upon co-transfection with hBcl-2, mitochondrial mBcl-2ΔBH1/2 dislocated to the nuclear/ER membrane, indicating that the two proteins could interact within cells (Figure 7B). This was not the case with the mBcl-2ΔBH1/2/3 mutant as it did not show co-localization with Bcl-2 on the nuclear envelope after co-transfection with Bcl-2 (Figure 7B). Thus, our data show that the deletion of the BH1/BH2 domains converts Bcl-2 into a molecule that can interact with wild-type Bcl-2 in a BH3-dependent manner, both *in vitro* and within cells.

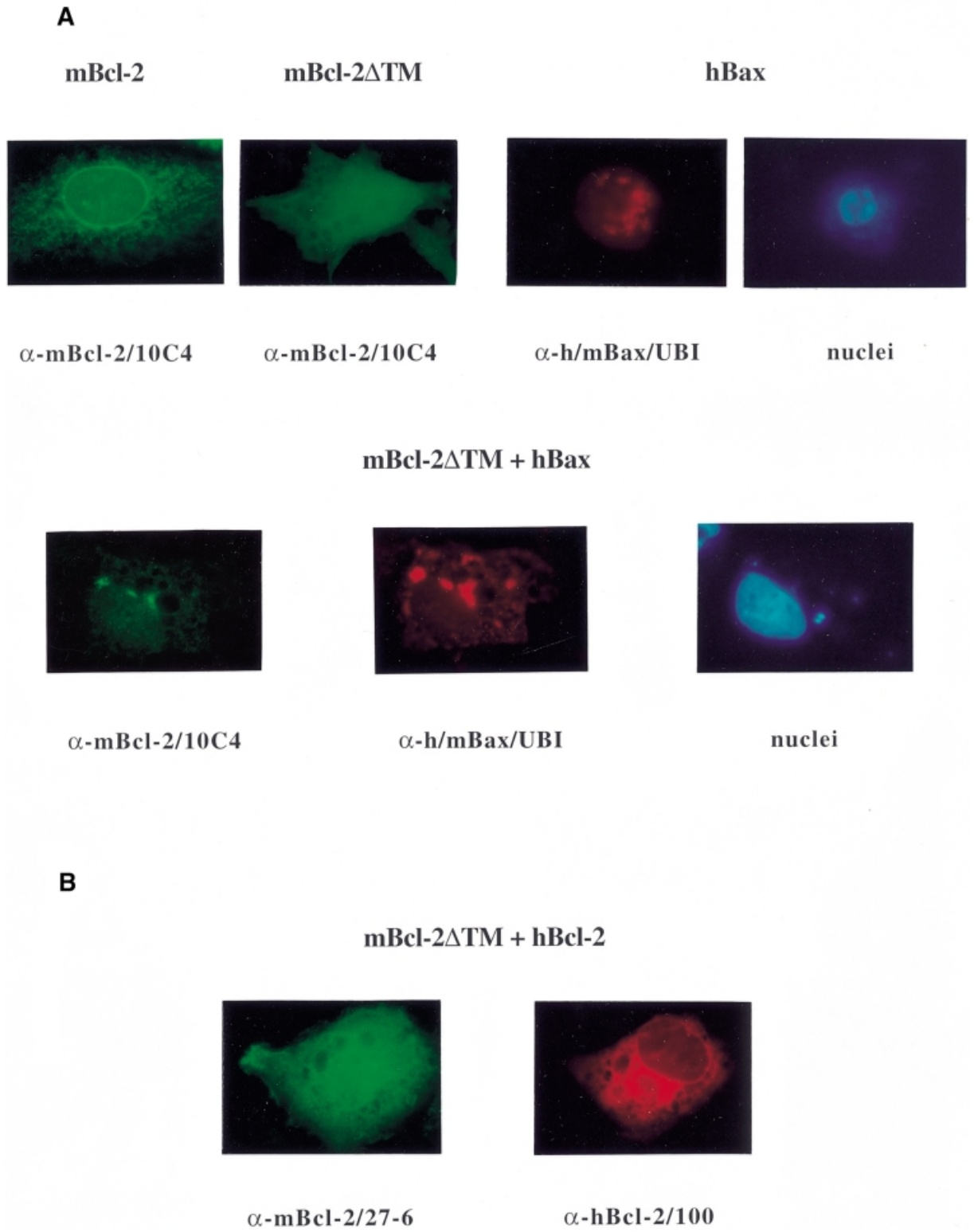

Fig. 5. Membrane recruitment of cytoplasmic Bcl-2ΔTM by Bax, but not by Bcl-2. (**A**) α-mBcl-2/10C4 (fluorescein), α-h/mBax/UBI (Texas Red) and Hoechst 33342 (UV, nuclei) fluorescence analysis on R6 cells transfected with the C-terminally deleted mBcl-2ΔTM mutant, hBax or both at 24 h post-transfection. For comparison a wild-type mBcl-2 transfectant is shown. (**B**) α-mBcl-2/27-6 (fluorescein) and α-hBcl-2/100 (Texas Red) fluorescence analysis on R6 cells co-transfected with mBcl-2ΔTM and hBcl-2 at 24 h post-transfection. Note that hBax attracts mBcl-2ΔTM from a diffuse to a punctated (mitochondrial) staining, accompanied by a block in nuclear condensation/fragmentation. Both hBcl-2 and mBcl-2 localize to the ER/nuclear membrane and do not attract mBcl-2ΔTM.

A Bcl-2 mutated in BH2 can attract wild-type Bcl-2 to mitochondria

It is known that blatant deletions in proteins can produce structural changes that may be artefactual. Although the mBcl-2ΔBH1/2 mutant is a perfect homolog of the naturally occurring Bcl-x_s splice variant (the variant of Bcl-x_L that lacks BH1 and BH2 domains) (Boise *et al.*, 1993), we were still worried that it might not fold correctly

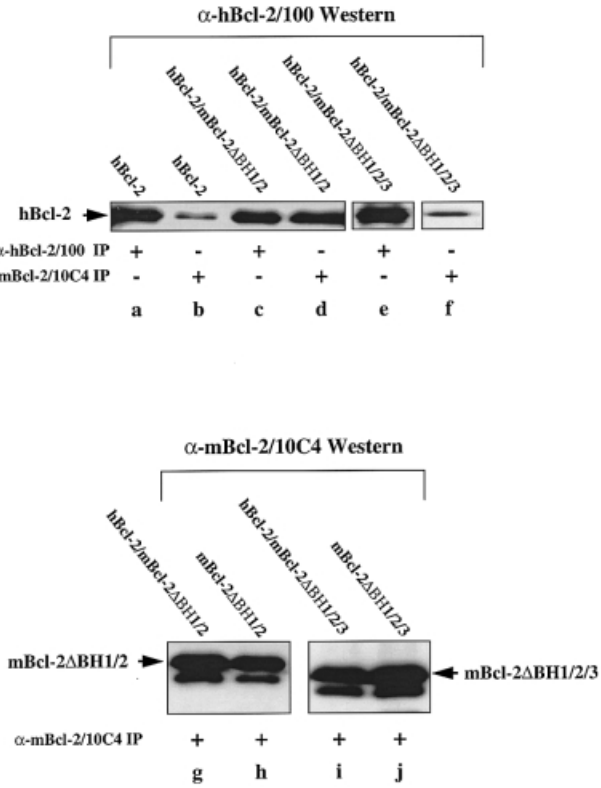

Fig. 6. BH3-dependent interaction between Bcl-2 and Bcl-2ΔBH1/2 in HEK extracts. α-hBcl-2/100 Western blot of α-hBcl-2/100 (lanes a, c and e) or α-mBcl-2/10C4 immunoprecipitates (lanes b, d and f) from unlabeled extracts of HEK cells transfected with hBcl-2 alone (lanes a and b) or together with mBcl-2ΔBH1/2 (lanes c and d) or mBcl-2ΔBH1/2/3 (lanes e and f). Note that there is more 26 kDa hBcl-2 in lane d than b or f, indicating that hBcl-2 co-precipitates with mBcl-2ΔBH1/2, but not with mBcl-2ΔBH1/2/3. As a control for equal expression/immunoprecipitation of the mutant proteins, we show an α-mBcl-2/10C4 Western blot of α-mBcl-2/10C4 immunoprecipitates from extracts of HEK cells transfected with mBcl-2ΔBH1/2 (lane h) or mBcl-2ΔBH1/2/3 (lane j) alone or together with hBcl-2 (lanes g and i).

inside cells and promote an aberrant interaction with wild-type Bcl-2. We therefore created a Bcl-2 variant that had retained a BH2 domain but in a mutated form. To avoid the introduction of unnatural mutations, we exchanged the BH2 domain and C-terminus of Bcl-2 with the corresponding regions of Bax. The resulting hBcl-2(BH2C-Bax) construct was expressed as a stable 25 kDa protein in HEK cells (our unpublished data) and exhibited a predominantly mitochondrial immunolocalization after transfection into R6 cells (Figure 8A). Like mBcl-2ΔBH1/2, this mutant Bcl-2 seemed to interact with wild-type Bcl-2 but at another intracellular site. Instead of being passively attracted by hBcl-2 to the ER/nuclear membrane (Figure 7B), the hBcl-2(BH2C-Bax) variant actively dislodged Bcl-2 from the ER/nuclear membrane to mitochondria (Figure 8B). This dislocation again depended on the BH3 domain, as an hBcl-2(BH2C-Bax) deleted in BH3 did not recruit Bcl-2 (our unpublished data). Thus, our findings indicate that not only blatant deletions but also subtle mutations in the BH2 region convert Bcl-2 into a molecule that can now interact with Bcl-2 in a BH3-dependent fashion.

Discussion

It has been proposed that Bcl-2 family members regulate apoptosis like a rheostat (Oltvai *et al.*, 1993). If pro-apoptotic factors such as Bax prevail, they form homodimers and cell death ensues. If anti-apoptotic factors such as Bcl-2 are more abundant, it is their turn to form homodimers and cells are protected from apoptotic insults. In between these two extremes is the formation of Bax–Bcl-2 heterodimers that keep additional cytotoxic and survival activities in check. Although various mutagenesis and expression studies have confirmed the existence and role of Bax–Bax and Bax–Bcl-2 dimerizations (Adams and Cory, 1998; Reed, 1998), those of Bcl-2 homodimers have remained hypothetical. In this study we challenge this issue and show by several methods that Bcl-2 does not form homodimers when it sets the rheostat to 'survival'.

The crystal structure of Bcl-x_L in the absence or presence of the Bak–BH3 peptide shows that Bcl-2 family members with several BH domains can be in two conformational states (Muchmore *et al.*, 1996; Aritomi *et al.*, 1997; Sattler *et al.*, 1997): a 'receptor state', where all BH domains join to form a hydrophobic face to which other proteins can bind, and a 'ligand state', where the BH3 domain of this hydrophobic face can turn outwards to interact with the hydrophobic face of another Bcl-2-like molecule. This nicely explains the formation of heterodimers between anti-apoptotic and pro-apoptotic partners, especially when the pro-apoptotic partner is a so-called 'BH3-only' protein. Such proteins have the BH3 domain as their only Bcl-2 homology domain and use non-homologous regions to localize to different subcellular compartments (Kelekar and Thompson, 1998). This has been elegantly demonstrated with the protein Bim, which usually binds to the microtubule-associated dynein motor complex via regions outside of BH3 but uses the BH3 domain to translocate to mitochondrial Bcl-2 in apoptotic cells (Puthalakath *et al.*, 1999).

An unresolved issue is whether Bax or Bcl-2 homodi- or multimers form via the same mechanism as Bax–Bcl-2 heterodimers. In favor of such a model is that homodimerization of Bax depends on both the BH3 and BH1 domains (Simonian *et al.*, 1996; Simonen *et al.*, 1997; Zha and Reed, 1997; Kelekar and Thompson, 1998), so that one Bax molecule would use its BH3 domain as a 'ligand', the other as part of the hydrophobic face 'receptor'. However, a previous study (Lewis *et al.*, 1998) and our study here show that Bax forms high-order structures (up to heptamers) that are inconsistent with such a dimerization scheme. Alternatively, Bax may not be able to maintain a hydrophobic face 'receptor' structure, but exposes its BH3 domain for homotypic interactions. There is, however, no reported evidence that BH3 peptides, either in their isolated form or when present in 'BH3-only' proteins have the tendency to dimerize or even multimerize. It therefore remains unknown how Bax multimers form.

Part of the reason for our lack of insight into the Bax structure is that this molecule undergoes a conformational change when it moves from an inactive state in the cytoplasm to an active state on the mitochondrial membrane (Wolter *et al.*, 1997; Goping *et al.*, 1998; Griffiths *et al.*, 1999). Analogous to diphtheria toxin or colicin,

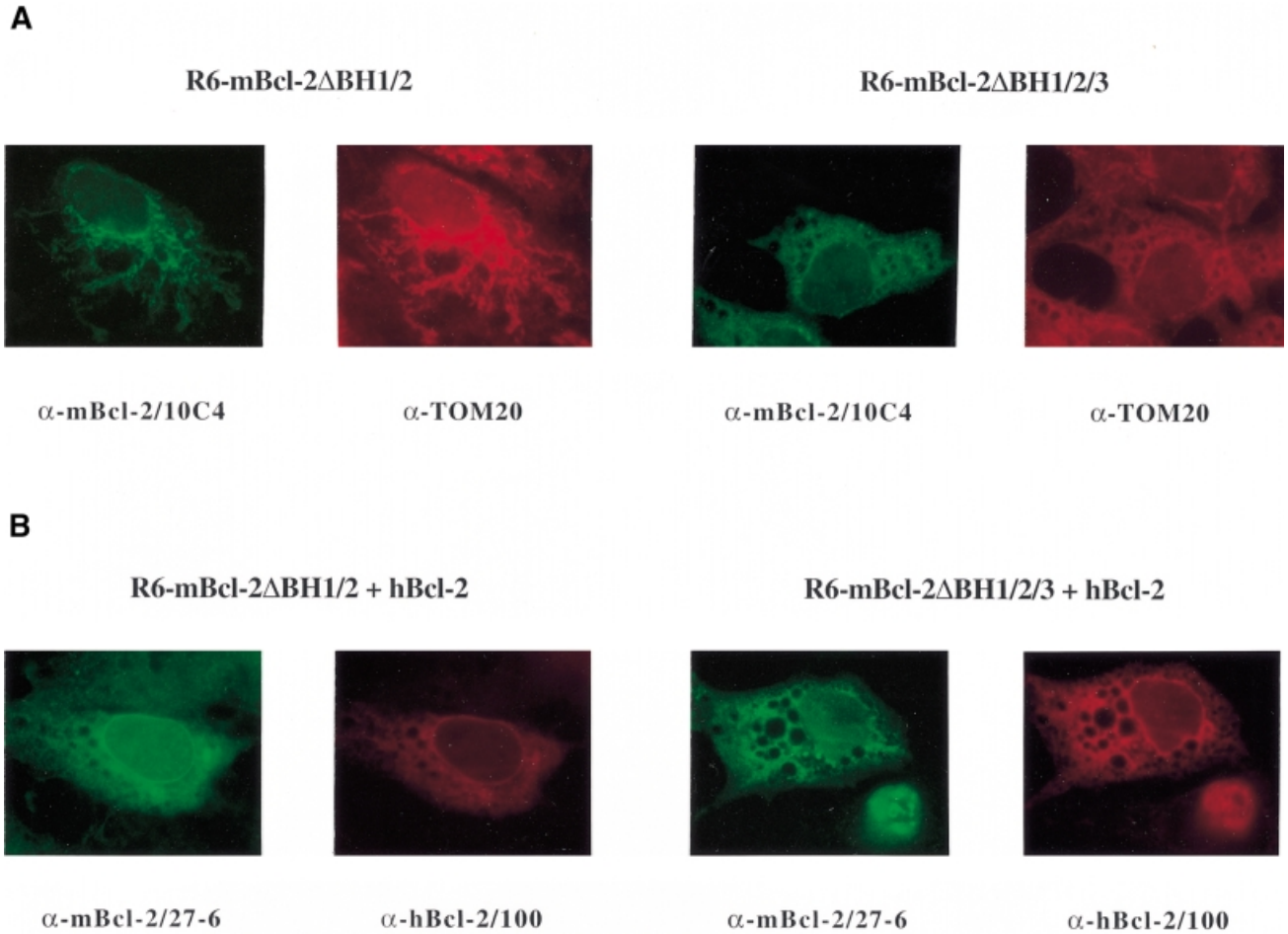

Fig. 7. Dislocation of mitochondria-associated Bcl-2ΔBH1/2, but not Bcl-2ΔBH1/2/3, to the ER/nuclear membrane by Bcl-2 in R6 cells. (**A**) α-mBcl-2/10C4 (fluorescein) and α-Tom20 (Texas Red) fluorescence analysis on R6 cells transfected with mBcl-2ΔBH1/2 or mBcl-2ΔBH1/2/3 at 24 h post-transfection. (**B**) α-mBcl-2/27-6 (fluorescein) and α-hBcl-2/100 (Texas Red) fluorescence analysis on R6 cells co-transfected with mBcl-2ΔBH1/2 and hBcl-2 or mBcl-2ΔBH1/2/3 and hBcl-2 at 24 h post-transfection. In this co-localization experiment α-mBcl-2/10C4 cannot be used because it is a monoclonal mouse antibody like α-hBcl-2/100. However, α-mBcl-2/27-6 and α-mBcl-2/10C4 exhibit the same immunostaining of mBcl-2ΔBH1/2 and mBcl-2ΔBH1/2/3 (our unpublished data). Note that mBcl-2ΔBH1/2 dislocates from its original mitochondrial to a nuclear envelope/ER pattern upon co-transfection with hBcl-2. In contrast, mBcl-2ΔBH1/2/3 does not co-localize with hBcl-2 on the nuclear envelope. The vacuoles are due to the transient transfection with Superfect.

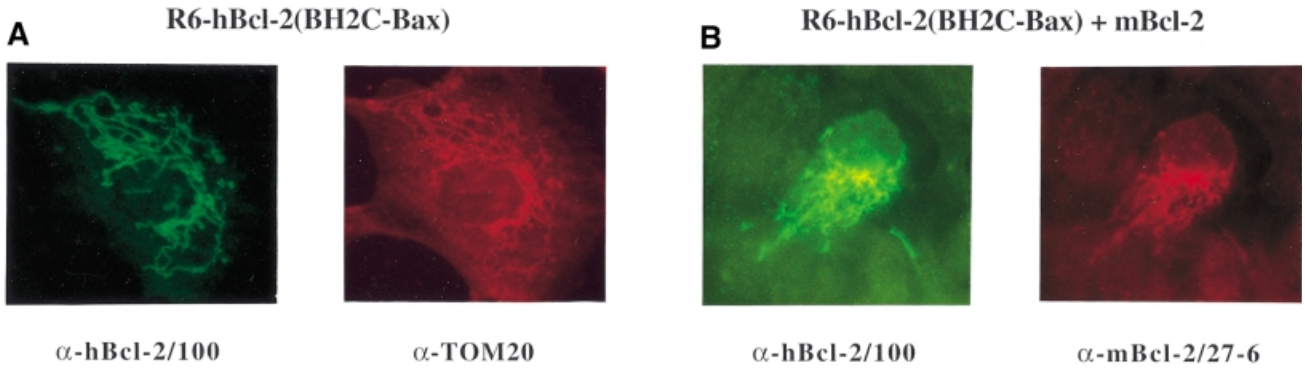

Fig. 8. Attraction of ER-localized Bcl-2 to mitochondria by Bcl-2(BH2C-Bax). (**A**) α-hBcl-2/100 (fluorescein) and α-Tom20 (Texas Red) fluorescence analysis on R6 cells transfected with hBcl-2(BH2C-Bax) at 24 h post-transfection. (**B**) α-hBcl-2/100 (fluorescein) and α-mBcl-2/27-6 (Texas Red) fluorescence analysis on R6 cells co-transfected with hBcl-2(BH2C-Bax) and mBcl-2 at 24 h post-transfection. Note that mBcl-2, which usually associates with nuclear/ER structures (Figure 5A), localizes to 'spaghetti-like' mitochondria upon co-expression with hBcl-2(BH2C-Bax).

this change may disrupt the hydrophobic face of Bax and expose its pore-forming BH1/BH2 (α5/α6) regions for membrane insertion. As a consequence, the Bax structure would open up like an umbrella (as proposed for the bacterial toxins) to allow other protein regions, such as the BH3 domain, to bind other proteins or to participate in multimerization reactions (Schendel *et al*., 1998). In this way, Bax could form a multimerized ion- or cytochrome *c*-releasing channel on the mitochondrial membrane. Although our data do not prove such a mechanism, they

show that Bax can form homodimers in immunoprecipitates of membrane extracts and even multimers as full-length recombinant protein *in vitro*. These Bax–Bax interactions are clearly mediated by the BH3 domain and not by regions that are artificially exposed due to the presence of detergent (NP-40 or LDAO), as recently suggested (Hsu and Youle, 1997).

The novel and quite surprising finding of this study is that Bcl-2 behaves differently from Bax under the same experimental conditions. Despite the fact that immunoprecipitations and FPLC analysis were also performed in detergent, neither cell-based, cell-extracted nor recombinant Bcl-2 homodi- or multimerized. Even in response to apoptotic stimuli such as MG132 or serum removal Bcl-2 remained in a monomeric form. These findings strongly indicate that Bcl-2 is structurally different from Bax and does not require homodimerization for its survival activity. Indeed, when Aritomi *et al.* built a homology model of Bax from the Bcl-x_L structure, they noted that Bax exhibited more hydrophobic patches and a better exposure of its α5/α6 helices than Bcl-x_L (Aritomi *et al.*, 1997). They suggested that Bax has a greater potential for membrane insertion and is more likely to form membrane pores than either Bcl-2 or Bcl-x_L. Moreover, there is no reported evidence that Bcl-2, like Bax, changes its conformation and translocates from the cytoplasm to membranes in response to apoptotic stimuli. Bcl-2 is rarely cytoplasmic, but if it is due to the removal of its hydrophobic C-terminus (e.g. mBcl-2ΔTM), it is still partially active (Borner *et al.*, 1994a) and looks like the known structure of C-terminally deleted Bcl-x_L. We therefore propose that Bcl-2 retains its hydrophobic face structure to bind crucial death-regulatory proteins (including BH3-containing proteins) both in the cytoplasm and on membranes. In this respect the C-terminal sequence would serve to anchor Bcl-2 to various different membranes to increase its access to crucial binding proteins (the 'docking activity' model) (Reed, 1997).

However, this model implies, as previously suggested (Aritomi *et al.*, 1997), that Bcl-2 would not necessarily form multimeric channels like Bax within cells. How can this be reconciled with the reported *in vitro* channel activity of Bcl-2 (Schendel *et al.*, 1998)? First, there is currently no proof that any member of the Bcl-2 family forms channels under physiological conditions. In fact, one report shows that Bcl-2 blocks the channel activity of Bax rather than being a channel itself (Antonsson *et al.*, 1997). Secondly, the non-physiological amounts of pure, recombinant Bcl-2 protein and phospholipid vesicles could force Bcl-2 into a conformation that facilitates membrane insertion and dimerizations like Bax. Thirdly, most of the recombinant Bcl-2 proteins used to measure channel activities *in vitro* were C-terminally deleted and/or tagged at their N-termini (His, GST, etc.) (Minn *et al.*, 1997; Schendel *et al.*, 1997). Although some fusion proteins such as Flag–Bcl-2 are active and stay in a monomeric form as shown here, others such as GST–Bcl-2 lose their capacity to protect cells from apoptosis and tend to dimerize (our unpublished data). Similarly, homodimerization of Bcl-2 in the yeast two-hybrid system may have been due to the presence of the LexA protein at the N-terminus of the Bcl-2 bait (Hanada *et al.*, 1995). By using untagged, full-length Bcl-2 proteins, we studied this molecule in its most natural form.

Our mutagenesis analyses indeed show that Bcl-2 needs to be in a correct conformation to perform its survival action. If the BH1 and BH2 regions of Bcl-2 are deleted, or the BH2 region is replaced by that of Bax, the hydrophobic face most likely changes its conformation in a way that Bcl-2 now homodimerizes in a BH3-dependent manner. That the dimerizations between the Bcl-2 mutants and Bcl-2 are not an artefact due to detergents can be shown by immunofluorescence analysis where the proteins are mutually attracted to mitochondria or ER/nuclear membrane sites. Why the Bcl-2 mutants are predominantly localized to mitochondria, as compared with the ER/nuclear association of wild-type Bcl-2, is not yet known. Perhaps there are exposed regions that now have a higher affinity for mitochondria than for other membranes, just as seen with Bax (Rossé *et al.*, 1998). Strikingly, both the Bcl-2ΔBH1/2 and Bcl-2(BH2C-Bax) mutants have lost their death-protective activity (Borner *et al.*, 1994a), indicating that the BH1/BH2 regions of Bcl-2 are indeed crucial for its function. Moreover, these mutants, but not the Bcl-2ΔBH1/2/3 version, can block the survival activity of Bcl-2 in a dominant-negative fashion (see Supplementary material available in *The EMBO Journal* Online), suggesting that their BH3 domains bind to the hydrophobic face of Bcl-2 like the BH3 domain of Bax. This reinforces the notion that the hydrophobic face of Bcl-2 is different from that of Bcl-2(BH2C-Bax) because only the former is maintained in a death-protective state while the latter is disrupted to expose its BH3 domain. It remains to be seen which region of Bcl-2 confers the stability of the hydrophobic face, and by what mechanism pro-apoptotic molecules such as Bax, Bak or the Bcl-2(BH2C-Bax) mutant change their conformation to di- or multimerize. A candidate region for regulation is the BH2 domain, in particular at its C-terminal half where it differs in sequence between Bcl-2 and Bax. This region, which we have called domain X, is absolutely crucial for the survival activity of Bcl-2 (Borner *etal.*, 1994b) but its role in homodimerization and cytotoxic activity of Bax is not yet known. Additional mutagenesis and crystallographic analysis will provide further insights into the structural differences between Bcl-2 and Bax and why they regulate apoptosis in an opposing fashion.

Materials and methods

Antibodies and cDNAs

The anti-Bcl-2 and anti-Bax antibodies used for immunoprecipitations and Western blots are described in the Supplementary material available in *The EMBO Journal* Online. The cDNAs for human Bax (hBax), human Bcl-2 (hBcl-2), mouse Bcl-2 (mBcl-2), mBcl-2 devoid of its C-terminus (mBcl-2ΔTM, formerly T3) and mBcl-2 devoid of the BH1/BH2 domains (mBcl-2ΔBH1/2, formerly DEL2) were generated and/or subcloned into the pcDNA3 or pcDNAamp vectors (Invitrogen) as previously described (Borner *et al.*, 1994a). The cDNAs for Flag-tagged hBcl-2, the mBcl-2 mutant lacking the BH1, BH2 and BH3 domains (mBcl-2ΔBH1/2/3) and the hBcl-2 mutant in which the BH2 and C-terminus were replaced by those of Bax [hBcl-2(BH2C-Bax)] were generated by PCR as described in the Supplementary material available in *The EMBO Journal* Online.

Cells

Human embryonic kidney 293 cells (HEK) and rat 6 (R6) embryo fibroblasts overexpressing Bcl-2, Flag–Bcl-2 and/or Bax were generated

and cultured as described in the Supplementary material available in *The EMBO Journal* Online.

Immunoprecipitations

HEK or R6 cell derivatives (3×10^6) were either non-labeled or radiolabeled with [^{35}S]methionine/cysteine (Tran^{35}S-label, Hartmann Analytic) and lysed for immunoprecipitations as previously described (Otter *et al.*, 1998). To disrupt Bax–Bax and Bax–Bcl-2 interactions, the cell lysate was supplemented with 100 μM wild-type (^{56}TKKLSE-CLKRIG**D**ELDSNM^{74}) or mutant (^{56}TKKLSECLKRIG**A**ELDSNM^{74}) Bax–BH3 peptide (kindly provided by Markus Wartmann and Carlos Garcia-Echeverria, Novartis Pharma Ltd, Basle, Switzerland). The lysates were then subjected to immunoprecipitation using 5 μl of α-mBcl-2/27-6, 3 μl of α-hBcl-2/100, 3 μl of α-mBcl-2/10C4, 6 μl of α-mBax or 2.5 μl of α-Flag/M5. Following antibody incubation for 2 h at 4°C, 50 μl of 50% (v/v) protein A–Sepharose (Sigma) or protein G–Sepharose (Amersham Pharmacia) were added, and the immunocomplexes were captured on an end-over-end wheel at 4°C for 1 h. Immunocomplexes were applied to 15% SDS–polyacrylamide gels and either blotted to a polyvinylidene difluoride membrane (PVDF) (Immobilon-P, Millipore) for Western blotting (unlabeled extracts) or analyzed by fluorography as described (Otter *et al*., 1998). For Bcl-2 immunodepletion, cell extracts were subjected to three rounds of immunoprecipitations using 5 μl of α-mBcl-2/10C4 and 50 μl of protein G–Sepharose for each round.

Western blotting

Western blot analysis of immunoprecipitates from unlabeled cell extracts was performed as described previously (Olivier *et al.*, 1997), using either α-hBcl-2/100 at a titer of 1:200, α-mBcl-2/10C4 at 1:500, α-mBcl-2/27-6 at 1:5000 or α-h/mBax/UBI at 1:10000. Secondary antibodies were Fcγ-specific, peroxidase-coupled goat anti-rabbit or anti-mouse antibodies (Jackson ImmunoResearch Laboratories).

Transient transfections

HEK cells were grown on 100 mm plates until ~80% confluence and transfected with 5 μg of one cDNA plus 5 μg of the pcDNA3 vector (single transfections) or 5 μg of each of two cDNAs (double transfections) using 25 μl of Superfect (Qiagen) as described by the manufacturer. After 3 h the Superfect–DNA complex was removed, and the cells were cultured for another 21 h in fresh medium before lysis for immunoprecipitation analysis.

Immunofluorescence analysis

Cells grown on 12 mm glass coverslips were transfected with 0.8 μg of one cDNA in 2.4 μl Superfect (single transfections) or 0.8 μg of each of two cDNAs in 4.8 μl Superfect as described above. At 24 h post-transfection, cells were washed twice in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde, and permeabilized with 0.05% saponin and acetone. The cells were treated with α-mBcl-2/27-6 (1:100), α-h/mBax/UBI (1:100), α-mBcl-2/10C4 (1:100) or α-hBcl-2/100 (1:100) in the absence or presence of the mitochondria marker antibody α-Tom20 (1:300) for 1 h followed by incubation with Texas Red- and fluorescein-conjugated goat anti-rabbit or anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories). After another fixation in 4% paraformaldehyde containing 2 μg/ml Hoechst 33342 dye (Molecular Probes), the anti-fading agent Slowfade (Molecular Probes) was added, and the cells were viewed under a Zeiss Axiovert fluorescence microscope at a magnification of 1000×. Pictures were taken with a Contax 167 MT camera.

FPLC analysis of Bcl-2/Bax multimers

mBcl-2 and hBax were expressed as GST fusion proteins in DH5α bacteria using the pGEX-based expression vectors as previously described (Olivier *et al*., 1997). After protein induction with 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG; Life Technologies) at 30°C for 4 h, bacterial extracts were prepared and applied to glutathione–Sepharose beads according to the manufacturer's protocol (Amersham Pharmacia). After washing in PBS plus 1% Triton X-100, the attached GST–mBcl-2 and GST–hBax were cleaved by 0.6 U of thrombin at 25°C for 1 h to release the mBcl-2 and hBax proteins into the supernatant. One hundred microliters (100 μg) of the cleaved proteins were applied to FPLC Superose 12 chromatography using 0.05% LDAO (Sigma) as detergent. Eluted protein was measured at OD_{280} on-line. For the analysis of Flag–mBcl-2, a cell extract of R6-Flag–Bcl-2mix cells was prepared in buffer A plus 0.2% NP-40 as described above for immunoprecipitations. The extract was applied to an anti-Flag M2 affinity column (Sigma), the column washed in buffer A and Flag–mBcl-2 eluted with 0.1 M glycine at pH 3.5. The eluted sample was immediately neutralized with NaOH and 100 μl (100 μg) subjected to FPLC chromatography as described for GST–mBcl-2.

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2.2 ARTICLE : Failure of Bcl-2 family members to interact with Apaf-1 in normal and apoptotic cells

Conus, S., Rossé, T. and Borner, C. (2000) *Cell Death Differ*. **7**, 947-954.

The molecular characterization of the Caenorhabditis elegans (*C. elegans*) cell death genes has been crucial in revealing some of the biochemical mechanisms underlying apoptosis in all animals. Four *C. elegans* genes, egl-1, ced-9, ced-4, and ced-3 are required for all somatic programmed cell death to occur. This genetic network is highly conserved during evolution. The pro-death gene egl-1 and the anti-death gene ced-9 have structural and functional similarities to the vertebrate Bcl-2 gene family. The killer gene ced-3 encodes a cysteine-aspartate protease (caspase), which is the archetype of a family of conserved proteins known as effectors of apoptosis in mammals. Zou *et al.* (1997) reported the biochemical identification of an apoptotic protease activating factor (Apaf-1), a human homologue of *C. elegans* CED-4, providing important clues to how CED-4 and its potential relatives could work. A number of proteins have been shown to interact with Apaf-1 or to be determinant for its activity as an apoptotic adapter (Cecconi, 1999). But it has remained controversial whether the CED-4 homologue Apaf-1 is a target and mediator of the death-protective activities of Bcl-2 and Bcl-xL. Two groups have reported a physical interaction between Bcl-xL and Apaf-1 in co-immunoprecipitates of HEK293 and MCF-7 cell lysates highly overexpressing the two proteins (Hu *et al.*, 1998; Pan *et al.*, 1998a). However another group could not find such Apaf-1 interactions when analyzing ten different Bcl-2 family members in their endogenous and overexpressed forms using cell lysates from normal and apoptotic cells (Moriishi *et al.*, 1999). All three studies used the same *in vitro* immunoprecipitation protocol where Apaf-1/Bcl-2 protein complexes may be disrupted during cell lysis.

With our results, we confirmed the failure of Bcl-2, Bcl-xL or Bax to co-immunoprecipitate with Apaf-1. In addition, we showed by immunofluorescence and subcellular fractionation analyses that while Flag-Apaf-1 and Apaf-1 are both cytoplasmic proteins, Bcl-2, Bcl-xL and Bax are predominantly bound to nuclear/ER and mitochondrial membranes. This pattern of localization is maintained when the proteins were co-overexpressed in normal or apoptotic cells in various combinations. Thus, neither Bcl-2, Bcl-xL nor Bax can sequester cytoplasmic Apaf-1 to intracellular membranes inside cells raising doubts that Apaf-1 is a direct physiological target of Bcl-2 family members.

While our paper was under review, Hausmann *et al.* (2000) published that Apaf-1 does not co-localize with Bcl-2 or Bcl-xL when analyzed by immunofluorescence and immunogold electron microscopy. Moreover, Newmeyer *et al.* (2000) and Haraguchi *et al.* (2000) used *in vitro* assays and cells deficient in Apaf-1 to show that Bcl-2 and Bcl-xL do not require Apaf-1 for their caspase-inhibiting and death protective function. These three papers confirmed our results showing that Bcl-2 family members does not directly interact with Apaf-1.

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Failure of Bcl-2 family members to interact with Apaf-1 in normal and apoptotic cells

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Abstract

CED-9 blocks programmed cell death (apoptosis) in the nematode *C. elegans* by binding to and neutralizing CED-4, an essential activator of the aspartate-directed cysteine protease (caspase) CED-3. In mammals, the CED-9 homologs Bcl-2 and Bcl-x_L also block apoptosis by interfering with the activation of CED-3-like caspases. However, it is unknown whether this occurs by binding to the CED-4 homolog Apaf-1. Whilst two groups previously detected an interaction between Bcl-x_L and Apaf-1 in immunoprecipitates,[1,2] another group found no interaction between Apaf-1 and any of ten individual members of the Bcl-2 family using the same experimental approach.[3] In this study, we aimed to resolve this discrepancy by monitoring the binding of Apaf-1 to three Bcl-2 family members within cells. Using immunofluorescence and Western blot analysis, we show that whilst Apaf-1 is a predominantly cytoplasmic protein, Bcl-2, Bcl-x_L and Bax mostly reside on nuclear/ER and mitochondrial membranes. This pattern of localization is maintained when the proteins are co-expressed in both normal and apoptotic cells, suggesting that Bcl-2, Bcl-x_L or Bax do not significantly sequester cytoplasmic Apaf-1 to intracellular membranes. In addition, we confirm that Apaf-1 does not interact with Bcl-2 and Bcl-x_L in immunoprecipitates. Based on these data, we propose that Apaf-1 is not a direct, physiological target of Bcl-2, Bcl-x_L or Bax. *Cell Death and Differentiation* (2000) **7,** 947–954.

Keywords: Apaf-1; apoptosis; Bax; Bcl-2; Bcl-x_L; CED-4

Abbreviations: HEK, human embryonic kidney; R6, rat 6 embryo fibroblasts; CED, product of cell-death-abnormal gene; caspase, cysteinyl aspartate-specific proteinase; Apaf-1, apoptotic protease activating factor-1; BH, Bcl-2 homology domain; COX, cytochrome c oxidase; NP-40, Nonidet P-40; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate; ER, endoplasmic reticulum; IP, immunoprecipitation

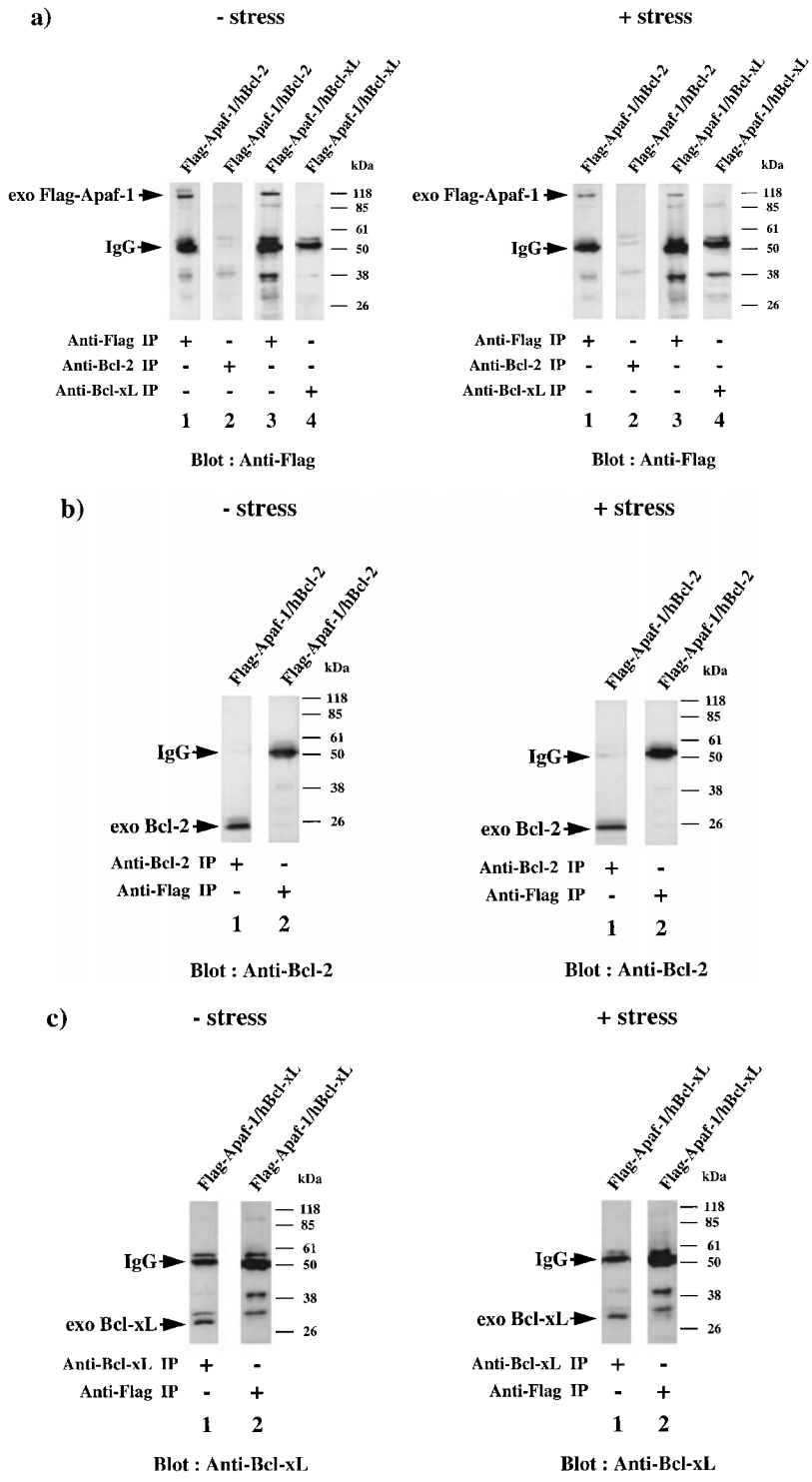
Introduction

Programmed cell death, or apoptosis, is the physiological process that removes superfluous and damaged cells from a multicellular organism.[4] Genetic studies in the nematode *C. elegans* revealed that programmed cell death during development is executed by the aspartate-directed cysteine protease (caspase) CED-3.[5] Activation of this protease occurs by binding an adaptor molecule CED-4[6,7] which oligomerizes and brings into proximity the inactive pro-form of CED-3 enabling it to autoprocess to the active protease.[8–10] Another protein, CED-9, blocks cell death by forming a complex with CED-4 and attenuating its ability to oligomerize and activate CED-3.[6,7,9,10,] The ability of CED-9, CED-4 and CED-3 to exist in a multiprotein complex has led to the 'apoptosome' model of cell-death regulation.[11]

Several counterparts of this genetic network have been identified in higher eukaryotes. Up to 14 mammalian CED-3-like caspases are known. Caspases-3 and -7 (and probably also caspase-6) function as executioner or effector caspases because they are activated in all types of apoptosis, their inhibition or chromosomal deletion interfere with apoptosis and/or they cleave most of the cellular substrates implicated in apoptosis.[12,13] The other caspases are so called initiator caspases which form a proteolytic cascade on selected signaling pathways (Fas/CD95, cytokine removal, etc.) to cleave and activate the inactive pro-forms of effector caspases.[12–14] The need for CED-4 homologs in caspase activation was not appreciated until the isolation of the first mammalian homolog Apaf-1.[15] Peculiarly, Apaf-1 does not directly activate effector caspases but forms oligomers to facilitate the activation of the initiator caspase-9 which in turn cleaves and activates effector caspases-3 and -7.[16–19] In addition, activation of Apaf-1 requires the binding of the mitochondrial component cytochrome c[15,20] which is released into the cytoplasm in response to many apoptotic stimuli.[21] Thus, like CED-4, Apaf-1 activates a CED-3-like caspase, but its function depends on mitochondria.

The mammalian homologs of CED-9 are a family of proteins that consists of anti-apoptotic (e.g. Bcl-2) and pro-apoptotic (e.g. Bax) members.[22–24] In analogy with *C. elegans*, anti-apoptotic proteins such as Bcl-2 and Bcl-x_L might function by binding to CED-4 homologs such as Apaf-1 to prohibit the activation of CED-3-like effector caspases. In support of this notion, Bcl-2 and Bcl-x_L have been shown to interfere with the activation of effector caspases,[25–27] and Bcl-x_L has been reported to form a complex with CED-4 upon co-overexpression in mammalian cells.[6,28] Moreover, two recent studies have shown that Bcl-x_L interacts with Apaf-1 in immunoprecipitates from lysates of cells overexpressing both proteins.[1,2] This interaction prevented caspase-9 activation either within a ternary caspase-9/Apaf-1/Bcl-x_L complex[2] or by disrupting

the binding of Apaf-1 to caspase-9.[1] Although this finding is in agreement with the nematodal 'apoptosome' model, it is inconsistent with the modes of action that have so far been described for Bcl-2 and Bcl-x_L. In contrast to CED-9, Bcl-2 and Bcl-x_L act by blocking the release of cytochrome c, i.e. at a step occurring before Apaf-1 activation.[29–31] Although the exact mechanism of this effect is yet unknown, it has been proposed that Bcl-2 and Bcl-x_L directly regulate the permeability of the mitochondrial membrane due to their association with this organelle[23] and/or their presumed pore forming activity.[32,33] Alternatively, they may sequester a cytoplasmic, cytochrome c-release factor such as the Bcl-2 family member BID.[34,35] Based on these propositions, Moriishi *et al* have recently challenged the idea that Apaf-



1 and Bcl-x_L interact by showing that Bcl-x_L or nine other Bcl-2 family members do not co-immunoprecipitate with Apaf-1 from lysates of mammalian cells.[3]

As Bcl-2 and Bcl-x_L are both integral membrane proteins, they have to be solubilized by detergents for immunoprecipitation studies. It has however been reported that detergents can either promote or disrupt interactions between Bcl-2 family members.[36,37] We therefore needed to investigate the interaction between Apaf-1 and Bcl-2 family members in an experimental system that does not require detergent extraction. We chose an immunofluorescence analysis on fixed, permeabilized rat embryo fibroblasts overexpressing the proteins and show that three Bcl-2 family members Bcl-2, Bcl-x_L, or Bax cannot attract cytoplasmic Apaf-1 to membranes in normal and apoptotic cells. These data raise doubts that Apaf-1 is a direct target of Bcl-2, Bcl-x_L or Bax in mammalian cells.

Results

We first studied interactions between Apaf-1 and two anti-apoptotic (Bcl-2 and Bcl-x_L) and one pro-apoptotic (Bax) Bcl-2 family members using the previously reported immunoprecipitation approach.[1–3] For that purpose, N-terminally, Flag-tagged Apaf-1 was transiently co-transfected with either Bcl-2, Bcl-x_L or Bax into human embryonic kidney 293 (HEK293) cells. In some cases, the cells were treated for different time periods with apoptotic agents such as the proteasomal inhibitor MG132 to determine whether interactions between Flag-Apaf-1 and Bcl-2 or Flag-Apaf-1 and Bcl-x_L are only

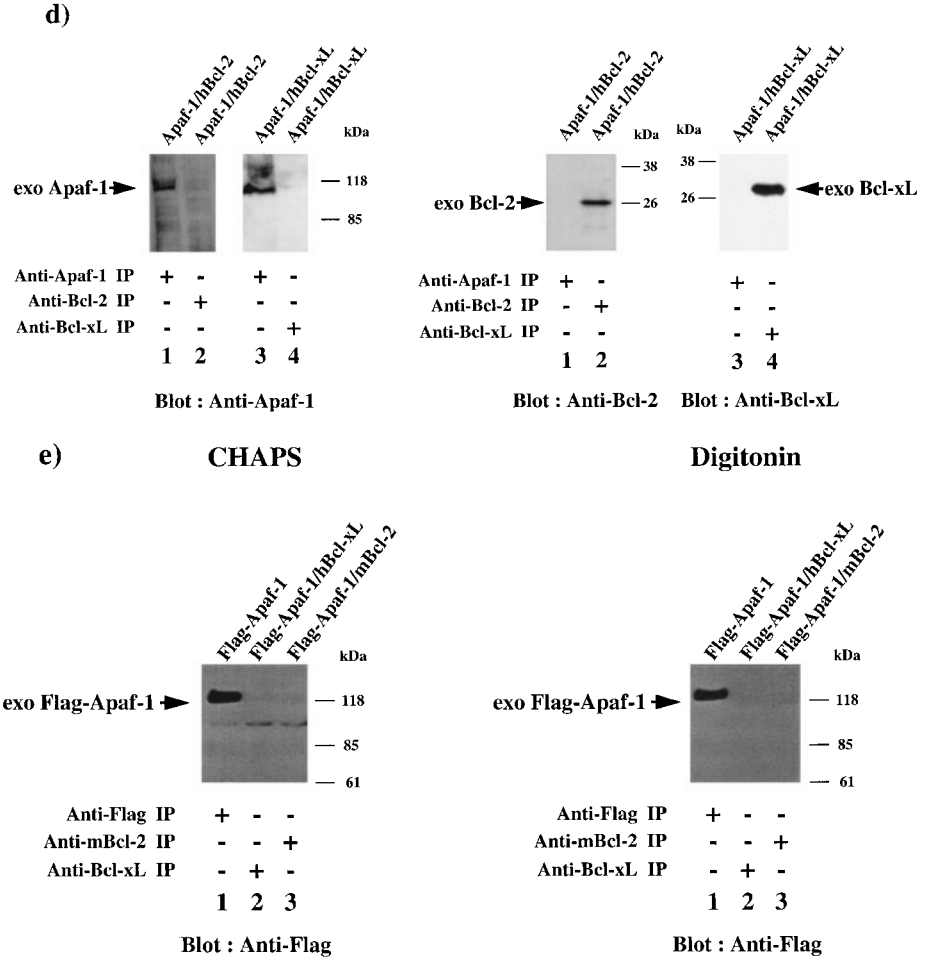


Figure 1 Flag-Apaf-1 or Apaf-1 do not interact with Bcl-2 or Bcl-x_L in immunoprecipitates. Anti-Flag (**a,e**), anti-Bcl-2 (**b,d**), anti-Bcl-x_L (**c,d**), or anti-Apaf-1 (**d**) Western blots of anti-Flag, anti-Bcl-2, anti-Bcl-x_L or anti-Apaf-1 immunoprecipitates (IP) from 0.2% NP-40 (**a–d**), 0.2% CHAPS (**e**) or 0.5% digitonin (**e**) lysates of HEK293 cells transfected with Flag-Apaf-1 alone or co-transfected with Flag-Apaf-1 and human Bcl-2 (hBcl-2), Flag-Apaf-1 and mouse Bcl-2 (mBcl-2), Flag-Apaf-1 and human Bcl-x_L (hBcl-x_L), Apaf-1 and human Bcl-2, or Apaf-1 and human Bcl-x_L, treated (+stress) or not (−stress) with 1 μM of the proteasome inhibitor MG132 for 24 h. Note that exogenous Flag-Apaf-1 is present in anti-Flag (**a**, lanes 1 and 3, **e**, lanes 1) but not in anti-Bcl-2 (**a**, lanes 2, **e**, lanes 3) or anti-Bcl-x_L (**a**, lanes 4, **e**, lanes 2) IPs from co-transfected cells. Similarly, Apaf-1 is present in anti-Apaf-1 (**d**, lanes 1 and 3, left panel), but not in anti-Bcl-2 (**d**, lane 2, left panel) or anti-Bcl-x_L (**d**, lane 4, left panel) IPs. By contrast, exogenous Bcl-2 is present in anti-Bcl-2 (**b**, lanes 1, **d**, lane 2, middle panel) but not in anti-Flag (**b**, lanes 2) or anti-Apaf-1 (**d**, lane 1, middle panel) IPs and exogenous Bcl-x_L is present in anti-Bcl-x_L (**c**, lanes 1, **d**, lane 4, right panel) but not in anti-Flag (**c**, lanes 2) or anti-Apaf-1 (**d**, lane 3, right panel) IPs from co-transfected cells. The two bands at 32 and 38 kDa detected in anti-Flag IPs (**c**, lanes 2) correspond to cross-reactive proteins as they are not detected in anti-Apaf-1 IPs (**d**, lane 3, right panel). Sometimes the secondary antibody reacted with the heavy IgG (ca. 50 kDa) of the primary antibody

formed when the latter two proteins act as death suppressors.[37] Cell lysates prepared in the presence of the detergent Nonidet P-40 (NP-40) were subjected to anti-Flag, anti-Bcl-2 or anti-Bcl-x_L immunoprecipitations, and the immunoprecipitates were Western-blotted for the detection of co-precipitating proteins. As shown in Figures 1 and 3, the proteins were similarly co-expressed so that interactions should have been seen in immunoprecipitates if they indeed occurred. An anti-Flag Western blot showed that Flag-Apaf-1 was present in anti-Flag (Figure 1a, lanes 1 and 3) but not in anti-Bcl-2 (Figure 1a, lane 2) or anti-Bcl-x_L (Figure 1a, lane 4) immunoprecipitates of HEK293 cell lysates co-overexpressing Flag-Apaf-1 and Bcl-2 or Flag-Apaf-1 and Bcl-x_L, respectively. Similarly, whilst Bcl-2 or Bcl-x_L could be

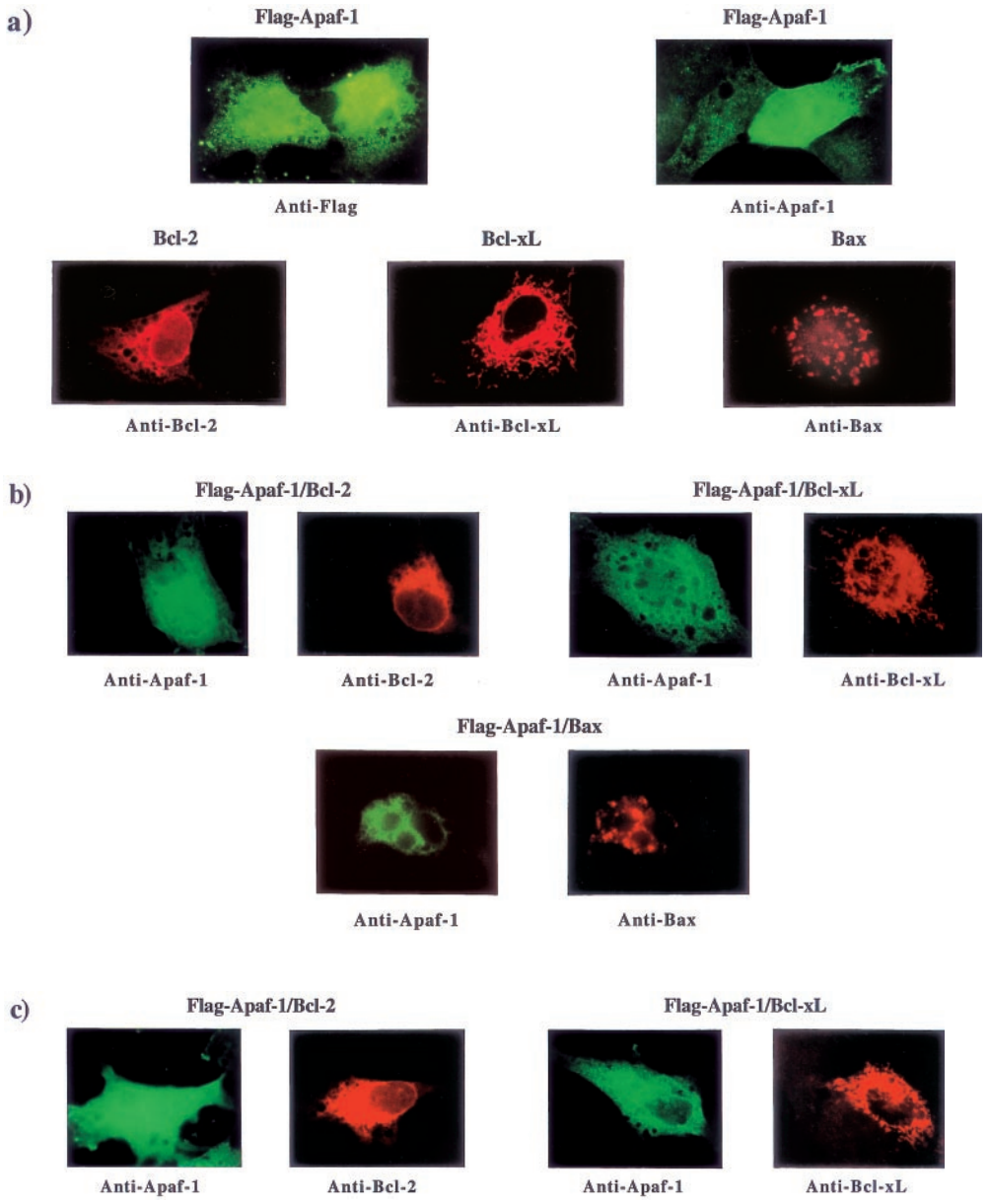


Figure 2 Bcl-2, Bcl-x_L or Bax cannot attract cytoplasmic Flag-Apaf-1 to intracellular membranes in both normal and apoptotically stressed cells. (**a**) Anti-Flag or anti-Apaf-1 (fluorescein), and anti-Bcl-2, anti-Bcl-x_L or anti-Bax (all Texas Red) immunofluorescence analysis on rat embryo fibroblasts (R6) transfected with Flag-Apaf-1, hBcl-2, hBcl-x_L or hBax for 15 h, respectively. (**b**) Anti-Apaf-1 (fluorescein) and anti-Bcl-2, anti-Bcl-x_L or anti-Bax (all Texas Red) immunofluorescence analysis on R6 cells co-transfected with Flag-Apaf-1 and hBcl-2 or Flag-Apaf-1 and hBcl-x_L or Flag-Apaf-1 and hBax for 15 h, respectively. (**c**) Immunofluorescence analysis of co-transfected R6 cells as in (**b**) but after treating the cells with 1 μM MG132 for 1 h (out of 15 h total transfection time). Note that Flag-Apaf-1 and Apaf-1 show a diffuse, cytoplasmic/nuclear staining irrespective of whether it is co-transfected with Bcl-2 (nuclear envelope/endoplasmic reticulum), Bcl-x_L (mitochondria, elongated) or Bax (mitochondria, punctuated) and/or whether the cells are stressed with the apoptotic agent MG132. The cells expressing Bax undergo apoptosis (nuclear fragmentation) (data not shown)

specifically immunoprecipitated with anti-Bcl-2 or anti-Bcl-x_L antibodies, respectively (Figure 1b, c, lanes 1), they did not co-immunoprecipitate with anti-Flag antibodies from the same cell extracts (Figure 1b, c, lanes 2). This was also the case for extracts prepared from cells exposed to MG132 for 0–24 h (Figure 1, compare −stress and +stress, 24 h). The anti-Flag antibody seemed to co-immunoprecipitate two proteins at 32 and 38 kDa which were detected by the anti-Bcl-x_L antibody (Figure 1c). However, upon close examination, the same proteins were also detected by anti-Flag and anti-Bcl-2 antibodies (Figure 1a, lanes 1 and 3 and Figure 1b, lane 2). By contrast, a highly specific anti-Apaf-1 antibody did not co-immunoprecipitate these proteins (Figure 1d, lane 3 in the anti-Bcl-x_L panel). This indicates that the 32 and 38 kDa proteins are most likely degradation products of IgG heavy chains of the anti-Flag antibody rather than modified (i.e. phosphorylated) forms of Bcl-x_L. The lack of interaction between Flag-Apaf-1 and Bcl-2 or Bcl-x_L was not due to the Flag-tag, as the same results were obtained by transfecting non-tagged Apaf-1 and using an anti-Apaf-1 specific antibody (Figure 1d). The latter experiment was also instructive because it did not reveal any interaction between Bcl-2 or Bcl-x_L and endogenous Apaf-1 (Figure 1d, lanes 2 and 4 in anti-Apaf-1 panel). Moreover, the failure of these molecules to form complexes was not due to the experimental conditions as anti-Bcl-2 effectively co-immunoprecipitated endogenous Bax, a known physiological partner of Bcl-2.[37,38] It was also highly unlikely that the putative Apaf-1/Bcl-2 complex was disrupted by the immunoprecipitation antibodies, because we used both polyclonal (data not shown) and monoclonal (Figure 1) antibodies to Bcl-x_L and Bcl-2. We also used two other detergents (digitonin, CHAPS) for cell lysis and immunoprecipitation and still did not find interactions between Apaf-1 and Bcl-2 or Apaf-1 and Bcl-x_L (Figure 1e). Unfortunately, we were unable to monitor the interaction between Apaf-1 and Bax as this combination killed most of the co-expressing cells before they could be lysed. Thus, our data confirm the recent report by Moriishi *et al*[3] that Apaf-1 does not form a stable complex with Bcl-2 or Bcl-x_L under the conditions used for immunoprecipitations.

Although we tested different detergents (NP-40, CHAPS, digitonin) for our *in vitro* experiments, it was still possible that Bcl-2 and Bcl-x_L interacted with Apaf-1 in cells, but that this interaction was disrupted during cell lysis and/or immunoprecipitation. To resolve this issue, we performed an immunofluorescence analysis on rat embryo fibroblasts (R6), Hela, HEK293 and SW480 human colon carcinoma cell lines which had previously been co-transfected with Flag-

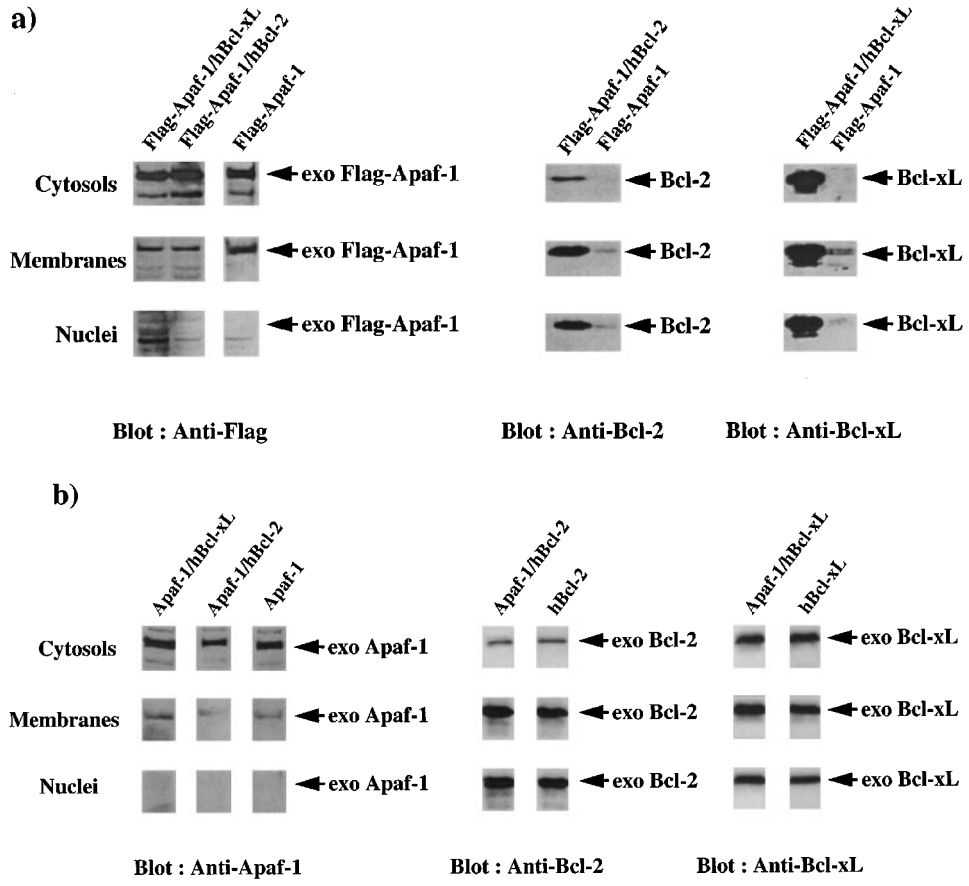


Figure 3 Apaf-1 is predominantly cytoplasmic while Bcl-2 and Bcl-x_L are mainly found in nuclear and membrane fractions. (**a**) Anti-Flag, anti-Bcl-2 or anti-Bcl-x_L Western blots of cytosolic, nuclear and membrane fractions of HEK293 cells transfected with Flag-Apaf-1 or co-transfected with Flag-Apaf-1 and hBcl-2 or Flag-Apaf-1 and hBcl-x_L. (**b**) Anti-Apaf-1, anti-Bcl-2 or anti-Bcl-x_L Western blots of cytosolic, nuclear and membrane fractions of HEK293 cells transfected with Apaf-1, hBcl-2 or hBcl-x_L or co-transfected with Apaf-1 and hBcl-2 or Apaf-1 and hBcl-x_L

Apaf-1 and Bcl-2, Flag-Apaf-1 and Bcl-x_L or Flag-Apaf-1 and Bax. Here we show the results in R6 cells but similar results were obtained in the other cell lines. After transient transfection, Flag-Apaf-1 displayed a diffuse immunostaining in the cytoplasm with both anti-Flag and anti-Apaf-1 antibodies (Figure 2a, Flag-Apaf-1). This staining was specific as non-transfected cells revealed only a low background staining with both antibodies (see neighboring cell in Figure 2a, anti-Apaf-1). The nuclear staining of Flag-Apaf-1 was probably due to the non-confocal type of immunofluorescence method used here as no Flag-Apaf-1 was detected in nuclear fractions isolated from Flag-Apaf-1-transfected cells (Figure 3a, nuclei). In contrast to the diffuse immunostaining of Apaf-1, Bcl-x_L was observed on elongated, 'spaghetti-like' structures that co-localized with the mitochondrial marker cytochrome c oxidase (COX) (Figure 2a, Bcl-x_L, and data not shown). Bax was also clearly mitochondrial, but in a punctated pattern, and the cells containing high levels of Bax were dying by apoptosis (Figure 2a, Bax, and ref[39]). Finally, Bcl-2 showed a broad subcellular localization pattern that included the nuclear envelope and the endoplasmic reticulum as previously reported (Figure 2a, Bcl-2).[40] Thus, if Flag-Apaf-1 interacted with Bcl-2 or Bcl-x_L upon co-overexpression, it should migrate to their sites of localization. However, despite effective and equal co-overexpression with Bcl-2, Bcl-x_L or Bax, Flag-Apaf-1 continued to display a predominantly cytoplasmic localization (Figure 2b). The same result was seen when the cells were stressed with MG132 or other apoptotic stimuli for different time periods (Figure 2c, 1 μM MG132 for 1 h is shown here). Thus, Apaf-1 does not seem to be attracted to intracellular sites where Bcl-2, Bcl-x_L or Bax reside.

To confirm that Apaf-1 was mostly cytoplasmic and Bcl-2 or Bcl-x_L were mostly membrane-bound even in co-transfected cells, we performed Western blots of cytosolic, membrane and nuclear fractions of HEK293 cells either transfected with Flag-Apaf-1, Apaf-1, Bcl-2 or Bcl-x_L alone or co-transfected with Flag-Apaf-1 (or Apaf-1) and Bcl-2 or Flag-Apaf-1 (or Apaf-1) and Bcl-x_L. Although some Flag-Apaf-1 and Apaf-1 were detected in the membrane fraction, most of these proteins resided in the cytosol, irrespective of the co-presence of overexpressed Bcl-2 or Bcl-x_L (Figure 3a, b). By contrast, both Bcl-2 and Bcl-x_L localized to membranes or nuclei, and this distribution did not change in the presence of overexpressed Flag-Apaf-1 (Figure 3a) or Apaf-1 (Figure 3b). Thus, Apaf-1 has a cytoplasmic localization distinct from Bcl-2 or Bcl-x_L.

Discussion

Despite intensive research, it has remained unknown how anti-apoptotic Bcl-2 family members prevent caspase activation. Two modes of action have been proposed, one which indirectly and another which directly affects Apaf-1 function. The indirect model suggests that Bcl-2 and/or Bcl-x_L act on the outer mitochondrial membrane to prevent the release of cytochrome c,[29–31] a necessary co-factor for Apaf-1 oligomerization and the subsequent activation of caspase-9 and -3.[15–17,20] The direct model suggests a physical interaction between Bcl-2 and Apaf-1 or Bcl-x_L and Apaf-1. Heterologous overexpressions of the *C. elegans* death regulators in mammalian cells have shown that CED-9, CED-4 and CED-3 exist in a multiprotein complex, dubbed the apoptosome, in which CED-9 inhibits apoptosis by binding to CED-4, thereby preventing it from activating CED-3.[6,7,9–11] Interestingly, such interactions have not only been detected by co-immunoprecipitation but also using immunofluorescence analysis where soluble CED-4 was sequestered to the perinuclear region by CED-9.[28] The latter study showed that it should be possible to detect a similar attraction between Apaf-1 and Bcl-2 or Bcl-x_L if the analogous mammalian apoptosome indeed consisted of these factors.

Our data disfavor the notion that Apaf-1 forms a mammalian apoptosome with Bcl-2 or Bcl-x_L. Why are our results in conflict with two previous studies that have reported direct interactions between Bcl-x_L and Apaf-1?[1,2] Both studies used immunoprecipitation approaches to determine protein-protein interactions. Although Moriishi *et al*[3] and we have used immunoprecipitation protocols that closely replicated those reported[1,2] it may be difficult to reach the exact same experimental conditions for reproducing the results. For example, we noticed that, when in abundance, Apaf-1, Bcl-2 and Bcl-x_L all bind non-specifically to Sepharose beads (data not shown), and thus part of the presumed Apaf-1/Bcl-2 or Apaf-1/Bcl-x_L complexes may originate from such artefacts. In addition, there remains the possibility that the quality of the detergents influences complex formations during cell lysis and immunoprecipitation. Our use of immunofluorescence to study the interaction between Apaf-1 and Bcl-2 family members within cells circumvents these in vitro problems. In several different cell types co-transfected with Flag-Apaf-1, Apaf-1, Bcl-2, Bcl-x_L or Bax and exposed to different apoptotic stimuli for various time periods, we have not seen any evidence that the diffusely stained, cytoplasmic Apaf-1 is sequestered to nuclear or mitochondrial membranes in a similar fashion that CED-9 sequesters CED-4.[28] Is it possible that Apaf-1 was recruited to Bcl-2 or Bcl-x_L at levels undetectable by immunofluorescence? Although such a possibility cannot be entirely excluded, we think that we should have seen a major sequestration by this technique as the apoptosome model in *C. elegans* accounts for a tight complex formation between CED-9 and CED-4 in healthy cells.[28,41] In addition, Western blot analysis of subcellular fractions of HEK293 cells co-transfected with Flag-Apaf-1/Bcl-2 or Flag-Apaf-1/Bcl-x_L revealed that both Apaf-1 and Flag-Apaf-1 were predominantly present in the cytosol rather than in membrane fractions. Thus, instead of being sequestered to membrane-associated Bcl-2 or Bcl-x_L, Apaf-1 seems to constitutively reside in the cytoplasm to await the release of cytochrome c for the activation of caspase-9. A novel CED-4 homolog, named Nod1/CARD4[42,43] has recently been identified, and it will be interesting to determine whether it fulfills the criteria to form an apoptosome with Bcl-2 or Bcl-x_L. Based on the findings that Bcl-2 and Bcl-x_L prevent the release of cytochrome c, it is expected that these proteins bind to and inhibit a CED-4 homolog that functions upstream of mitochondria to initiate the release of cytochrome c and other apoptogenic factors.

Materials and Methods

Subcellular fractionation, immunoprecipitations and Western blot analysis

HEK293 cells were co-transfected with 5 μg of each human Flag-Apaf-1/pcDNA3 (kindly provided by V Dixit), human Apaf-1/pcDNA3.1(−) (kindly provided by X Wang), human Bcl-2/pcDNA3, mouse Bcl-2/pcDNA3 or human Bcl-x_L/pcDNA3 (kindly provided by G Nuñez) using 25 μl of Superfect (Qiagen) as described by the manufacturer. In some cases, the cells were treated with 1 μM of MG132 (Alexis) for 0–24 h. The cells were lysed in 0.2% NP-40, 0.2% CHAPS or 0.5% digitonin and subjected to immunoprecipitation using 3 μl of mouse monoclonal anti-hBcl-2/100 (Neomarker), 5 μl of rabbit polyclonal anti-m/rBcl-2/27-6,[37] 3 μl of mouse monoclonal anti-Bcl-x_L/2H12 (Zymed), 2.5 μl of mouse monoclonal anti-Flag/M5 (Sigma) or 5 μl of rabbit polyclona anti-Apaf-1/IP14 (Zymed) and 50 μl of 50% (v/v) protein G-Sepharose (PharmaciaAmersham) or protein A-Sepharose (Sigma) as previously described.[37] Immunocomplexes were subjected to Western blot analysis using either anti-hBcl-2/100 at a titer of 1 : 200, anti-Bcl-x_L/2H12 at a titer of 1 : 1000, anti-Flag/M5 at a titer of 1 : 1000 or anti-Apaf-I/IP14 at a titer of 1 : 1000. Secondary antibodies were Fcγ-specific, peroxidase-coupled goat anti-rabbit or anti-mouse antibodies (Jackson ImmunoResearch Laboratories), and the detection system was enhanced chemiluminescence (ECL, AmershamPharmacia). For subcellular fractionation analysis, the co-transfected HEK293 cells were lysed in buffer A/sucrose (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.25 M sucrose, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 0.4 mM Pefabloc, 1 μg/ml pepstatin) using a tight fitting Dounce homogenizer. The lysates were centrifuged at $750 \times g$ to obtain a nuclear pellet. The postnuclear supernatant was further centrifuged at $100\ 000 \times g$ to obtain a membrane pellet and the cytosol. Thirty μg of each fraction was loaded on polyacrylamide gels for Western blot analysis.

Immunofluorescence analysis

R6 cells were transfected with 0.8 μg of each Flag-hApaf-1/pcDNA3, hBcl-2/pcDNA3, hBcl-x_L/pcDNA3 or hBax/pcDNA3 in 2.4 μl (single transfections) or 4.8 μl (double transfections) Superfect as described by the manufacturer (Qiagen). The cells were fixed in 4% paraformaldehyde, and permeabilized with 0.05% saponin and acetone as previously described.[39] The cells were treated with mouse monoclonal anti-Flag (1 : 100), rabbit polyclonal anti-Apaf-1/IP14 (1 : 50), mouse monoclonal anti-hBcl-2/100 (1 : 100), mouse monoclonal anti-Bcl-x_L/2H12 (1 : 100) or mouse monoclonal anti-hBax/2D2 (1 : 100) (Zymed) for 1 h followed by incubations with fluorescein- and Texas Red-conjugated goat anti-rabbit and anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories). After postfixation in 4% paraformaldehyde, the antifading agent Slowfade (Molecular Probes) was added, and the cells were viewed under a regular Zeiss Axiovert fluorescence microscope at a magnification of $1000\times$. Pictures were taken with a Contax 167 MT camera.

Note added in proof

While this paper was under review, Hausmann *et al*[44] published that Apaf-1 does not colocalize with Bcl-2 or Bcl-x_L when analyzed by immunofluorescence and immunogold electron microscopy. Moreover, Newmeyer *et al*[45] and Haraguchi *et al*[46] used *in vitro* assays and cells deficient in Apaf-1, respectively, to show that Bcl-2 and Bcl-x_L do not require Apaf-1 for their caspase-inhibiting and death protective function.

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2.2.1 Additional results

The goal was to complete Figure 1 of the paper, specially the panel **e**, by performing some additional experiments and controls to confirm that Bcl-2 family members cannot directly interact with Apaf-1. For that purpose, we used exactly the same reagents, cells and previously reported immunoprecipitation approach (see "Materials and Methods" in the article).

Flag-Apaf-1 does not interact with human Bcl-2 in immunoprecipitates and Bcl-2/Bcl-xL are readily extracted from membranes using CHAPS or Digitonin as detergent.

To address the small possibility that Flag-hApaf-1 interacted with human Bcl-2 in a species-specific manner (we showed only the results obtained with mouse Bcl-2 in the paper) and to make sure that Bcl-2 and Bcl-xL could be extracted from ER/nuclear and mitochondrial membranes (it was a missing control), we transiently co-transfected N-terminally, Flag-tagged human Apaf-1 (Flag-Apaf-1) with either human Bcl-2 (hBcl-2) or human Bcl-xL (hBcl-xL) into human embryonic kidney 293 (HEK293) cells. Cell lysates were prepared in the presence of CHAPS or Digitonin as detergent and subjected to anti-human Bcl-2 (anti-Bcl-2) or anti-human Bcl-xL (anti-Bcl-xL) immunoprecipitations (IP). The immunoprecipitates were analyzed on a Western blot for the detection of the Bcl-2 and Bcl-xL proteins or the co-precipitating protein Flag-Apaf-1 (Figure 9).

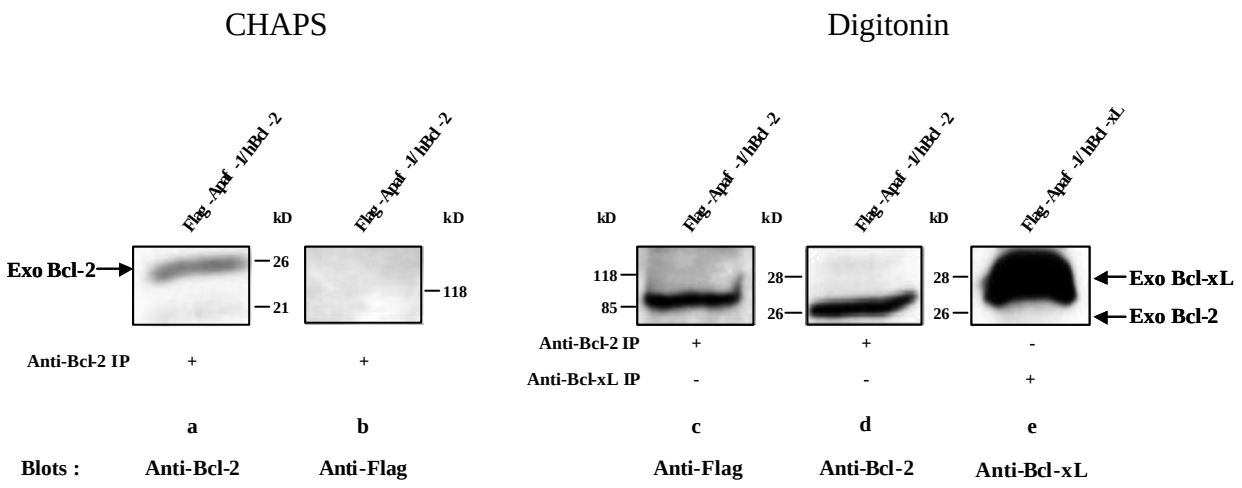

Figure 9 : Flag-Apaf-1 does not interact with Bcl-2 and Bcl-2/Bcl-xL are pulled out of their target membranes.
Anti-Bcl-2 (lanes a and d), anti-Flag (lanes b and c), and anti-Bcl-xL (lane e) Western blots of anti-Bcl-2 or anti-Bcl-xL immunoprecipitates (IP) from 0.2% CHAPS (lanes a and b) or 0.5% Digitonin (lanes c, d and e) lysates of HEK 293 cells co-transfected with Flag-Apaf-1 and human Bcl-2 (hBcl-2) or Flag-Apaf-1 and human Bcl-xL (hBcl-xL). Note that exogenous Bcl-2 is present in anti-Bcl-2 (lanes a and d) IPs from co-transfected cells. Similarly exogenous Bcl-xL is present in anti-Bcl-xL (lane e) IP. However, Flag-Apaf-1 is not present in anti-Bcl-2 (lanes b and c) IPs from co-transfected cells, because the detected band corresponds to a non-specific protein (compare lane c with Figure 1, panel **a**, lanes 3 in the article).

As shown in Figure 9, Bcl-2 and Bcl-xL were specifically immunoprecipitated with anti-Bcl-2 (lanes a and d) or anti-Bcl-xL (lane e) antibodies in the presence of CHAPS or Digitonin as detergent. Moreover, we previously demonstrated that Flag-Apaf-1 immunoprecipitated with anti-Flag antibody in the presence of the same detergents (paper, Figure 1, panel **e**, lanes 1). These results indicated that if there was an interaction between Flag-Apaf-1 and Bcl-2 or Bcl-xL, we should be able to detect it. But even under these conditions, Flag-Apaf-1 did not interact with hBcl-2 (Figure 9, lanes b and c), mBcl-2 (paper, Figure 1, panel **e**, lanes 3) or hBcl-xL (paper, Figure 1, panel **e**, lanes 2). Although we tested different detergents (NP-40, CHAPS, Digitonin) for our *in vitro* experiments, it was still possible that an eventual cellular interaction between Bcl-2/Bcl-xL and Apaf-1 was disrupted upon cell lysis and/or immunoprecipitation. To solve this issue, we confirmed our results by immunofluorescence (paper, Figure 2) and subcellular fractionation (paper, Figure 3) analyses.

Apaf-1 does not interact with Bcl-2 or Bcl-xL, even in the presence of dATP.

It has been reported that dATP (25 times more active than ATP, Genini *et al.*, 2000) is an important factor for the *in vitro* binding of pro-caspase-9, cytochrome c (cyt. c) and Apaf-1 which in turn leads to caspase-9 activation (Li *et al.*, 1997).
To test the possibility that the interaction between Apaf-1 and Bcl-2/Bcl-xL could only occur in the presence of sufficient levels of dATP in the extract, we performed an anti-Flag Western blot of anti-Bcl-xL, anti-Flag or anti-Bcl-2 immunoprecipitates (IP) from 0.2% NP-40 and 1 mM dATP lysates of HEK293 cells transiently co-transfected with Flag-Apaf-1 and hBcl-xL or with Flag-Apaf-1 and hBcl-2 for the detection of the co-precipitating protein Flag-Apaf-1 (Figure 10).

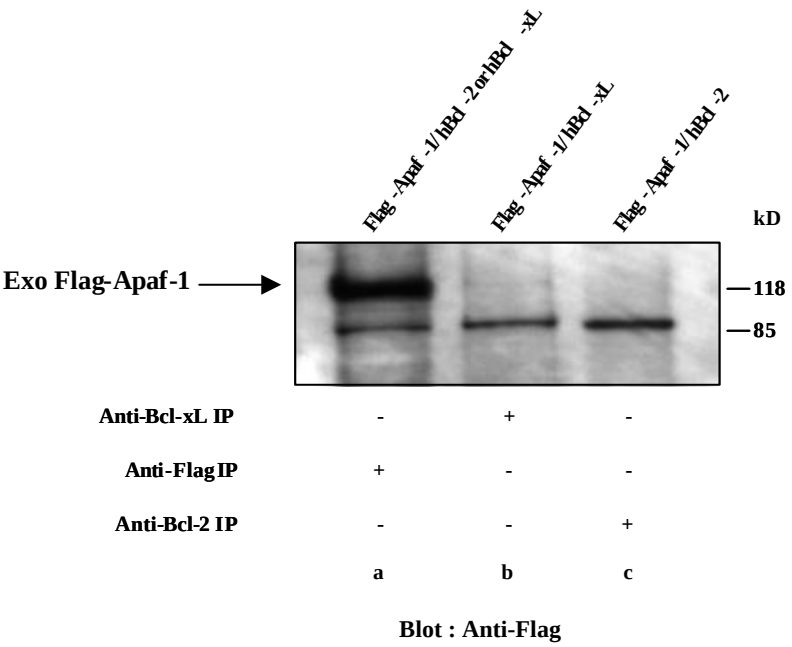

Figure 10 : No interaction between Flag-Apaf-1 and Bcl-2 or Bcl-xL in the presence of dATP.
Anti-Flag Western Blot of anti-Bcl-xL, anti-Bcl-2 or anti-Flag immunoprecipitates (IP) from 0.2% NP-40 and 1 mM dATP lysates of HEK 293 cells co-transfected with Flag-Apaf-1 and human Bcl-2 or Flag-Apaf-1 and hBcl-xL. Note that exogenous Flag-Apaf-1 is present in anti-Flag (lane a) but not in anti-Bcl-xL (lane b) or anti-Bcl-2 (lane c) IPs from co-transfected cells. Moreover, the non-specific band is still detected at about 85 kD (lanes a, b and c).

As seen from the absence of co-immunoprecipitated Flag-Apaf-1 in anti-Bcl-xL (Figure 10, lane b) or anti-Bcl-2 (Figure 10, lane c) IPs from co-transfected cells, Flag-Apaf-1 is not able to interact with Bcl-2 or Bcl-xL under the conditions used. This is not due to the degradation of the different proteins because we could detect them as full-length, entire proteins as seen in anti-Flag IPs (Figure 10, lane a), in anti-Bcl-2 IPs (paper, Figure 1, panel **b**, lanes 1) or in anti-Bcl-xL IPs (paper, Figure 1, panel **c**, lanes 1). Even, when we stressed the cells to force Bcl-2 and Bcl-xL to act as survival factors we could not detect any interaction with Apaf-1. This was also the case when we added dATP or cytochrome c to the extraction buffer (data not shown, but exactly the same as in Figure 10).
Taken together, our additional data confirm that Apaf-1 is not a direct, physiological target of Bcl-2, Bcl-xL or Bax.

2.3 ARTICLE : The binding properties and biological activities of Bcl-2 and Bax in cells exposed to apoptotic stimuli

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Notice :

My contribution to this work was the co-immunoprecipitation analyses to show that Bax is the only protein that specifically co-immunoprecipitates with Bcl-2 and that Bcl-2 is the only protein that associates stably with Bax in non-stressed mammalian cells (Figure 1A, middle panel, Figures 1C and 1D, Figures 2A and 2B). Moreover, I participated in the experiments showing that cell death protection by Bcl-2 does not require Bax and is most effective when Bcl-2 is in excess of Bax by performing again co-immunoprecipitation analyses (Figures 7B and 8B).

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The Binding Properties and Biological Activities of Bcl-2 and Bax in Cells Exposed to Apoptotic Stimuli*

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The oncogene product Bcl-2 protects cells from apoptosis whereas its homolog Bax functions to kill cells. Several binding partners of Bcl-2 and Bax have been isolated, but none of them has yet provided clues as to exactly how Bcl-2 and Bax work. According to one view, Bcl-2 and Bax interact with survival and death effector molecules, respectively, and neutralize each other through heterodimerization. Alternatively, Bcl-2 requires Bax for death protection, and additional proteins bind to the heterodimer to regulate its activity. Here we used a co-immunoprecipitation strategy to distinguish between these two possibilities. We show that the Bcl-2-Bax heterodimer is maintained, and no other protein associates stably in detectable amounts with Bcl-2, Bax, or the heterodimer in anti-Bcl-2 and anti-Bax immunoprecipitates from normal cells and cells exposed to apoptotic stimuli. Analysis of cells expressing various levels of Bcl-2 and Bax, however, revealed that the degree of protection against apoptosis does not correlate with the number of Bcl-2-Bax heterodimers but the amount of Bcl-2 that is free of Bax. In addition, the survival activity of Bcl-2 is unaffected when Bax expression is ablated by an antisense strategy. Our findings suggest that the Bcl-2-Bax heterodimer is a negative regulator of death protection, and that Bcl-2 requires neither Bax nor major, stable interactions with other cellular proteins to exert its survival function. We therefore propose that Bcl-2 acts as an enzyme (capturing substrates in a transient way), as a homodi- or multimer, or through the interaction with non-proteaceous targets (lipids, ions).

Bcl-2 is an oncogene product originally isolated from human follicular lymphomas harboring a t(14;18) chromosomal translocation (1). Compared with other transforming genes it has the unusual property of increasing cell numbers by preventing programmed cell death (apoptosis) rather than stimulating cell multiplication (2). The death-protective activity of wild-type Bcl-2 seems to be proportional to its expression level (3). No natural activating mutant of Bcl-2 has yet been found in normal or transformed cells. Thus, to study the molecular action of Bcl-2, the protein has been overexpressed in many mammalian cells and shown to counteract programmed cell death induced by various stimuli (2). Both endogenous and exogenous Bcl-2 are anchored via their carboxyl-terminal hydrophobic tails to the outer membranes of mitochondria, nuclei, and the endoplasmic reticulum with the bulk of the protein facing the cytoplasm (4–7). Whereas a cytosolic Bcl-2 mutant remains partially active, membrane localization is required for full activity (8–10).

The mechanisms underlying the survival activity of Bcl-2 are still defined vaguely. It has been shown that Bcl-2 acts as an antioxidant (8, 11), preserves the potential of mitochondrial membranes (12, 13), and blocks the release of calcium from internal stores into the cytoplasm (14–16). It also interferes with the apoptotic effect of cell-permeable analogs of ceramide (17, 18), retards cell proliferation by prolonging the G_1 phase of the cell cycle (19–21), enhances gene amplification and recombination (22), and prevents activation of a subclass of cysteine proteases called caspases (formely ced-3/ICE (interleukin-1β-converting enzyme)-like proteases) which have become largely implicated in the execution phase of apoptosis (23–25). Moreover, the Bcl-2 homolog Bcl-x_L has recently been shown to be structurally related to bacterial, pore-forming proteins (26) and to form ion channels in synthetic phospholipid bilayers (27). Thus, Bcl-x_L, and by analogy Bcl-2, may directly or indirectly affect the permeability and/or transport capacity of organelles and regulate their homeostasis during apoptotic processes. Indeed, very recently Bcl-2 was shown to inhibit the release of holocytochrome *c* and an apoptogenic protease from the intermembrane space of mitochondria into the cytoplasm (28–30). Both proteins were shown to contribute to nuclear fragmentation and apoptosis via the activation of cytoplasmic caspases.

To understand better the molecular function of Bcl-2, it is necessary to identify the molecules with which Bcl-2 interacts. So far, such molecules have been searched only under non-apoptotic situations. Using interaction cloning, yeast two-hybrid and recombinant baculovirus systems, various Bcl-2-binding proteins have been discovered. Among them are R-Ras, a Ras-related protein implicated in cell adhesion and apoptosis (31–33); c-Raf-1, a transducer of growth factor-elicited signals via the mitogen-activated protein kinase pathway (34–36); BAG-1, a ubiquitin-like protein enhancing the survival action of Bcl-2 (37); Nip1–3, three proteins of unknown function also interacting with the survival factor E1B (38); a p53-binding protein called 53BP2 (39); the cellular prion protein PrP (40); the mitochondrial carnitine palmitoyltransferase (41); and more recently, calcineurin (42) and the *Caenorhabditis elegans* death gene product ced-4 (43). Although all of these proteins could be forced to interact with Bcl-2 when mixed in recombinant forms *in vitro* or co-overexpressed at high levels in intact cells, there has been no conclusive evidence that they are partners and/or regulators of Bcl-2 under physiological concentrations/conditions. We have shown recently that, in contrast to previous reports (34–36), Bcl-2 does not co-immunoprecipitate with Raf from normal cellular extracts and extracts of cells

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exposed to apoptotic agents (44). Moreover, using various approaches to down-regulate Raf activity, we demonstrated that Bcl-2 does not use a Raf/mitogen-activated protein kinase signaling pathway to confer cell survival (44).

Bcl-2 belongs to a family of related proteins. Whereas some members such as Bcl-2, Bcl-x_L, Bcl-w, Bfl-1, Mcl-1, A1, and BHRF act as death repressors (anti-apoptotic subfamily) (45, 46; for review, see Ref. 47), others such as Bax, Bad, Bak, Bik, and Bid exert the opposite function, *e.g.* induce apoptosis when overexpressed in a variety of eukaroytic cells (pro-apoptotic subfamily) (48; for review, see Ref. 47). Selective pairs of anti-apoptotic and pro-apoptotic proteins, such as for example Bcl-2 and Bax, dimerize via highly homologous regions (BH[1] regions) present in all family members (49, 50). Mutagenesis analyses have shown that the BH1 and BH2 regions of Bcl-2 interact with the BH3 region of Bax (51–54). The same BH regions are also required for the anti-apoptotic and pro-apoptotic activities of Bcl-2 and Bax, respectively, as if survival/death activity and heterodimerization capacity were non-separable functions (51–54). These findings suggest two models for the regulation of apoptosis by Bcl-2 and Bax. (i) Bcl-2 binds unknown factors crucial for its survival activity via the BH1/BH2 regions, and Bax binds unknown factors crucial for its killing activity via the BH3 region. The Bcl-2-Bax heterodimer disrupts these interactions and serves a negative regulatory role for both Bcl-2 and Bax. (ii) Alternatively, the Bcl-2-Bax heterodimer is the active component for death protection (in analogy to the Myc-Max complex that stimulates transcription; see Ref. 55), and additional proteins bind to it to regulate its survival function.

To distinguish between the two possibilities, we embarked on a co-immunoprecipitation strategy to detect the proteins that bind to Bcl-2, Bax, or the heterodimer in response to various apoptotic stresses. We show that Bcl-2 and Bax co-localize in intact cells and are firm partners during four different apoptotic stresses. However, no additional proteins associate stably in significant amounts with Bcl-2, Bax, or the heterodimer under these conditions.

EXPERIMENTAL PROCEDURES

Reagents—Tumor necrosis factor α, hygromycin B, and protein G-agarose were purchased from Juro Supply/Calbiochem, Lucerne, Switzerland. Lipofectin was from Life Technologies, Basel, Switzerland. Dithiothreitol (DTT), protein A-Sepharose, and peroxidase-labeled goat anti-rabbit antibodies were from Sigma Chemical Co., St. Louis, MO. Rhodamine-conjugated goat anti-mouse and fluorescein-conjugated goat anti-rabbit IgG were from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA. Polyvinylidene difluoride membranes (Immobilon-P) were from Millipore, Volketswil, Switzerland. Enhance was from DuPont-Nemours, Geneva, Switzerland. [^{35}S]Methionine/cysteine (Tran^{35}S-label) was bought from ICN, Zürich, Switzerland, and the enhanced chemiluminescence (ECL) detection system was from Amersham, Buckinghamshire, U. K. Brefeldin A (BFA) (dissolved in methanol) was from Alexis Corp., Läufelfingen, Switzerland, and dithiobis(succinimidylpropionate) was from Pierce, Rockford, IL. MG132 (dissolved in Me_2SO) was a gift from Proscript Inc., Boston (formerly known as Myogenics Inc.). Staurosporine (dissolved in Me_2SO), okadaic acid (dissolved in Me_2SO), and the antisense phosphorothioate oligonucleotide against Bax were kindly provided by Novartis Ltd. The monoclonal anti-human Bcl-2 antibody clone 100 was generously given by D. Y. Mason, John Radcliffe Hospital, Oxford, Great Britain.

cDNAs—The Flag-Bcl-2 cDNA was constructed by inserting the mouse Bcl-2 cDNA into the *Eco*RI site of the Flag-P1 plasmid (Kodak). For stable and transient expressions in rat fibroblasts, the human Bax, human Bcl-2, and Flag-Bcl-2 cDNAs were inserted into the *Eco*RI site of the pcDNA3 plasmid.

Antibodies—Rabbit polyclonal antisera against amino acids 41–54 (27-6) and against amino acids 95–111 (28-5) of murine Bcl-2 were used in this study (9). To detect Bax the following antibodies were available: Ab 06-499, a rabbit polyclonal antibody against the NH_2-terminal amino acids 1–21 of human Bax cross-reacting with mouse and rat Bax, kindly provided by Upstate Biotechnology, Inc., Lake Placid, NY; Ab 13666E, a rabbit polyclonal antibody against amino acids 43–61 of human Bax, non-reactive with rat/mouse Bax, purchased from Pharmingen, Hamburg, Germany; and Ab 13686E, a rabbit polyclonal antibody against amino acids 43–61 of mouse Bax, cross-reactive with rat, but not human Bax, purchased from Pharmingen. The mouse monoclonal anti-Flag antibody M5 was bought from Kodak/Integra Biosciences.

Cells—Vector control and Bcl-2-overexpressing rat 6 embryo fibroblasts (R6) and murine L929 fibroblasts were generated by retroviral transduction of the pMV12hygro plasmid lacking or containing the murine Bcl-2 cDNA as described previously (9, 19). Cell lines expressing intermediate (R6-Bcl-2#5) and high amounts of Bcl-2 (R6-Bcl-2#9) were used for further studies. R6 cells overexpressing Flag-Bcl-2 were produced by transfecting the Flag-Bcl-2/pcDNA3 construct using Lipofectin. Following selection in 400 μg/ml G418, clones were picked, expanded into cell lines, and analyzed for Flag-Bcl-2 expression by anti-Flag immunoblotting. For further studies, a cell line expressing low amounts of Flag-Bcl-2 (R6-Flag-Bcl-2#15) and a mixed cell population expressing high amounts of Flag-Bcl-2 (R6-Flag-Bcl-2mix) were used. In addition, the Flag-Bcl-2/pcDNA3 vector was transfected into R6-Bcl-2#9 cells to yield a cell line (R6-Bcl-2/Flag-Bcl-2#7) that overexpressed both Bcl-2 and Flag-Bcl-2. R6 cells co-overexpressing mouse Bcl-2 and human Bax were generated by transfecting the Bax/pcDNA3 construct into the R6-Bcl-2#9 cell line. Following selection in 400 μg/ml G418, two cell lines were isolated which expressed high amounts of Bcl-2 together with low (R6-Bcl-2-Bax#1) or high amounts of human Bax (R6-Bcl-2-Bax#9). All R6 cell derivatives were grown in Dulbecco's modified Eagle's medium containing 5% fetal calf serum and low amounts (50 μg/ml) of hygromycin (to maintain Bcl-2 expression) or G418 (to maintain Flag-Bcl-2 or Bax expression). Vector control and Bcl-2-overexpressing JILY B lymphoblasts were generated as described previously (19). These were mixed cell populations carrying the episomal pMEPhygro vector (Invitrogen Corp., NV Leek, The Netherlands) lacking or containing the murine Bcl-2 cDNA. They were cultured in RPMI 1640 containing 10% fetal calf serum and 50 μg/ml hygromycin (to maintain Bcl-2 expression) at 5% CO_2 and 37 °C.

Drug Treatments and Cell Viability Assay—Cells were seeded at 2×10^5 in triplicate into 35-mm wells. The next day, the cells were treated with the solvent Me_2SO (0.1%), 1 μM staurosporine, or 1 μM MG132 to induce apoptosis. After 24–96 h, viable cells were counted on triplicate plates using either the trypan blue exclusion assay or the LIVE/DEAD fluorometric assay as described by the manufacturer (Molecular Probes, Eugene, OR). The results are the means of six to eight independent experiments.

Protein Extraction and Immunoblotting—Total protein extraction and immunoblotting were performed as described previously (24). To detect the co-immunoprecipitation of Bcl-2 and Bax, anti-Bcl-2 (27-6) immunoprecipitates from unlabeled cell extracts of R6-Bcl-2#9 were analyzed by anti-Bax Ab 06-499 immunoblotting at a titer of 1:10,000. Mouse Bcl-2 was immunodetected by the rabbit polyclonal 27-6 antibody at a titer of 1:5,000. Secondary antibodies were peroxidase-coupled goat anti-rabbit antibodies. The detection system was ECL.

Immunoprecipitations—3×10^6 cells were labeled with 50 μCi/ml [^{35}S]methionine/cysteine in 5 ml of methionine/cysteine-free RPMI medium overnight. 10 min to 72 h before extraction, cells were treated with 0.1% solvent (Me_2SO or methanol), 1 μM staurosporine, 1 μM okadaic acid, 1 μM MG132, or 5 μg/ml BFA. All cells (viable and dead) were harvested by centrifugation, washed once in phosphate-buffered saline and once in buffer A (10 mM Hepes, pH 7.2, 143 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 100 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin, 0.2 mM phenylmethylsulfonyl fluoride), and lysed in buffer A plus 0.2% Nonidet P-40. After leaving on ice for 30 min, the cell lysates were cleared by centrifugation, supplemented with 2.5 mg/ml ovalbumin, and adjusted to the same amount of radioactivity. 600 μl of the lysates was precleared on 50 μl of 50% protein A-Sepharose at 4 °C for 1 h and then subjected to immunoprecipitation using 5 μl of affinity-purified anti-mouse Bcl-2 antibody 27-6 or 28-5, 3 μl of anti-human Bax antibody Ab 13666E, 3 μl of anti-mouse/rat Bax antibody Ab 13686E, or 2.5 μl of the anti-FLAG antibody. Following antibody incubation for 2 h at 4 °C, 50 μl of a 50% protein A-Sepharose suspension (protein G-agarose for anti-FLAG antibody) was added, and the immunocomplexes were captured on an end-over-end wheel at 4 °C for 60 min. Immunocomplexes were pelleted by centrifugation, washed three times in buffer A,

[1] The abbreviations used are: BH, Bcl-2 homology; DTT, dithiothreitol; BFA, brefeldin A; ECL, enhanced chemiluminescence; Me_2SO, dimethyl sulfoxide; Ab, antibody; PAGE, polyacrylamide gel electrophoresis.

and then boiled in SDS-sample buffer for 6 or 12% SDS-PAGE analysis. The gels were fixed in 40% methanol and 10% acetic acid, treated with Enhance, washed in H_20, dried, and subjected to fluorography.

For mild cell disruptions, cells were washed twice in phosphate-buffered saline and exposed to five cycles of freezing in dry ice/ethanol and thawing at 30 °C. Subsequently, 0.2% Nonidet P-40 was added either directly or after cross-linking in the presence of dithiobis(succinimidylpropionate). For certain immunoprecipitations, buffer A was modified as follows. The pH was adjusted to 5–8, or phosphatase inhibitors (50 mM NaF, 100 μM Na_3VO_4, 25 mM β-glycerophosphate), an ATP regeneration system (1 mM DTT, 2 mM ATP, 10 mM phosphocreatine, and 50 μg/ml creatine kinase), 1 mM $CaCl_2$ in the absence of EGTA, or various amounts of KCl (0–500 mM) were added. To account for disulfide-linked protein-protein interactions, immunoprecipitates were boiled in SDS-sample buffer in the absence or presence of 30 mM DTT. To detect high stringency interactions, RIPA buffer (buffer A plus 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) was used for cell disruption, immunoprecipitation, and washes.

Transient Transfection and Immunocytochemistry—R6 cells were grown on sterile 12-mm glass coverslips until 60–70% confluence. 1 μg each of human Bax/pcDNA3 and human Bcl-2/pcDNA3 was combined with 3 μl (3 μg) of Lipofectin in Dulbecco's modified Eagle's medium (without serum and antibiotics) and transfected into the cells as described by the manufacturer (Life Technologies). After 6 h, the Lipofectin was removed, replaced by fresh Dulbecco's modified Eagle's medium plus 10% fetal calf serum in the absence or presence of 5 μg/ml BFA, and incubated at 37 °C for another 22 h. At 28 h post-transfection, cells were washed twice in phosphate-buffered saline, fixed in 4% paraformaldehyde, and permeabilized with 0.05% saponin and acetone. The cells were treated with the monoclonal anti-human Bcl-2 antiserum, clone 100 (1:10) and the polyclonal anti-human Bax antiserum Ab 06-499 (1:200) for 1 h followed by an incubation with rhodamine-conjugated goat anti-mouse and fluorescein-conjugated goat anti-rabbit secondary antibodies. The antifading agent SlowFade® (Molecular Probes) was added, and the cells were viewed under a Zeiss Axiovert fluorescence microscope at a magnification of × 1,000.

Antisense Experiments—2 × 10^6 R6-Bcl-2#9 cells were transfected with a 1 μM concentration of the antisense phosphorothioate oligonucleotide 5′-TGCTCCCCGGACCCGTCCAT-3′ targeted against the RNA sequence 1–20 of rat Bax using Lipofectin. As a control, a 1 μM concentration of the corresponding sense oligonucleotide 5′-ATGGACGGGTCCGGGGAGCA was transfected. After each day, a total cell extract was prepared and analyzed for Bax expression by Ab 06-499 immunoblotting. At 3 days post-transfection when endogenous Bax expression was ablated, the cells were treated with 1 μM staurosporine or 1 μM MG132, and viability was monitored for another 24 h by the trypan blue exclusion assay. The down-regulation of Bax expression lasted at least until day 5 post-transfection.

Quantitation of Proteins—Autoradiography of Western blots (ECL) and immunoprecipitations were scanned with a Bio-Rad GS-700 densitometer, and the protein bands were quantified by imaging densitometry (Molecular Analyst® 2.1). Arbitrary integrated volume units (outer diameter × area) are presented in the graphics. The values were corrected against the background of the autoradiography, and the protein amount on the polyvinylidene difluoride membrane was determined by amido black staining.

Statistical Analysis—All quantitative measurements were performed at least three times. Results are shown as means ± S.E.

RESULTS

Bax Is the Only Protein That Specifically Co-immunoprecipitates with Bcl-2 under Various Extraction and Immunoprecipitation Conditions—To identify novel Bcl-2-binding proteins, we followed exactly the protocol used for the co-immunoprecipitation and isolation of Bax, *i.e.* direct cell lysis in an isotonic buffer containing 0.2% Nonidet P-40 (50). This has been considered mild conditions retaining protein-protein interactions (56). First, we used an antibody (27-6) against an epitope shown to be irrelevant for the survival activity of Bcl-2 according to mutagenesis data (amino acids 41–60) (26, 57). This antibody immunoprecipitated a high amount of Bcl-2 from [^{35}S]methionine/cysteine-labeled extracts of the Bcl-2-overexpressing R6 cell line R6-Bcl-2#9 (Fig. 1*A*, *middle panel*). Compared with immunoprecipitates from extracts of R6-pMV12 vector control cells, only a protein of 21 kDa was specifically co-immunoprecipitated with Bcl-2 from R6-Bcl-2#9 cell extracts (Fig. 1*A*, *middle panel*). Similar findings were obtained with anti-27-6 immunoprecipitates from extracts of Bcl-2-overexpressing JILY B lymphoblasts and L929 fibroblasts (Fig. 1*B*) and U937 monocytes (data not shown) as well as with immunoprecipitates using an anti-Bcl-2 antibody against another epitope (28-5; amino acids 95–111) (Fig. 1*A*, *right panel*). To resolve high molecular mass proteins better, immunoprecipitates were run on 6% SDS-PAGE. However, no major protein above 26 kDa co-precipitated with Bcl-2 (Fig. 1*A*, *left panel*). The co-precipitating 21-kDa protein was identified as Bax on an anti-Bax Western blot of the anti-Bcl-2 immunoprecipitates (Fig. 1*D*). Because Bcl-2 and Bax contained a similar number of radiolabeled methionine and cysteine residues (12 in mouse Bcl-2, 10 in rat Bax) (50) and had similar protein turnover rates (data not shown), we could estimate the stoichiometry of the two proteins in the heterodimer. Bcl-2 was 3–10 times more abundant than Bax in anti-Bcl-2 immunoprecipitates from L929-Bcl-2#5, JILY-Bcl-2mix, and R6-Bcl-2#9 cell extracts (Fig. 1, *A* and *B*). Less than 20% of the total cellular level of rat Bax remained in the supernatant of the anti-Bcl-2 immunoprecipitates (Fig. 2*C*), indicating that Bcl-2 co-immunoprecipitated almost all of the endogenous Bax. Thus, most of the overexpressed Bcl-2 appeared to be free of Bax and did not associate stably with detectable amounts of other cellular proteins.

Because the anti-Bcl-2 antibodies 27-6 and 28-5 may have interacted with the same sites as Bcl-2-binding proteins, we overexpressed in R6 cells a NH_2-terminally tagged (Flag) Bcl-2 that could be immunoprecipitated with an anti-tag (Flag) antibody recognizing an epitope outside of the Bcl-2 molecule. Again, Bax was the only protein that specifically co-immunoprecipitated with Flag-Bcl-2 from extracts of cells that overexpressed Flag-Bcl-2 alone (R6-Flag-Bcl-2mix) or Flag-Bcl-2 together with Bcl-2 (R6-Flag-Bcl-2/Bcl-2#7) (Fig. 1*C*).

To account for the possibility that the experimental conditions were not optimal for detecting Bcl-2-binding proteins other than Bax, we performed anti-Bcl-2 immunoprecipitations under variable pH (5–8) and salt (0–500 mM) conditions and added phosphatase inhibitors, calcium, or an ATP regeneration system to extraction, immunoprecipitation, and wash buffers. Despite these modifications, no protein other than Bax was specifically co-immunoprecipitated with Bcl-2 (see Fig. 4*B* and data not shown). In addition, disruption of the immunocomplexes in the presence or absence of DTT revealed that Bcl-2 did not interact with a binding partner via disulfide bonds (data not shown).

Bcl-2 Is the Only Protein That Associates Stably with Bax in Non-stressed Mammalian Cells—To identify novel Bax-binding proteins we performed anti-Bax immunoprecipitations of radiolabeled R6-pMV12 and R6-Bcl-2#9 extracts using a polyclonal antibody recognizing amino acids 64–78 of the mouse/rat Bax protein (Ab 13686E). The extraction and immunoprecipitation conditions were as described for Bcl-2 (direct lysis in 0.2% Nonidet P-40). Anti-Bax immunoprecipitates of R6-pMV12 cell extracts contained endogenous Bax along with other cellular proteins (Fig. 2*A*, *lane b*). These proteins did not bind specifically to Bax because they also co-precipitated when endogenous Bax was down-regulated by an antisense strategy (see below) and when immunoprecipitations were performed in the presence of the Bax peptide antigen (data not shown). By contrast, anti-Bax antibodies specifically co-immunoprecipitated overexpressed Bcl-2 from R6-Bcl-2#9 cell extracts (Fig. 2*A*, *lane d*). The amount of co-precipitated Bcl-2 was similar to that of Bax but significantly lower than the amount of Bcl-2 that can be immunoprecipitated by anti-Bcl-2 antibodies from these extracts (Fig. 2*A*, compare *lanes c* and *d*). These data indicate

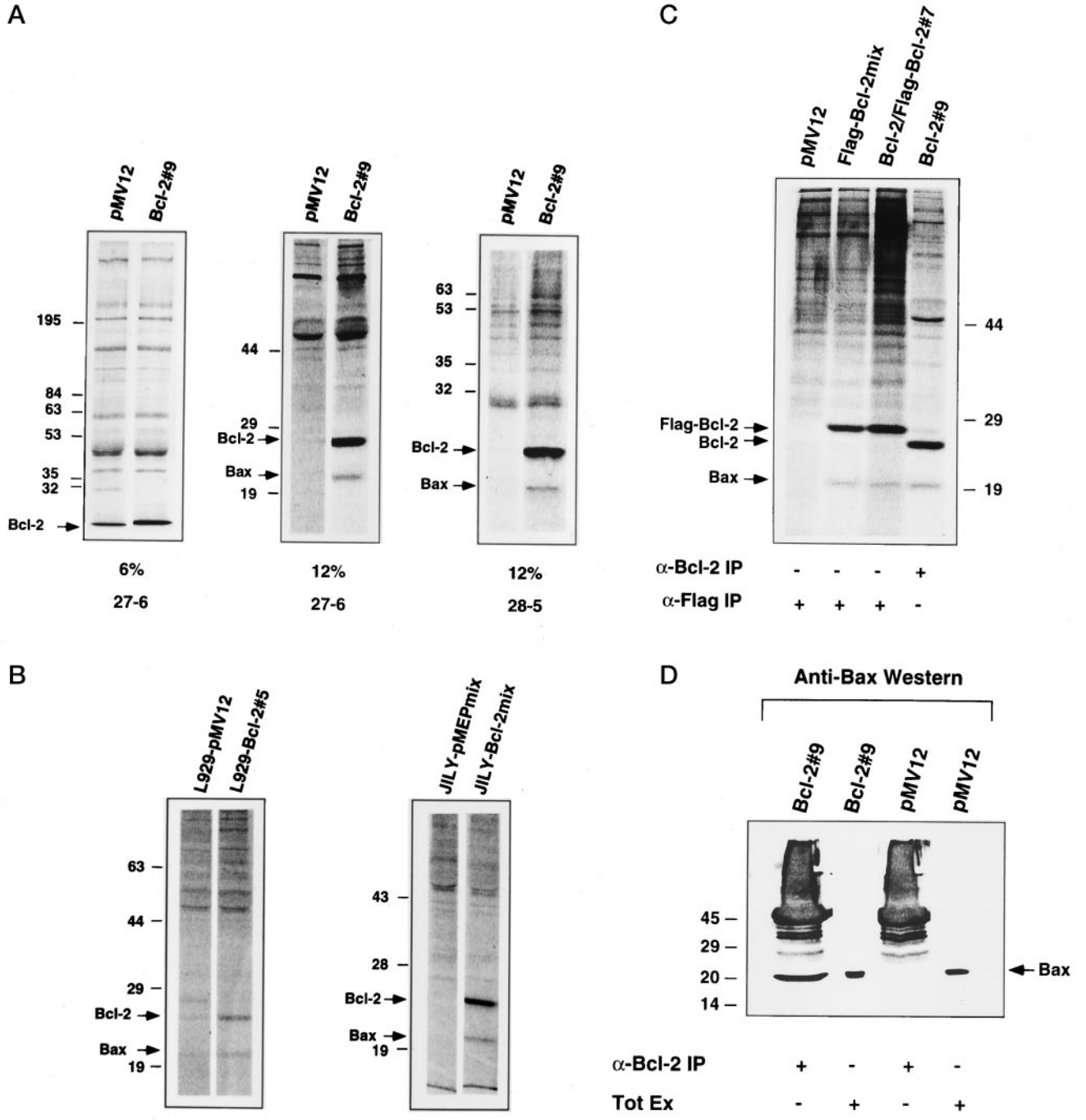

FIG. 1. **Bax specifically co-immunoprecipitates with Bcl-2.** Cells were labeled with [^{35}S]methionine/cysteine overnight, disrupted in buffer A containing 0.2% Nonidet P-40, and the lysate was subjected to anti-Bcl-2 or anti-Flag immunoprecipitations. *Panel A*, anti-Bcl-2/27-6 (*left* and *middle panels*) or anti-Bcl-2/28-5 (*right panel*) immunoprecipitates from vector control R6-pMV12 and Bcl-2 overexpressing R6-Bcl-2#9 cell extracts, analyzed on 6% (*left panel*) or 12% (*middle* and *right panels*) SDS-PAGE. *Panel B*, anti-Bcl-2/27-6 immunoprecipitates from vector control L929-pMV12 and JILY-pMEPmix and Bcl-2 overexpressing L929-Bcl-2#5 and JILY-Bcl-2mix cell extracts. *Panel C*, anti-Flag/M5 immunoprecipitates (*α-Flag IP*) from vector control R6-pMV12, Flag-Bcl-2 overexpressing R6-Flag-Bcl-2mix, and Flag-Bcl-2/Bcl-2 co-overexpressing R6-Bcl-2/Flag-Bcl-2#7 cell extracts. For comparison an anti-Bcl-2/27-6 immunoprecipitate (*α-Bcl-2 IP*) from R6-Bcl-2#9 cell extracts is shown. *Panel D*, total extracts (*Tot Ex*) and anti-Bcl-2 immunoprecipitates (27-6, *α-Bcl IP*) from unlabeled R6-pMV12 and R6-Bcl-2#9 cells were analyzed by anti-Bax (Ab 06-499) Western blotting as described under "Experimental Procedures."

that Bcl-2 and Bax form equimolar heterodimers until most of the endogenous Bax is bound. Any excessive Bcl-2 is free of Bax and may thus be available for the interaction with other molecules. However, as shown above for anti-Bcl-2 immunoprecipitations (Fig. 1), no proteins other than Bcl-2 and Bax specifically immunoprecipitated in detectable amounts with anti-Bax antibodies. This was true for low molecular mass (12% SDS-PAGE, Fig. 2*A*) as well as high molecular mass proteins (6% SDS-PAGE, data not shown). We also did not detect any protein that is released from endogenous Bax once Bcl-2 was bound, indicating that Bcl-2 did not compete for proteins constitutively bound to Bax (Fig. 2*A*). These findings were confirmed by another anti-Bax antibody directed against an NH_2-terminal epitope of Bax (Ab 06-499) as well as by anti-Bax immunoprecipitations from L929 and JILY cell extracts (data not shown).

To continue our search for novel proteins interacting with Bcl-2 and/or Bax, we overexpressed human Bax in R6-Bcl-2#9

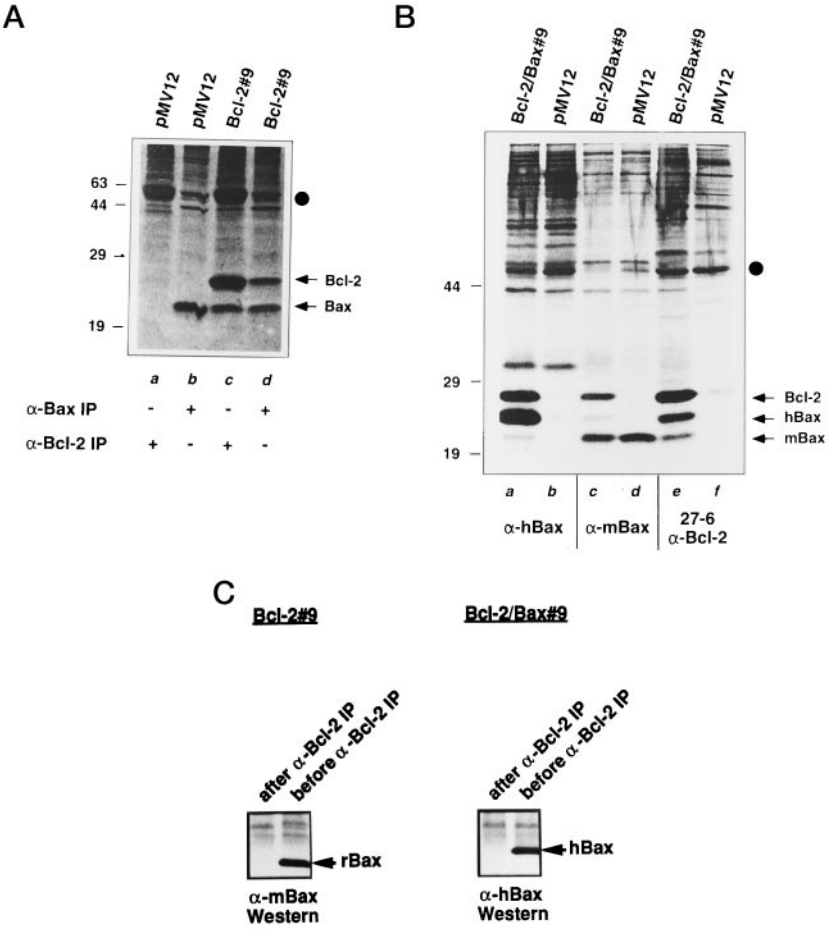

FIG. 2. **Bcl-2 specifically co-immunoprecipitates with endogenous and overexpressed Bax.** Cells were labeled with [^{35}S]methionine/cysteine overnight, disrupted in buffer A containing 0.2% Nonidet P-40, and the lysate was subjected to anti-Bcl-2 or anti-Bax immunoprecipitations. *Panel A*, anti-Bcl-2/27-6 (*α-Bcl-2 IP*) or anti-mouse/rat Bax/Ab 13686E (*α-Bax IP*) immunoprecipitates from R6-pMV12 (*lanes a* and *b*) and R6-Bcl-2#9 (*lanes c* and *d*) cell extracts. *Panel B*, anti-Bcl-2/27-6 (*27-6 α-Bcl-2*), anti-mouse/rat Bax/Ab 13686E (*α-mBax*), or anti-human Bax/Ab 13666E (*α-hBax*) immunoprecipitates from R6-pMV12 (*lanes b*, *d*, and *f*) and R6-Bcl-2-Bax#9 (*lanes a*, *c*, and *e*) cell extracts. *Arrows* indicate the positions of the overexpressed 26-kDa mouse Bcl-2, the overexpressed 24-kDa human Bax, and the endogenous 21-kDa rat Bax. The major, co-precipitating 45-kDa protein is actin (*closed circles*). *Panel C*, *left panel*, equal amounts of unlabeled R6-Bcl-2#9 cell extracts were probed with anti-mouse/rat Bax/Ab13868E on Western blots (*α-mBax*) for the presence of endogenous rat Bax (*rBax*) before and after anti-Bcl-2/27-6 immunoprecipitation; *right panel*, same experiment but using anti-human Bax/Ab 13666E (*α-hBax*) to monitor extracts of R6-Bcl-2-Bax#9 cells for the presence of overexpressed human Bax (*hBax*).

cells yielding the R6-Bcl-2-Bax#9 cell line. In these cells the molecular mass of the overexpressed Bax was 3 kDa higher (24 kDa) than that of endogenous Bax, allowing a distinction between the two protein species (Fig. 2*B*). The introduced 24-kDa human Bax had the following effects on endogenous rat Bax and overexpressed murine Bcl-2. (i) It did not disrupt the 1:1 complex between Bcl-2 and endogenous rat Bax (compare Fig. 2, *panel A*, *lane d*, with *panel B*, *lane c*), indicating that preformed Bcl-2-Bax heterodimers are not disturbed by incoming Bax. (ii) It bound to the Bcl-2 that was in excess of endogenous Bax and therefore formed more Bcl-2-Bax heterodimers than in the R6-Bcl-2#9 cell line (Fig. 2*B*, *lane a*). This is best illustrated with anti-Bcl-2 immunoprecipitates from R6-Bcl-2-Bax#9 cells (Fig. 2*B*, *lane e*) where Bcl-2 formed heterodimers with both overexpressed human 24-kDa Bax and endogenous rat 21-kDa Bax (compare Fig. 2, *panel A*, *lane c*, with *panel B*, *lane e*). Again, less than 20% of the overexpressed human Bax remained in the supernatant after an anti-Bcl-2 immunoprecipitation, indicating that most of the human Bax was complexed to and co-precipitated by Bcl-2 (Fig. 2*C*). Similarly, when comparing anti-human Bax (Fig. 2*B*, *lane a*) and anti-Bcl-2 (Fig. 2*B*, *lane e*) immunoprecipitates it became evident that almost all Bcl-2 was bound to Bax in R6-Bcl-2-Bax#9 cells. This may explain the diminished death-protection capacity of this cell line (see below). (iii) The newly formed human Bcl-2-Bax heterodimers did not co-immunoprecipitate novel proteins, irrespective of whether anti-human Bax (Fig. 2*B*, compare *lanes a* and *b*) or anti-Bcl-2 immunoprecipitates (Fig. 2*B*, compare *lanes e* and *f*) were analyzed. In addition, it seemed that some of the overexpressed human 24-kDa Bax was not bound to Bcl-2 in R6-Bcl-2-Bax#9 cells (Fig. 2*B*, compare *lanes a* and *e*). This free form of Bax did not interact stably with new protein partners either.

Taken together, our data show that Bcl-2 and Bax have a strong affinity for each other, and neither the Bcl-2-Bax heterodimer nor the free Bcl-2 and Bax molecules appear to bind stably to other cellular proteins, at least as judged from immunoprecipitations.

Bcl-2 and Bax Co-localize in Intact Cells—To show that Bcl-2 and Bax may also be partners in intact cells, we studied the intracellular localization of the two proteins by immunocytochemistry. Equimolar amounts of human Bcl-2 and human Bax cDNAs were transiently transfected into R6 cells. Bcl-2 was detected with a monoclonal anti-human Bcl-2 antiserum and a rhodamine-conjugated secondary antibody, Bax by a polyclonal anti-human Bax antiserum and a fluorescein-conjugated secondary antibody. Bcl-2 and Bax co-localized perfectly in a punctuated subcellular structure (Fig. 3*A*) reminiscent of mitochondria (data not shown). Although this analysis does not prove heterodimer formation of Bcl-2 and Bax *in vivo*, it clearly demonstrates that the two proteins localize to the same subcellular compartment.

Bcl-2-Bax Heterodimers Are Maintained, and Neither Bcl-2 nor Bax Associates Stably with New Proteins under Apoptotic Stresses—Interactions between Bcl-2 and Bax and putative binding proteins have so far been studied in non-apoptotic cellular systems. It is however possible that Bcl-2 and/or Bax acquires or loses binding partners only following cellular treatments with apoptotic agents. We therefore exposed R6-pMV12 and R6-Bcl-2#9 cells to the protein kinase inhibitor staurosporine (1 μM), the secretion-inhibiting drug BFA (5 μg/ml), the proteasome inhibitor MG132 (1 μM), or the protein phosphatase inhibitor okadaic acid (1 μM) for different time periods. Under all of these conditions R6-pMV12 rapidly underwent apoptosis,

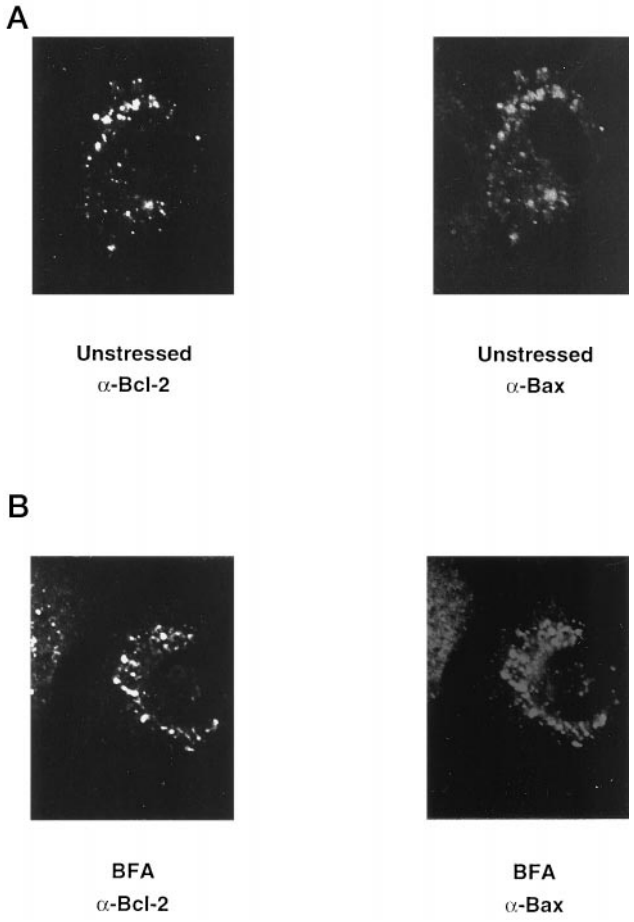

FIG. 3. **Bcl-2 and Bax co-localize in intact normal cells and cells exposed to BFA.** R6 cells on 12-mm glass coverslips were transfected with human Bcl-2 and human Bax cDNAs as described under "Experimental Procedures." After 6 h, the cells were incubated with fresh medium in the absence (*unstressed*) or presence (*BFA*) of 5 μg/ml BFA and left for another 22 h. 28 h post-transfection, the cells were fixed, permeabilized, incubated with the anti-human Bcl-2 antibody, clone 100 (*α-Bcl-2*), and the anti-Bax antibody Ab 06-499 (*α-Bax*) and then with secondary rhodamine-conjugated (*α-Bcl-2*) and fluorescein-conjugated (*α-Bax*) antibodies. Photographs were taken under a Zeiss fluorescence microscope at a magnification of × 1,000.

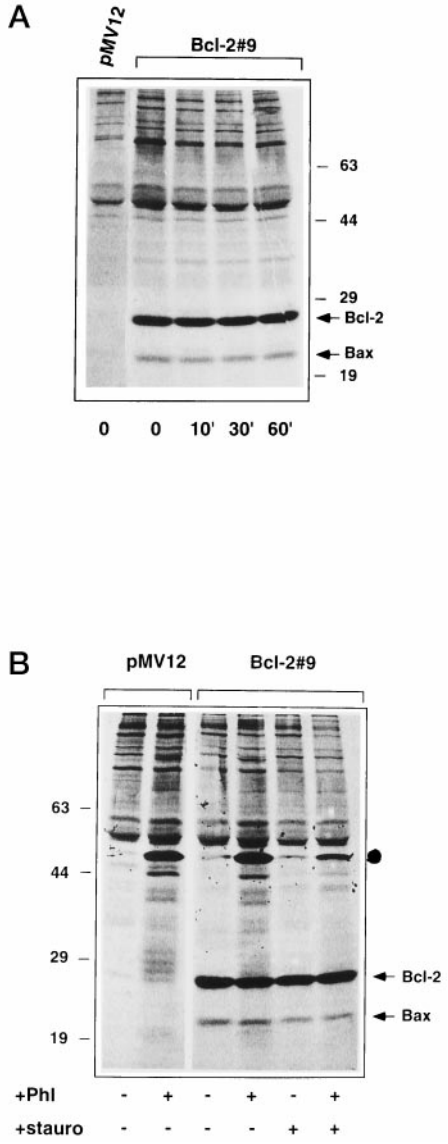

FIG. 4. **Stable Bcl-2-Bax heterodimers in anti-Bcl-2 immunoprecipitates from cells exposed to staurosporine.** R6-pMV12 and R6-Bcl-2#9 cells were labeled with [^{35}S]methionine/cysteine overnight. 10–60 min (*panel A*) or 48 h (*panel B*) before extraction, the cells were treated with 1 μM staurosporine. Cell disruption was in buffer A plus 0.2% Nonidet P-40 in the presence or absence of phosphatase inhibitors (±*PhI*). Immunoprecipitations were performed with and without phosphatase inhibitors using the anti-Bcl-2/27-6 antibody. The positions of endogenous Bax and overexpressed Bcl-2 are indicated by *arrows*. The major 45-kDa protein that co-precipitates in the presence of the phosphatase inhibitors is actin (*closed circle*).

whereas R6-Bcl-2#9 cells were protected efficiently for up to 48–72 h (see Figs. 7*C* and 8*C*, and data not shown).

We first investigated Bcl-2 binding properties at early time points (10–60 min) of a cellular exposure to staurosporine. The amounts of free and heterodimerized Bcl-2 and Bax proteins were not altered in anti-Bcl-2 immunoprecipitates from stressed R6-Bcl-2#9 cells, nor was any new protein partner of Bcl-2 detected under these conditions (Fig. 4*A*). This was also true for cell extracts from R6-Bcl-2#9 cells stressed for up to 48 h with staurosporine irrespective of the presence of absence of phosphatase inhibitors in the extraction and immunoprecipitation buffers (Fig. 4*B*). Similar results were obtained with the apoptotic stimuli MG132, okadaic acid, or BFA (data not shown).

Next, we tested if Bax bound a novel protein following a treatment of cells with apoptotic agents. It has been reported recently that Bax formed disulfide-linked homodimers in response to the apoptotic agent camptothecin (58). We therefore analyzed anti-Bax immunoprecipitates in the presence or absence of the reducing agent DTT. We did not detect any Bax homodimer formation nor the interaction of new proteins with Bax in anti-Bax immunoprecipitates from R6-pMV12 cells stressed with staurosporine or BFA for up to 48 h (Fig. 5). Moreover, anti-Bax immunoprecipitates from stressed R6-Bcl-2#9 cells revealed that Bax still interacted with Bcl-2 in an equimolar ratio (Fig. 5). Occasionally, higher levels of proteins at 55 and 75 kDa were detected in anti-Bax immunoprecipitates of staurosporine or BFA-treated compared with non-treated cell extracts (Fig. 5, *filled* and *open circles*). This however varied between different experiments and occurred in R6-pMV12 and R6-Bcl-2#9 cells, excluding the possibility that these proteins were real Bax-binding proteins released in the presence of Bcl-2. Thus, as judged from immunoprecipitations, the only consistent and firm partner of Bax during apoptotic stresses appeared to be Bcl-2. Indeed, immunofluorescence analysis showed that Bcl-2 and Bax still co-localized to a punctuated subcellular site in intact cells after an apoptotic stress with 5 μg/ml BFA for 22 h (Fig. 3*B*).

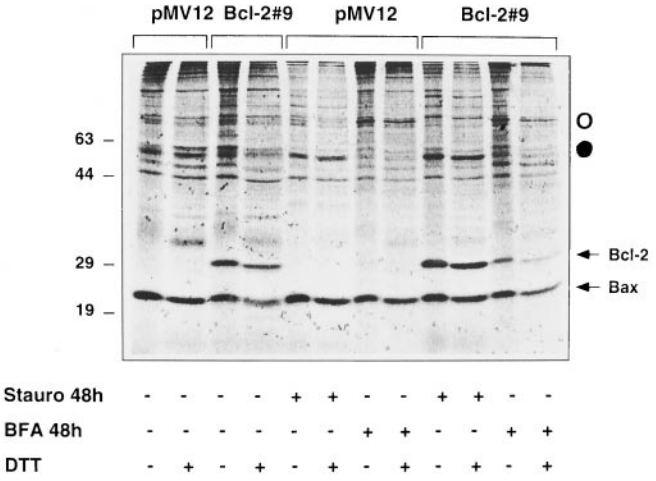

FIG. 5. **Stable Bcl-2-Bax heterodimers in anti-Bax immunoprecipitates from cells exposed to staurosporine or BFA.** R6-pMV12 and R6-Bcl-2#9 cells were labeled with [^{35}S]methionine/cysteine overnight. 48 h before extraction, the cells were treated with 1 μM staurosporine or 5 μg/ml BFA. Cell disruption was in buffer A plus 0.2% Nonidet P-40. Immunoprecipitations were performed using the anti-mouse Bax antibody 13686E. Immunocomplexes were denatured in SDS-sample buffer in the presence or absence of 30 mM DTT. The positions of endogenous Bax and overexpressed Bcl-2 are indicated by *arrows*. *Filled* and *open circles* mark 55- and 75-kDa proteins that occasionally co-immunoprecipitate with Bax. This, however, varied among different experiments and occurred in R6-pMV12 and R6-Bcl-2#9 cells, excluding the possibility that these proteins were real Bax-binding proteins released in the presence of Bcl-2.

Cell Death Protection by Bcl-2 Does Not Require Bax and Is Most Effective When Bcl-2 Is in Excess of Bax—The stability of the Bcl-2-Bax heterodimer in immunoprecipitates from cells exposed to apoptotic stimuli suggested that Bcl-2 may require Bax for its death-protection activity. To investigate this issue further, we (i) down-regulated endogenous Bax expression in R6-Bcl-2#9 cells by an antisense Bax strategy and (ii) generated R6 cell lines that expressed various levels of Bcl-2 and Bax. All cells were monitored for their survival capacity in response to the apoptotic agents staurosporine and MG132.

Transfection of R6-Bcl-2#9 cells with a 20-mer antisense Bax phosphorothioate oligonucleotide encompassing the ATG start codon of rat Bax led to a time-dependent decrease of endogenous Bax expression (Fig. 6*A*). This was complete after 3 days and persisted for another 2 days (Fig. 6*A* and data not shown). A sense Bax oligonucleotide encompassing the same sequence did not affect endogenous Bax expression (Fig. 6*A*). When sense-transfected and antisense-transfected R6-Bcl-2#9 cells were compared for their capacity to resist staurosporine- or MG132-induced apoptosis, no difference was detected over a period of 24 h when Bax was down-regulated entirely (between day 3 and 4 post-transfection) (Fig. 6*B*). These results indicate that Bcl-2 can act as a survival factor in the absence of Bax expression.

To study whether the survival activity of Bcl-2 was directly proportional to its expression level, we constructed R6 cell lines that expressed the 26-kDa Bcl-2 and/or the 27-kDa Flag-Bcl-2 protein at low (R6-Flag-Bcl-2#15), intermediate (R6-Bcl-2#5), or high levels (R6-Bcl-2#9, R6-Flag-Bcl-2mix, R6-Bcl-2/Flag-Bcl-2#7) (Fig. 7*A*). Importantly, Flag-Bcl-2 protected cells against apoptosis as efficiently as Bcl-2 when expressed at similar levels (data not shown). In addition, human 24-kDa Bax protein was expressed at low (R6-Bcl-2-Bax#1) and high amounts (R6-Bcl-2-Bax#9) in the R6-Bcl-2#9 cell line to study the effect of exogenous Bax on death protection by Bcl-2 (Fig. 8*A*).

Similar amounts of endogenous Bax were co-immunoprecipitated with anti-Bcl-2 antibodies from the extracts of the various Bcl-2-overexpressing cell lines (Fig. 7*B*), indicating that all of these cells had the endogenous Bax complexed to Bcl-2 and differed in the amount of the Bcl-2 protein that was free of Bax.

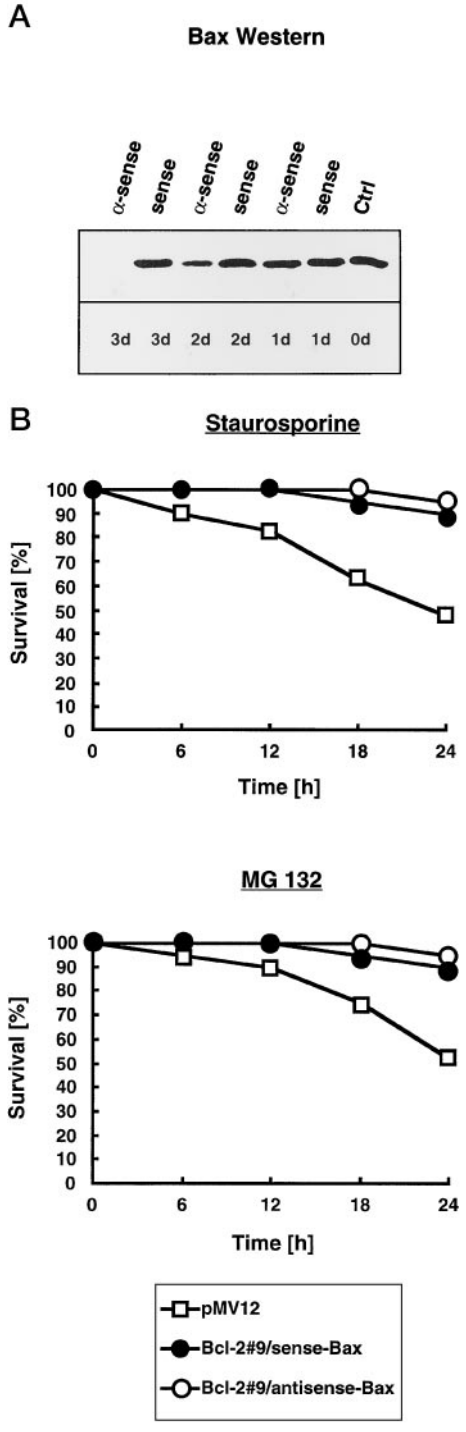

FIG. 6. **Bcl-2 does not require Bax expression for its death-protective activity.** R6-Bcl-2#9 cells were transfected with a 1 μM concentration of the antisense (*α-sense*) or sense phosphorothioate Bax oligonucleotide 1–20 using Lipofectin. Control cells (*Ctrl*) received only Lipofectin. *Panel A*, every day total cell extracts were prepared and analyzed for endogenous Bax expression by anti-Bax Western blotting (Ab 06-499). *Panel B*, at day 3 post-transfection, antisense (○)- and sense-transfected R6-Bcl-2#9 (● as well as R6-pMV12 vector control cells (□) were treated with 1 μM staurosporine or 1 μM MG132 for 24 h. Cell viability was monitored by counting trypan blue-excluded cells in a Neubauer chamber. The displayed cell counts are the means of three independent experiments.

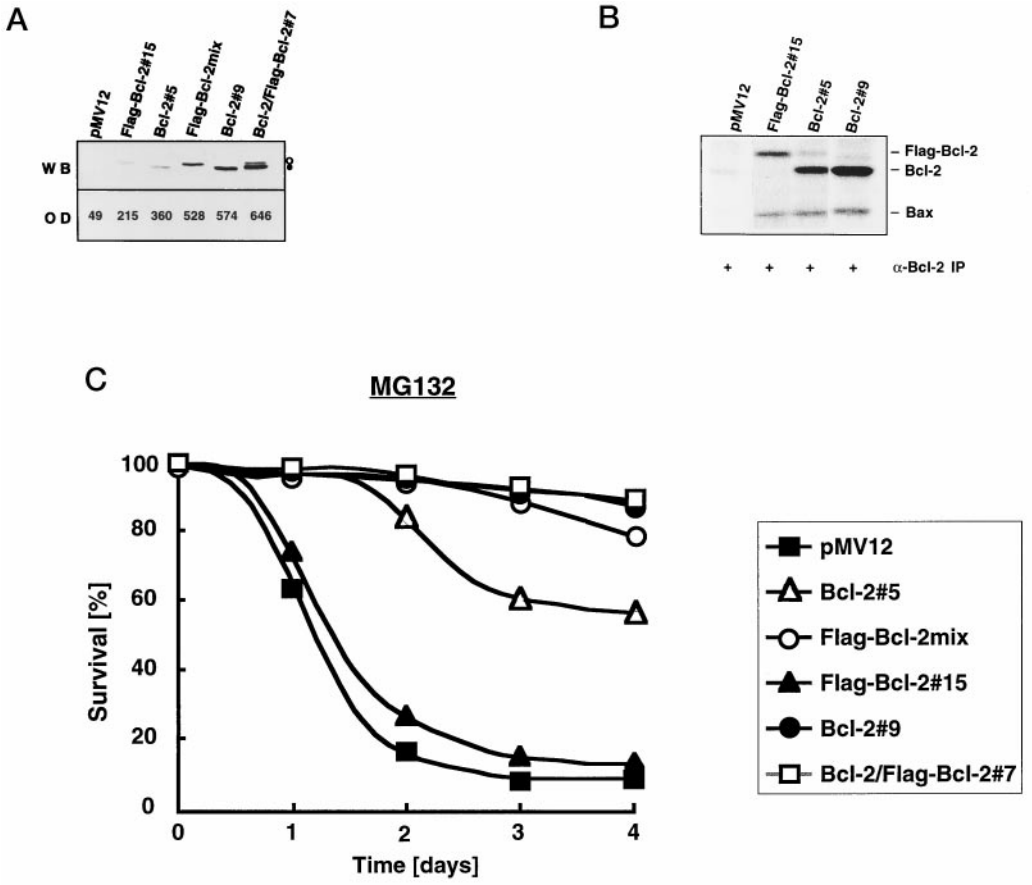

FIG. 7. **Protection against apoptosis induced by MG132 positively correlates with the amount of Bcl-2 free of Bax.** *Panel A*, unlabeled, total protein extracts of vector control R6-pMV12, Flag-Bcl-2 overexpressing R6-Flag-Bcl-2#15 and R6-Flag-Bcl-2mix, Bcl-2 overexpressing R6-Bcl-2#5 and R6-Bcl-2#9, and Bcl-2/Flag-Bcl-2 co-overexpressing R6-Bcl-2/Flag-Bcl-2#7 cells were analyzed by anti-Bcl-2/27-6 Western blotting (*WB*) followed by quantifying the expression levels of the 26-kDa Bcl-2 (*closed circle*) and the 27-kDa Flag-Bcl-2 (*open circle*) by densitometric scanning of the ECL autoradiographs (*OD*). Each OD value represents the sum of the intensities obtained from Bcl-2 and Flag-Bcl-2. *Panel B*, [^{35}S]methionine/cysteine-labeled buffer A and 0.2% Nonidet P-40 protein extracts of the various cell derivatives were subjected to anti-Bcl-2/27-6 immunoprecipitations. The positions of the endogenous 21-kDa rat Bax and the overexpressed 26-kDa Bcl-2 and 27-kDa Flag-Bcl-2 are indicated. *Panel C*, subconfluent cell derivatives were treated with 1 μM MG132 for 4 days, and each day the cell viability was determined by trypan blue exclusion or the LIVE/DEAD kit. The data are the means of six independent experiments. ■, pMV12; △, Bcl-2#5; ○, Flag-Bcl-2mix; ▲, Flag-Bcl-2#15; ●, Bcl-2#9; □, Bcl-2/Flag-Bcl-2#7.

As judged from anti-Bcl-2 immunoprecipitates (Fig. 7*B*), the level of free Bcl-2 correlated positively with the degree of death protection against apoptosis induced by MG132 (Fig. 7*C*). Whereas R6-FLAG-Bcl-2#15 cells only slightly resisted apoptosis, this was enhanced in R6-Bcl-2#5 and best in R6-Bcl-2#9 and R6-Bcl-2/Flag-Bcl-2#7 cells (Fig. 7*C*). Conversely, the expression of exogenous human Bax resensitized R6-Bcl-2#9 cells for MG132- or staurosporine-induced apoptosis in a dose-dependent manner (Fig. 8*C*). This was accompanied by the appearance of more Bcl-2-Bax heterodimers in both anti-Bcl-2 and anti-Bax immunoprecipitates of R6-Bcl-2-Bax#9 compared with R6-Bcl-2#9 cells (Fig. 8*B*), suggesting that the Bcl-2-Bax heterodimer neutralized rather than favored the death-protective activity of Bcl-2. The resensitization was somehow smaller with the R6-Bcl-2-Bax#1 cell line (Fig. 8*C*) because less human Bax was overexpressed (Fig. 8*A*) and/or co-precipitated in anti-Bcl-2 immunoprecipitates compared with the R6-Bcl-2-Bax#9 cell line (data not shown).

DISCUSSION

To our knowledge this is the first study describing the binding properties, subcellular localizations, and biological activities of Bcl-2 and Bax in cells exposed to apoptotic stimuli. It shows that (i) Bcl-2 and Bax co-localize to the same subcellular compartment and form strong, equimolar heterodimers under both normal and apoptotic conditions; (ii) the protection against apoptosis does not necessitate Bax expression and correlates with the amount of the Bcl-2 protein that is free of Bax; and (iii) no other cellular protein associates stably with Bcl-2, Bax, or the Bcl-2-Bax heterodimer in anti-Bcl-2 or anti-Bax immunoprecipitates.

The conditions used for cell fractionations and immunoprecipitations were as physiological as possible (143 mM KCl, pH 7.5). However, because both Bcl-2 and Bax are integral membrane proteins they had to be solubilized in 0.2% Nonidet P-40. Although such a treatment might disrupt protein-protein interactions, several membrane complexes including Bcl-2-Bax have been isolated previously in the presence of 0.2–1% non-ionic detergent (50, 56). In addition, it has been reported recently that non-ionic detergents promoted rather than disrupted dimerizations among members of the Bcl-2 family (59). Disruption of the Bcl-2-Bax complex, however, occurred when 0.5% deoxycholate and/or 0.1% SDS (RIPA buffer conditions) were added, suggesting an intermediate binding affinity between the two proteins (Ref. 50 and data not shown). Since we did not detect any Bcl-2-binding protein under the stringent RIPA buffer conditions, no cellular protein seems to bind stronger to Bcl-2 than Bax and *vice versa*.

There was no obvious reason why proteins with an affinity similar to Bax would not have been co-immunoprecipitated with anti-Bcl-2 antibodies, especially in cells where a high

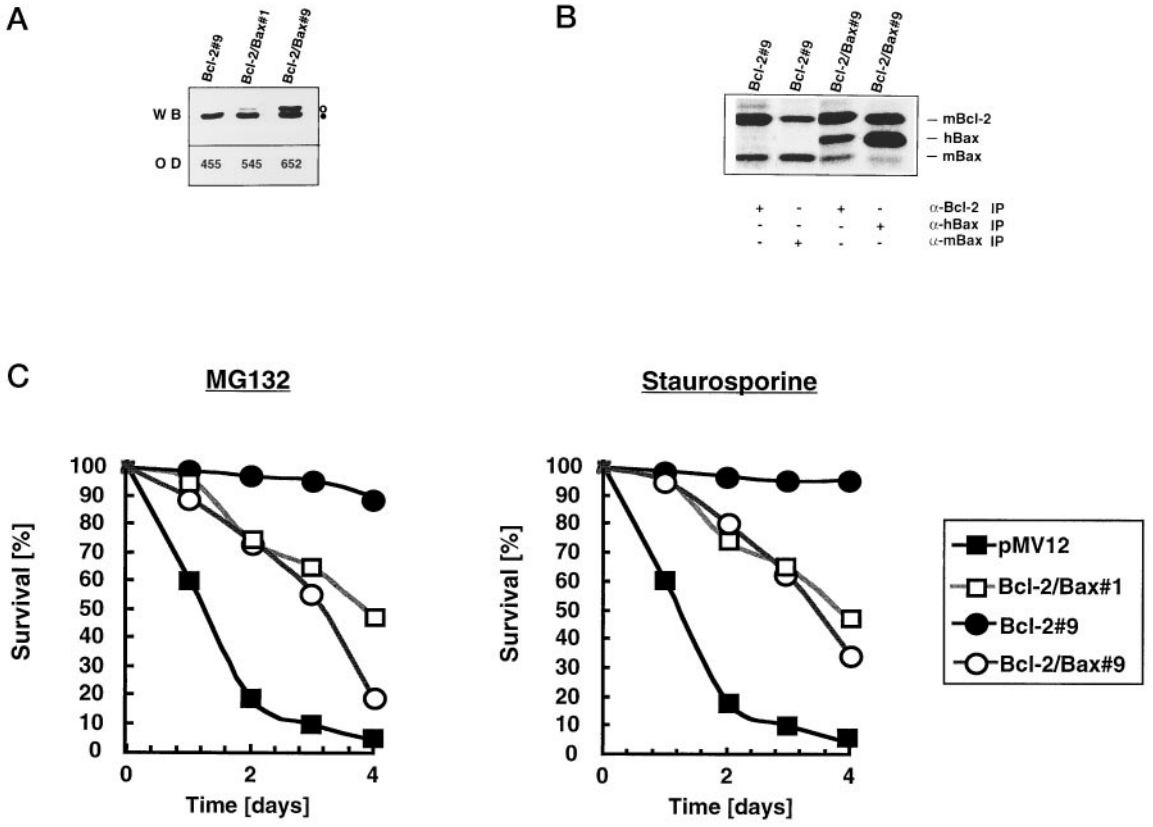

FIG. 8. **Overexpression of Bax resensitizes Bcl-2 overexpressing cells for apoptosis induced by MG132 or staurosporine.** *Panel A*, unlabeled, total protein extracts of Bcl-2 overexpressing R6-Bcl-2#9 and Bcl-2-Bax co-overexpressing R6-Bcl-2-Bax#1 and R6-Bcl-2-Bax#9 cells were analyzed by anti-Bax/Ab 06-499 Western blotting (*WB*) followed by quantifying the expression levels of the endogenous 21-kDa rat Bax (*closed circle*) and the overexpressed 24-kDa human Bax (*open circle*) by densitometric scanning of the ECL autoradiographs (*OD*). Each OD value represents the sum of the intensities obtained from the two Bax species. *Panel B*, [^{35}S]methionine/cysteine-labeled buffer A/0.2% Nonidet P-40 protein extracts of the various cell derivatives were subjected to anti-Bcl-2/27-6 (α-Bcl-2-IP), anti-Bax/Ab 13686E (α-mBax-IP), or anti-Bax/Ab 13666E (α-hBax-IP) immunoprecipitations. The positions of the endogenous 21-kDa rat Bax (*mBax*), the overexpressed 24-kDa human Bax, and the 26-kDa mouse Bcl-2 are indicated. *Panel C*, subconfluent cell derivatives were treated with 1 μM MG132 or 1 μM staurosporine for 4 days, and each day the cell viability was determined by trypan blue exclusion or the LIVE/DEAD kit. The data are the means of six independent experiments.

amount of the Bcl-2 protein was accessible, *i.e.* free of Bax (R6-Bcl-2#9). Interference by the antibodies used was considered minimal as both the anti-Bcl-2 antibody 27-6 and the anti-Flag antibody detect epitopes outside of the BH1–4 domains essential for the survival activity of Bcl-2 (26, 57). In addition, we have reported recently that Bax was the only cellular protein that was specifically retained by a GST-Bcl-2 affinity column incubated with radiolabeled extracts from nonstressed or apoptotically stressed cells, although this method was antibody-independent (44).

Compared with other binding techniques (yeast two-hybrid, interaction cloning) co-immunoprecipitation is the method of choice if one wants to study protein-protein interactions that may only occur in response to cellular treatments with apoptotic stimuli. However, the following disadvantages should be considered. First, proteins that bind transiently or with low affinities are difficult to co-immmunoprecipitate. Although we tried to optimize buffer conditions for extraction and/or immunoprecipitation, complexes of Bcl-2 or Bax with other cellular proteins may not have been maintained after cell disruption. To overcome this problem we lysed cells by repeated freeze-thawing cycles and added the bifunctional cross-linker dithiobis(succinimidylpropionate) to the extract before solubilization in detergent. However, even under these conditions no additional Bcl-2-binding proteins were found (data not shown). Second, the binding between Bcl-2 and its partners may depend on post-translational modifications. It has been reported previously that the binding of Bcl-2 to c-Raf-1 and/or Ras is phosphorylation-sensitive (35, 60). Moreover, Bax was shown to form disulfide-linked dimers in response to chemotherapeutic drugs (58). However, the absence or presence of phosphatase inhibitors or DTT had only a minor effect on the protein pattern in our immunoprecipitates, and no disulfide-linked Bax homodimers were found. Third, the binding partners of Bcl-2 and/or Bax may not be sufficiently abundant to be detected by immunoprecipitations because they exhibit a high turnover rate. We have recently obtained evidence for the involvement of short lived proteins in apoptosis induced by decreased ubiquitination (61). Fourth, the binding proteins may not contain enough methionine and/or cysteine residues to be radiolabeled and detected on autoradiographs after immunoprecipitation (for example, heat shock protein 27; Ref. 62). This issue is currently being addressed by labeling cellular proteins with other amino acids or detecting them in immunoprecipitates by silver staining.

It was surprising that none of the previously reported Bcl-2-binding proteins was detected in our anti-Bcl-2 immunoprecipitates (31–43, 60). Interestingly, with the notably exception of Bax (50), all so far known Bcl-2-binding proteins have been isolated by interaction cloning or yeast two hybrid techniques (31–43) and have not yet been shown to bind to Bcl-2 at endogenous expression levels. By contrast, interactions of these proteins with Bcl-2 were detected readily when they were mixed as recombinant proteins *in vitro* or immunoprecipitated following forced co-overexpressions in mammalian, insect, or yeast cells (31–43, 60). Although such experiments provide

first clues as to whether a given protein-protein interaction is possible at all, they increase the chances of nonspecific interactions and should therefore be accompanied by analyses under physiological conditions. This might be especially important for proteins that have been reported to bind to the NH_2-terminal BH4 region of Bcl-2, such as c-Raf-1, BAG-1, and calcineurin (34–37, 42). Recent molecular modeling has shown that this region can adopt a helical conformation that contains five hydrophobic amino acids clustered on one surface of the helix (63). Thus, protein binding to the BH4 of Bcl-2 may be based on nonspecific hydrophobic interactions that confer to Bcl-2 the "stickiness" that has been suggested previously (64).

Based on site-directed mutagenesis studies it has remained controversial whether Bcl-2 requires Bax for its death-protective function (51). By using an antisense Bax strategy and increasing the amount of exogenous Bax in Bcl-2-overexpressing cells, we show here that Bcl-2 exerts its survival function independent of Bax and that higher levels of Bcl-2-Bax heterodimers diminish rather than enhance the death-protective capacity of Bcl-2. The same result has been obtained recently from studying the activities of Bcl-2 and Bax in Bax and/or Bcl-2 knock-out mice (65). We also find that the degree of Bcl-2-Bax heterodimer formation depends on the expression levels of each partner in unstressed cells. This in turn dictates the amount of Bcl-2 that will be available to protect the cell once it is exposed to apoptotic stimuli. Thus, our data favor a model in which putative effectors of Bcl-2 do not disrupt Bcl-2-Bax heterodimers but bind to and/or modulate the activity of Bcl-2 that is free of Bax.

How might Bcl-2 and Bax regulate apoptosis in the absence of a stable interaction with other cellular proteins? Bcl-2 and/or Bax may be enzymes that bind to proteins or other substrates crucial for death or survival in a transient fashion. Alternatively, Bcl-2 and/or Bax may interact with small peptides or non-proteaceous molecules such as lipids that are difficult to be detected by SDS-PAGE. In this regard it is worth noting that Bcl-2 protects cells from apoptosis induced by membrane-permeable ceramide analogs (17, 18), presumably by preventing these analogs from activating the death effector machinery (caspase 3 activation) (66). Studies are in progress to investigate whether Bcl-2 binds directly to ceramide. Finally, Bcl-2 and/or Bax may form homodimers or oligomers on intracellular membranes. It has been reported that Bcl-2 can homodimerize *in vitro* in the yeast two-hybrid system as well as in immunoprecipitates (50, 51, 67, 68). Site-directed mutagenesis revealed that the dimers are formed in a head-to-tail fashion and involve the same domains that are crucial for the survival activity of Bcl-2 (BH4 interacts with BH1/BH2) (9, 51, 67, 68). The head-to-tail arrangement would allow Bcl-2 to form multimers, although such structures have not yet been detected biochemically. Homodimers or multimers of Bcl-2 or Bax could create ion channels that maintain the correct ion permeability during apoptotic stresses. In fact, the three-dimensional structure of the Bcl-2 homolog Bcl-x_L resembles that of pore-forming bacterial toxins diphtheria toxin and colicin (26), and purified Bcl-x_L (27) and Bcl-2 (69) reconstituted in a synthetic phospholipid bilayer generate ion fluxes in the absence of any other cellular protein. However, it remains to be determined whether Bcl-x_L, Bcl-2, or Bax indeed forms ion channels on biological membranes under physiological conditions and whether homodimerizations or multimerizations play a role in this respect. Further studies are required to validate the proposed concepts, in particular to determine the kind of effector molecules, if any, which mediate the biologic activities of Bcl-2 and Bax.

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2.4 ARTICLE : Bcl-2 prolongs cell survival after Bax-induced release of cytochrome c

Rossé, T., Olivier, R., Monney, L., Rager, M., Conus, S., Fellay, I., Jansen, B. and Borner, C. (1998) *Nature* **391**, 496-499.

Notice :

My contribution to this article was the co-immunoprecipitation analyses from radiolabelled extracts of cells that stably overexpressed Bcl-2 and Bax to show that these two proteins interact in our conditions confirming the fact that Bcl-2 and Bax co-localized in immnuofluorescence analyses (data not shown but cited as reference).

Slimb-mediated proteolysis and accumulate to high levels. For example, Ci and Arm may normally be phosphorylated by PKA and glycogen synthase kinase 3 (GSK3; reviewed in refs 5, 17), and signalling by Hh and Arm may change the state or degree of their phosphorylation, blocking their ability to interact with Slimb.

Slimb has ~80% amino-acid identity to β-TRCP, a *Xenopus* protein of unknown function[25]. In *Xenopus* and mammalian cells, β-catenin, a homologue of *Drosophila* Arm, seems to be targeted for degradation by the ubiquitin/proteasome pathway in the absence of Wg/Wnt signalling[26,27]. Hence, the close structural homology between β-TRCP and Slimb raises the possibility that the two proteins have a conserved role in destabilizing Arm/β-catenin. Mutations in other genes which allow β-catenin to escape from degradation are associated with a wide range of metastatic disorders[28]. We therefore speculate that mammalian homologues of *slimb* may be tumour-suppressor genes. □

Methods

Mutations and transgenes. *slmb*^{1} and *slmb*^{2} are EMS-induced mutations isolated from a screen for mutations that cause abnormal adult patterns in somatic clones[10]. *slmb*^{1} and *slmb*^{2} are lethal *in trans* to each other and *in trans* to deficiency *Df(3R)3-R1*, which eliminates the chromosome interval 93B3-5 to 93D2-4. *slmb*^{1}/*slmb*^{2} and *slmb*^{1}/*Df(3R)3-R1* animals survive to the pupal stage; imaginal discs isolated from *slmb*^{1}/*slmb*^{2} larvae show ectopic Dpp and Wg expression as well as high levels of Ci and excessive proliferation in anterior compartment cells. *slmb*^{2}/*Df(3R)3-R1* animals die during early larval life. *slmb*^{P1493} is a P-element insertion mutation at cytological position 93B10-11, originally called *l(3)00295*. *slmb*^{P1493} behaves like *Df(3R)3-R1* in complementation tests with *slmb*^{1} and *slmb*^{2}, resulting in pupal and early larval lethality, respectively. Wild-type function can be restored by precise P-element excision. The *smo*^{3}, *dpp*^{d12} and *PKA*$^{-}$ mutations, as well as the *dpp-lacZ3.0* and *ci-lacZ* reporter genes, have been described[10,11,29]. *Tubα1-slmb* is a transgene containing the entire *slimb* ORF under the control of the constitutive *Tubulinα1* promoter[11] inserted on the left arm of the second chromosome (see below). *hsp70-Myc-tagged GFP* is a transgene containing the coding sequence for a Myc-tagged form of green fluorescent protein (GFP) under the control of the heat-shock inducible promoter of the *hsp70* gene (A. Adachi and G. Struhl, unpublished).

Cloning of *slimb*. The genomic DNA sequence flanking the insertion site of *slmb*^{P1493} was isolated by plasmid rescue and used as a probe to screen an ovarian cDNA library. A full-length sequence for the *slimb* ORF was assembled from several partial cDNA clones. The identity of the *slimb* ORF is verified by rescue experiments: *slmb*^{2} or *slmb*^{P1493} homozygotes as well as *slmb*^{1}/*slmb*^{2} heterozygotes that carry a single copy of *Tubα1-slmb* transgene are viable and show normal morphology, except that 5–10% of the adults exhibit duplications of the haltere.

Generating clones of marked mutant cells. Clones of mutant cells were generated by FLP/FRT-mediated mitotic recombination as described[10]. Genotypes for generating clones are as follows. *slmb* mutant clones: *y hsp-flp.1/y* or *Y; FRT82E slmb*$^{1,2\ \text{or}\ P1493}$/*FRT82E hsp-CD2, y*$^{+}$ (or *hsp70-myc-gfp, w*$^{+}$) with or without *dpp-lacZ 3.0* on the second chromosome. *slmb dpp* clones: *y hsp-flp.1/y or Y; dpp*^{d12} *stc FRT 39E/Tubα1-slmb FRT39E; slmb*^{1}/*slmb*^{2}. *slmb smo* mutant clones: *y hsp-flp.1/y or Y; smo*^{3} *stc FRT 39E/Tubα1-slimb FRT39E; slmb*^{1}/*slmb*^{2}.

Imaginal disc staining and western blot analysis. Standard protocols for immunofluorescence and immunohistochemistry of imaginal discs were used[10]. Western blot analysis was performed as described[4].

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Bcl-2 prolongs cell survival after Bax-induced release of cytochrome *c*

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Following exposure of cells to stimuli that trigger programmed cell death (apoptosis), cytochrome *c* is rapidly released from mitochondria into the cytoplasm where it activates proteolytic molecules known as caspases that specifically cleave the amino-acid sequence DEVD and are crucial for the execution of apoptosis[1–4]. The protein Bcl-2 interferes with this activation of caspases by preventing the release of cytochrome *c*[2–4]. Here we study these molecular interactions during apoptosis induced by the protein Bax, a pro-apoptotic homologue of Bcl-2 (refs 5, 6). We show that

in cells transiently transfected with *bax*, Bax localizes to mitochondria and induces the release of cytochrome *c*, activation of caspase-3, membrane blebbing, nuclear fragmentation, and cell death. Caspase inibitors do not affect Bax-induced cytochrome *c* release but block caspase-3 activation and nuclear fragmentation. Unexpectedly, Bcl-2 also fails to prevent Bax-induced cytochrome *c* release, although it co-localizes with Bax to mitochondria. Cells overexpressing both Bcl-2 and Bax show no signs of caspase activation and survive with significant amounts of cytochrome *c* in the cytoplasm. These findings indicate that Bcl-2 can interfere with Bax killing downstream of and independently of cytochrome *c* release.

Rat (R6) embryo fibroblasts and human SK2 melanoma transfected with *bax* were monitored for Bax expression and cytochrome *c* release by immunocytochemistry and for nuclear condensation/fragmentation by Hoechst staining. Immunostaining of endogenous rat Bax was filamentous (Fig. 1a), whereas that of transfected human Bax was punctuated (Fig. 1c, e). However, both forms of Bax

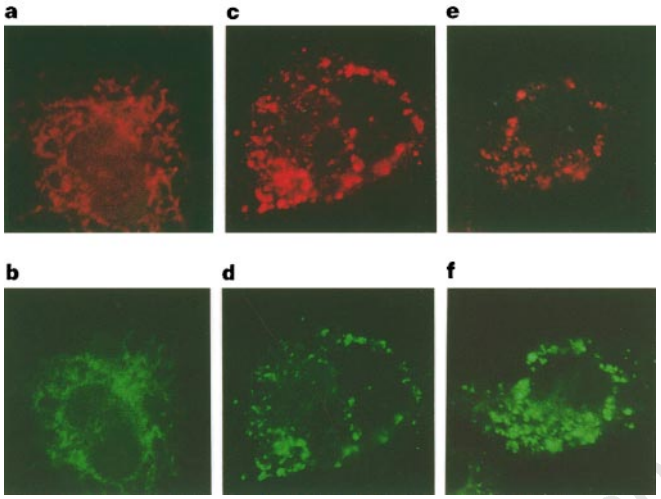

Figure 1 Bax co-localizes with mitochondrial cytochrome *c* oxidase VIc (COX) and Bcl-2. Anti-Bax (**a**, **c**, **e**), anti-COX (**b**, **d**) and anti-Bcl-2 (**f**) immunocytochemical analysis on non-transfected (**a**, **b**), *bax*-transfected (**c**, **d**) and *bax/bcl-2*-transfected (**e**, **f**) R6 cells at 8 h post-transfection. Magnification, ×1,000.

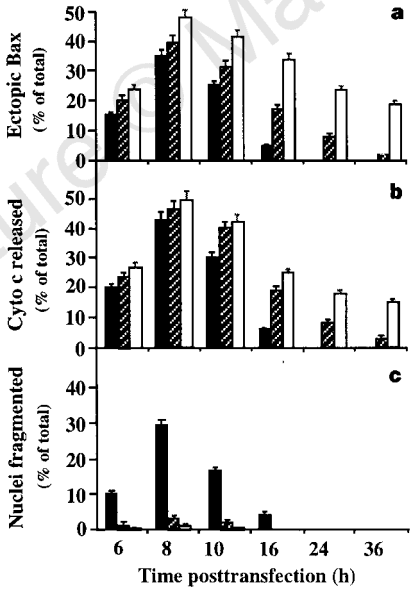

Figure 2 Quantification of ectopic Bax expression, cytosolic cytochrome *c* and fragmented nuclei following *bax* transfection into R6 and SK2 cells. The cells were transfected with *bax* (black bars) in the absence or presence of Z-DEVD-fmk (hatched bars) or co-transfected with *bax* and *bcl-2* (white bars) and subjected to anti-Bax, anti-cytochrome *c* and Hoechst 33342 analysis at various times post-transfection as for Fig. 3. The percentages of cells displaying punctuated Bax (**a**), diffuse cytochrome *c* (**b**) or fragmented nuclei (**c**) was determined by counting fluorescent cells on 20 randomly selected fields under the microscope. 100% represents total cells of the respective fluorescence per field. Data are depicted as means ± s.d. from two independent transfections into R6 and SK2 cells, respectively (total of 80 fields analysed).

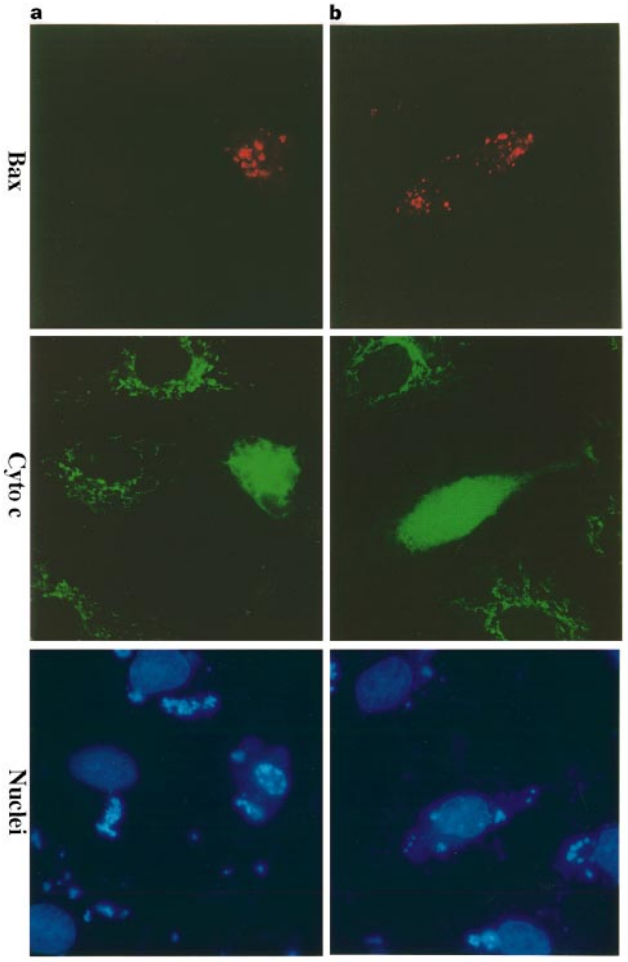

Figure 3 Bax induces cytochrome *c* release irrespective of Bcl-2 inhibition. Anti-Bax (rhodamine), anti-cytochrome *c* (fluorescein) and Hoechst 33342 (UV) fluorescence analysis on *bax*-transfected (**a**) and *bax/bcl-2*-transfected (**b**) R6 cells at 8 h post-transfection. Hoechst-stained aggregates around the nuclei are DNA/Superfect complexes that could not be entirely removed after transfection. Magnification, ×1,000.

co-localized with the mitochondrial cytochrome *c* oxidase subunit VIc (COX) (Fig. 1b, d), indicating that ectopic Bax changed the mitochondrial morphology from a filamentous to a punctuated pattern. This change was specific for Bax overexpression as it occurred in only 0.1% of non-transfected cells (data not shown). The strong anti-Bax immunostaining allowed cells overexpressing Bax to be counted by fluorescence microscopy. At 6 h post-transfection, about 15% of total cells were positive for ectopic Bax (Fig. 2a). This number increased to 35% at 8 h post-transfection but decreased thereafter because of cell loss by apoptosis (Fig. 2a).

We next studied the fate of cytochrome *c* in *bax*-transfected R6 and SK2 cells. The immunostaining of cytochrome *c* was filamentous in non-transfectants but was diffuse in cells expressing punctuated Bax, suggesting that ectopic Bax may have provoked a redistribution of cytochrome *c* from the mitochondria to the cytoplasm (Fig. 3a). Cells displaying diffuse cytochrome *c* staining were all apoptotic because their nuclei were condensed and/or fragmented (Figs 2b, c and 3a). To study the role of caspases in this type of cell death, we treated *bax* transfectants with cell-permeable caspase inhibitors, Z-DEVD-fmk (Fig. 2) or Z-VAD-fmk (data not shown). As shown in Fig. 2, such treatments prolonged the survival of cells overexpressing Bax after release of cytochrome *c*. The nuclei of these cells were not fragmented but remained condensed, and all cells were detached from the coverslip at 36 h post-transfection (Fig. 2). These results indicate that caspases act downstream of cytochrome *c* to trigger Bax-induced nuclear fragmentation, but that there is a pathway to cell death that cannot be blocked by caspase inhibitors.

To confirm our results, we transfected human *bax* into HEK 293T cells. Owing to the very high efficiency of transfection, these cells allowed a biochemical analysis of caspase-3 activation and DNA fragmentation. Transfectants were identified by X-Gal blue staining following co-transfection with *bax* and β-galactosidase (*β-gal*) as previously described[7]. Apoptotic cells were round and blebbing, whereas surviving cells were elongated in shape (Fig. 4a). Between 6 and 10 h post-transfection, elongated blue cells (Fig. 4b, white bars) showed increased blebbing (Fig. 4b, black bars) and were shedded into the medium. Thus, only a few blue cells remained attached to the plate by 16 h post-transfection (Fig. 4b). Western blot analysis revealed that between 6 and 10 h post-transfection, 20–40% of total cytochrome *c* was redistributed from a membrane to a soluble fraction (Fig. 4c). This coincided with the conversion of the inactive 32K pro-caspase-3 into the active 17K protease (Fig. 4d) and was followed by the appearance of nucleosome-sized DNA fragments at 8–16 h post-transfection (Fig. 4e). At 16–24 h post-transfection, cytosolic cytochrome *c*, p17 caspase-3, and DNA fragments had all completely disappeared, presumably because apoptotic cells were lost (Fig. 4c–e). Again, the caspase inhibitors Z-DEVD-fmk (Fig. 4c–e) and Z-VAD-fmk (data not shown) did not inhibit cytochrome *c* release but blocked both caspase-3 activation and DNA fragmentation. Thus, a large number of blue cells still adhered to the plate (Fig. 4b) and expressed cytosolic levels of cytochrome *c* at 16 h post-transfection (Fig. 4c). Most of these cells displayed a blebbing phenotype (Fig. 4b, filled bars), however, and shedded off the plate later on, confirming that caspase inhibitors can delay but not prevent Bax-induced cytoxocity.

Bcl-2 interacts with Bax and blocks Bax-induced apoptosis[5,6]. We therefore co-transfected equal amounts of *bcl-2* and *bax* into R6 and SK2 cells and studied the effect of Bcl-2 on Bax-induced cytochrome *c* release, caspase activation and nuclear fragmentation. As previously seen with Bax and mitochondrial COX (Fig. 1c, d), Bcl-2 and Bax co-immunolocalized in a punctuated pattern (Fig. 1e, f), indicating that overexpressed Bcl-2 encountered Bax on the mitochondria. Consistent with this, Bcl-2 and Bax co-immunoprecipitated from radiolabelled extracts of cells that stably overexpressed Bcl-2 and Bax[8]. At any time post-transfection, Bcl-2 coexpression increased the number of cells positive for punctuated, ectopic Bax

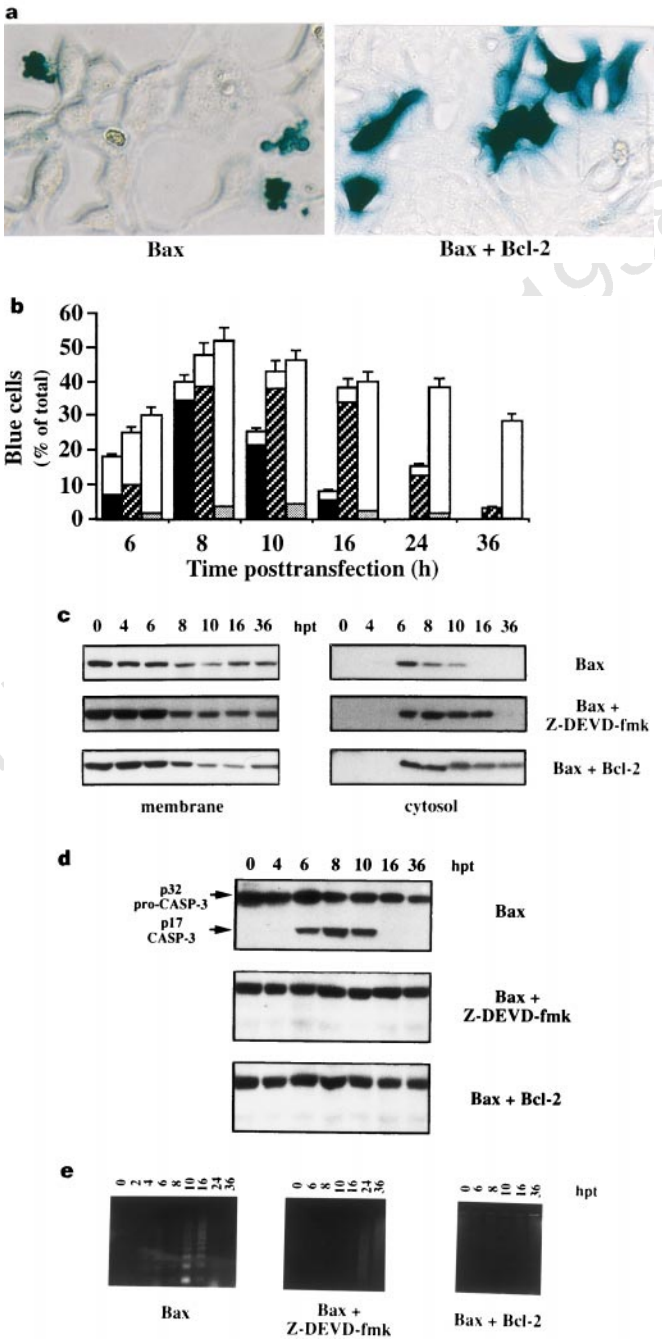

Figure 4 Apoptotic morphology, cytochrome *c* release, caspase-3 activation and DNA fragmentation in response to Bax or Bax/Bcl-2 expression in HEK 293T cells. At various times after transfection with *bax/β-gal* or *bax/bcl-2/β-gal* in the presence or absence of Z-DEVD-fmk, 293T cells were stained with X-Gal and counted, or disrupted and analysed for cytochrome *c* expression, caspase-3 activation and DNA fragmentation as described in Methods. **a**, Blue-stained, *bax*-transfected 293T cells are round and blebbing (left), whereas the respective *bax/bcl-2*-transfectants show an intact, elongated morphology (right). Pictures were taken at 10 h post-transfection. **b**, Quantification of blue cells with blebbing and with normal morphology. The percentage of blebbing blue cells per plate (out of total cells) are represented by filled bars (black for *bax* transfectants, hatched for *bax* transfectants with Z-DEVD-fmk, shaded for *bax/bcl-2* co-transfectants); results for the respective normal blue cells are represented by white bars. The data are means ± s.d. from 20 field countings of three independent transfections. **c**, Anti-cytochrome *c* immunoblot of cytosolic and membrane fractions. **d**, Anti-caspase-3 immunoblot of total protein. The positions of the inactive 32K pro-form and the active 17K form are indicated by arrows. **e**, DNA fragmentation analysis. hpt, Hours post-transfection.

(Fig. 2a), and the nuclei of these cells were not fragmented (Figs 2c, 3b). Some of the *bax/bcl-2* co-transfectants even survived for 36 h (Fig. 2a). Unexpectedly, however, Bcl-2 was incapable of blocking Bax-induced cytochrome *c* release because all cells overexpressing Bax displayed a diffuse immunostaining of cytochrome *c* (Figs 2b, 3b). Western blot analysis of extracts from HEK 293T cells co-transfected with *bcl-2* and *bax* confirmed that significant levels of cytochrome *c* remained in the cytoplasm for up to 36 h (Fig. 4c). Both caspase-3 activation and DNA fragmentation were blocked and only a few blebbing cells were detected after X-Gal staining of *bcl-2/bax/β-gal* triple transfectants at any time post-transfection (Fig. 4a–e). Even at 36 h post-transfection, we still noted blue adherent 293T cells with an intact, elongated morphology (Fig. 4b). Thus, Bcl-2 can act after cytochrome *c* release to prevent Bax-induced caspase-3 activation, nuclear fragmentation and cytotoxicity.

We have shown that Bax triggers the same sequence of cellular events as previously described for other apoptotic agents[1–4]: the release of cytochrome *c* from mitochondria into the cytoplasm, followed by the activation of death-effector caspases, nuclear fragmentation and apoptosis. However, two features are different: (1) Bax provokes a pathway to cell death not blocked by the caspase inhibitors Z-DEVD-fmk and Z-VAD-fmk[9,10], and (2) Bcl-2 seems to have a caspase-inhibiting activity downstream or aside from cytochrome *c* release. The latter may be due to an interaction of Bcl-2 with the cytochrome *c* receptor Apaf-1/CED-4 (ref. 11), a recently identified mediator of caspase-3 activation by cytochrome *c*. □

Methods

Transient transfection into R6 and SK2 cells and immunofluorescence analysis. 5×10^4 rat R6 embryo fibroblasts or SK2 human melonoma cells grown on glass coverslips were transfected with either 1 μg of each Bax/pcDNA3 and pcDNA3 or Bax/pcDNA3 and Bcl-2/pcDNA3 using 10 μg of Superfect (Qiagen) as described by the manufacturer. In some experiments, 50 μM of the caspase inhibitors Z-VAD-fmk or Z-DEVD-fmk (Bachem) were added at the beginning and at every 12 h post-transfection. At various times post-transfection, cells were fixed and permeabilized in 4% paraformaldehyde/0.05% saponin containing 4 μg ml^{-1} Hoechst 33342 dye (Molecular Probes) to visualize nuclei and DNA. Cells were then treated with hamster monoclonal anti-Bcl-2 3F11 (Pharmingen), rabbit polyclonal anti-Bax 06-499 (Upstate Biotechnology), mouse monoclonal anti-cytochrome *c* 6H2.B4 (Pharmingen) or mouse monoclonal anti-cytochrome *c* oxidase subunit VIc (COX) (Molecular Probes) antibodies, and then incubated with rhodamine- and fluorescein-conjugated secondary antibodies (Jackson Laboratories). The antifading agent SlowFade (Molecular Probes) was added and cells were viewed under a Zeiss Axiovert fluorescence microscope. Fluorescent cells were counted on 20 randomly selected fields (40–50 cells per field) under the microscope and the number of cells positive for punctuated Bax, diffusely stained (cytosolic) cytochrome *c*, and fragmented nuclei (per cent of total) were determined.

Transient transfection into HEK 293T cells and analysis by X-Gal staining. 3×10^5 human embryonic kidney (HEK) 293T cells grown on 35-mm plastic plates were transfected with either 1 μg each of *bax*/pcDNA3, *β-gal*/pcDNA3 and pcDNA3 or *bax*/pcDNA3, *β-gal*/pcDNA3 and *bcl-2*/pcDNA3 using 15 μg Superfect. After various times, cells were stained with 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (X-Gal) and examined by phase-contrast microscopy as described[7]. To determine the transfection efficiency, we counted trypsinized blue and white cells in a Neubauer chamber. To determine the extent of apoptosis, we counted blue cells that were round and blebbing on 20 randomly selected fields under a Nikon inverted microscope at a magnification of ×400 (phase contrast).

Protein extraction and western blotting. At 6–36 h post-transfection, HEK 293T cells were either directly lysed in a Tris/SDS buffer as described[12] (total extract) or disrupted by Dounce homogenization using buffer STE (10 mM HEPES, pH 7.4, 1 mM EDTA, 0.25 M sucrose, 2 μg ml^{-1} leupeptin, 1 mM PMSF, 1 μg ml^{-1} pepstatin), followed by ultracentrifugation to separate cytosol and membranes. Equal amounts of protein were subjected to immunoblotting using either mouse monoclonal anti-cytochrome *c* 7H8.2C12 (Pharmingen) or rabbit polyclonal anti-caspase-3 (gift from D. Nicholson[13]) antibodies, followed by horseradish peroxidase-coupled secondary antibodies (Jackson Laboratories) as described[12]. Immunodetection was by enhanced chemiluminescence (Pierce). Cytochrome *c* was quantified by densitometric scanning of autoradiographs.

DNA fragmentation. At 6–36 h post-transfection, HEK 293T cells of a 35-mm well were washed three times in phosphate-buffered saline and then immediately lysed in phenol:chloroform:isoamylalcohol (25:24:1). The aqueous phase containing nucleic acids was treated with RNase and loaded onto a 2% agarose gel containing ethidium bromide in order to visualize DNA fragmentation.

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The role of transferrin-receptor variation in the host range of *Trypanosoma brucei*

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***Trypanosoma brucei*[1] is a unicellular parasite transmitted between African mammals by tsetse flies. *T. brucei* multiplies freely in the bloodstream of many different mammals, and survives by antigenic variation of the main component of its surface coat, variant surface glycoprotein (VSG)[2,3]. Trypanosomes take up transferrin through a heterodimeric transferrin receptor[4–9], the genes for which are expressed in telomeric expression sites along with the VSG gene. There are up to 20 of these expression sites per trypanosome nucleus[3,10–15], but usually only one is active at a time. Different expression sites encode transferrin receptors that are similar but not identical[16]. Here we show that these small differences between transferrin receptors can have profound effects on the binding affinity for transferrins from different**

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2.5 ARTICLE : Apoptotic crosstalk between the endoplasmic reticulum and mitochondria controlled by Bcl-2

Häcki, J., Egger, L., Monney, L., Conus, S., Rossé, T., Fellay, I. And Borner, C. (2000) *Oncogene* **19**, 2286-2295.

Notice :

My contribution to this paper was the preparation and purification of the plasmid cytochrome b5/pcDNA3 (cb5/pcDNA3) used to generate the plasmid ER-targeted Bcl-2/cyt.b5 pcDNA3 and create stable rat 6 embryo fibroblasts cell lines (R6Bcl-2/cb5#6). With these cells, studies were performed to show that the cytochrome c release by Brefeldin A is not only blocked by wild-type Bcl-2, but also by this Bcl-2 variant which is exclusively targeted to the endoplasmic reticulum (Bcl-2/cb5).

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Apoptotic crosstalk between the endoplasmic reticulum and mitochondria controlled by Bcl-2

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Apoptosis involves mitochondrial steps such as the release of the apoptogenic factor cytochrome c which are effectively blocked by Bcl-2. Although Bcl-2 may have a direct action on the mitochondrial membrane, it also resides and functions on the endoplasmic reticulum (ER), and there is increasing evidence for a role of the ER in apoptosis regulation as well. Here we uncover a hitherto unrecognized, apoptotic crosstalk between the ER and mitochondria that is controlled by Bcl-2. After triggering massive ER dilation due to an inhibition of secretion, the drug brefeldin A (BFA) induces the release of cytochrome c from mitochondria in a caspase-8- and Bid-independent manner. This is followed by caspase-3 activation and DNA/nuclear fragmentation. Surprisingly, cytochrome c release by BFA is not only blocked by wild-type Bcl-2 but also by a Bcl-2 variant that is exclusively targeted to the ER (Bcl-2/cb5). Similar findings were obtained with tunicamycin, an agent interfering with N-linked glycosylations in the secretory system. Thus, apoptotic agents perturbing ER functions induce a novel crosstalk between the ER and mitochondria that can be interrupted by ER-based Bcl-2. *Oncogene* (2000) **19,** 2286–2295.

Keywords: apoptosis; cytochrome c; caspases; Bcl-2; ER stress; BFA

Introduction

Apoptosis involves a subset of effector caspase proteases (caspases-3,-6,-7) which dismantle the cell by cleaving structural proteins essential for maintaining nuclear and cytoplasmic architecture and enzymes essential for repair of damaged cell components (Nuñez *et al*., 1998; Stroh and Schulze-Osthoff, 1998). Activation of these caspases occurs via mitochondria-independent and -dependent mechanisms. In the former case, an adaptor protein such as FADD (Fas-associated death domain) clusters an initiator caspase such as caspase-8 so that the protease can autoactivate and cleave and activate effector caspases (Ashkenazi and Dixit, 1998; Kumar and Colussi, 1999). In the latter case, some unknown factors or a caspase-8-mediated cleavage and mitochondrial translocation of the Bcl-2 homolog Bid damage mitochondria to release cytochrome c into the cytoplasm (Green and Reed, 1998; Li *et al*., 1998; Luo *et al*., 1998). Cytochrome c then binds to cytoplasmic Apaf-1 which recruits and oligomerizes the initiator caspase-9 for autoactivation and subsequent cleavage and activiation of effector caspases (Hu *et al*., 1998b; Li *et al*., 1997; Srinivasula *et al*., 1998).

The activation of effector caspases can be blocked by anti-apoptotic members of the Bcl-2 family such as Bcl-2 or Bcl-x_L (Adams and Cory, 1998; Reed, 1998). Both proteins reside on the outer mitochondrial membrane where they prevent the release of cytochrome c in response to many apoptotic stimuli (Kharbanda *et al*., 1997; Kluck *et al*., 1997; Yang *et al*., 1997). The mechanism of this interference is still unknown. On one hand it was proposed that Bcl-2 and Bcl-x_L function as docking proteins (Reed, 1997), forming multiprotein complexes with apoptosis regulatory molecules including caspase activators such as Apaf-1 (Hu *et al*., 1998a; Pan *et al*., 1998) or components of the mitochondrial permeability transition pore such as adenine nucleotide translocator (ANT) or voltage-dependent anion channel (VDAC) (Marzo *et al*., 1998; Shimizu *et al*., 1999). On the other hand it was shown that Bcl-x_L is structurally homologous to pore-forming bacterial toxins (Muchmore *et al*., 1996) and can form ion channels on membranes *in vitro* (Minn *et al*., 1997; Schendel *et al*., 1998). Such an activity may maintain the ion homeostasis across the mitochondrial membranes and thus prevent major mitochondrial damage.

However, apart from mitochondria, Bcl-2 and Bcl-x_L associate with other cytoplasmic membranes, notably those of the nuclear envelope and the ER (Givol *et al*., 1994; Krajewski *et al*., 1993) raising the possibility that they also act at extramitochondrial sites. Indeed, it was recently reported that a Bcl-2 targeted to the ER via the C-terminus of cytochrome b5 was capable of blocking most, albeit not all types of apoptosis (Lee *et al*., 1999; Zhu *et al*., 1996). It has however remained enigmatic how Bcl-2 performs its survival function on the ER.

Studying ER abnormalities during apoptosis has clearly lagged behind that of mitochondria. The ER surveys the correct folding, glycosylation and sorting of the proteins in the secretory system (Aridor and Balch, 1999). When these processes are perturbed, two signaling pathways can be activated; the unfolded protein response (UPR) pathway leading to the induction of ER chaperones such as grp78/Bip via the C/EBP homologous transcription factor CHOP/Gadd153 (Chapman *et al*., 1998; Wang *et al*., 1996)

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and the ER overload response (EOR) pathway leading to the production of cytokines via the transcription factor NFκB (Pahl and Baeuerle, 1997). Both pathways help the cell to cope with incorrectly folded or accumulated proteins in the ER but may also contribute to its elimination when abnormalities become too extensive. Consistent with this idea, both CHOP/Gadd153 (Brenner *et al*., 1997; Zinszner *et al*., 1998) and NFκB (Baichwal and Baeuerle, 1997) have been implicated in apoptosis regulation. Moreover, several ER membrane proteins have been reported which interact with Bcl-2 family members such as Bax inhibitor I (Xu and Reed, 1998), Bap31 (Ng *et al*., 1997), calnexin (Torgler *et al*., 1997) and the calcium pump SERCA (Kuo *et al*., 1998) although the purpose of these interactions is yet unknown.

Even though we are getting a better picture of the role of ER perturbations in apoptosis induction, we do

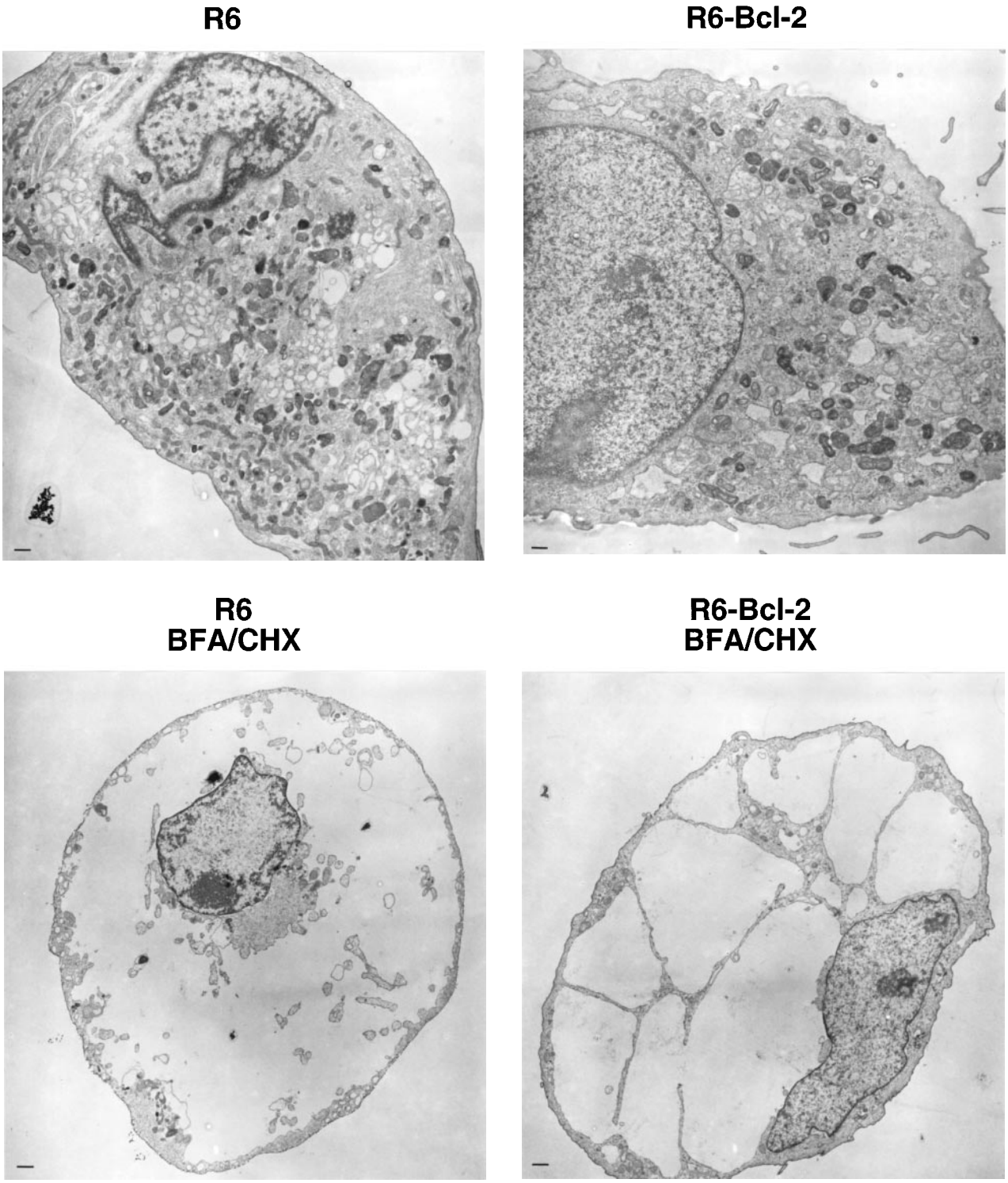

Figure 1 BFA/CHX provoked dramatic ER dilation. Electron microscopy imaging (magnification 5500 ×) of R6 and R6-Bcl-2#9 cells non-treated or treated with BFA/CHX for 6 h. Note that at this time the BFA/CHX-treated cells display a massively vacuolarized ER but no typical signs of apoptosis such as plasma membrane blebbing or nuclear condensation/fragmentation. Scale bar in the lower left corner corresponds to 1 μm

not yet know how they impinge on the apoptotic machinery. Here we apply two apoptotic stresses that produce aberrant protein in the secretory system; brefeldin A (BFA), which blocks retrograde transport of Golgi-derived vesicles, leading to a stop in secretion and the fusion of the Golgi/ER compartments (Chardin and McCormick, 1999; Fujiwara *et al*., 1998) and tunicamycin which inhibits the first reaction in the dolichol pathway of N-glycosylations of proteins in the ER lumen (Olden *et al*., 1982; Tkacz and Lampen, 1975). Interestingly, both stresses provoke a release of cytochrome c before or at the time of effector caspase-3 activation suggesting a crosstalk between the perturbed ER and mitochondria. This crosstalk does not involve caspase-8 mediated cleavage and/or mitochondrial translocation of Bid, but a yet unknown, caspase-independent mechanism. Moreover, Bcl-2 can interrupt this crosstalk at the level of the ER and does not need to be associated with mitochondria to fulfill its function.

Results

When applied to various cell types in different doses, BFA induced rapid cell death that was not clearly apoptotic and difficult to monitor biochemically (data not shown). This was probably due to the massive accumulation of aberrant proteins in the collapsed Golgi-ER hybrid compartment. We therefore diminished the amount of aberrant proteins by slowing down their synthesis and subsequent transport into the ER lumen using a low dose of cycloheximide (1 μg/ml) (CHX) in combination with BFA. This dose of CHX alone did not induce apoptosis within the time frame of our experiments (data not shown).

Electron microscopy analysis showed that while healthy, untreated rat embryo fibroblasts (R6 cells) contained only few and small vacuoles, their treatment with 5 μg/ml BFA and 1 μg/ml CHX led to a dramatic vacuolarization of the ER/Golgi network (Figure 1). This effect became visible between 4–6 h of the drug treatment, at a time when the cells were still viable (Figure 2a) and did not display typical signs of apoptosis such as nuclear condensation (Figure 1) or DNA fragmentation (Figure 2b). However, after 6 h of BFA/CHX-treatment, the cells gradually died (Figure 2a) and accumulated nucleosome-sized DNA fragments (Figure 2b). At 24 h posttreatment, up to 70% of the cells were shrunken and had condensed and/or fragmented nuclei (Figure 3a). In the presence of a fivefold excess of Bcl-2 (R6-Bcl-2#9), BFA/CHX still provoked massive ER dilation as compared to untreated cells (Figure 1), but cell shrinkage (Figure 3a) and DNA/nuclear fragmentation (Figures 2b and 3a) were effectively blocked, and cell survival was prolonged (Figure 2a). Similar findings were obtained when R6 cells were exposed to tunicamycin (Figure 2a and data not shown). These data show that ER damaging agents kill cells by apoptosis, and that Bcl-2 prevents the killing at a step distal to the damage.

To uncover how the perturbed ER communicates with the apoptotic machinery, we first determined the activation of the effector caspase-3. For that purpose, we subjected BFA/CHX-treated cells to immunofluorescence analysis using an antibody specific for the active p17 form of caspase-3. In addition, we prepared from these cells a cytosolic extract to detect p17 on

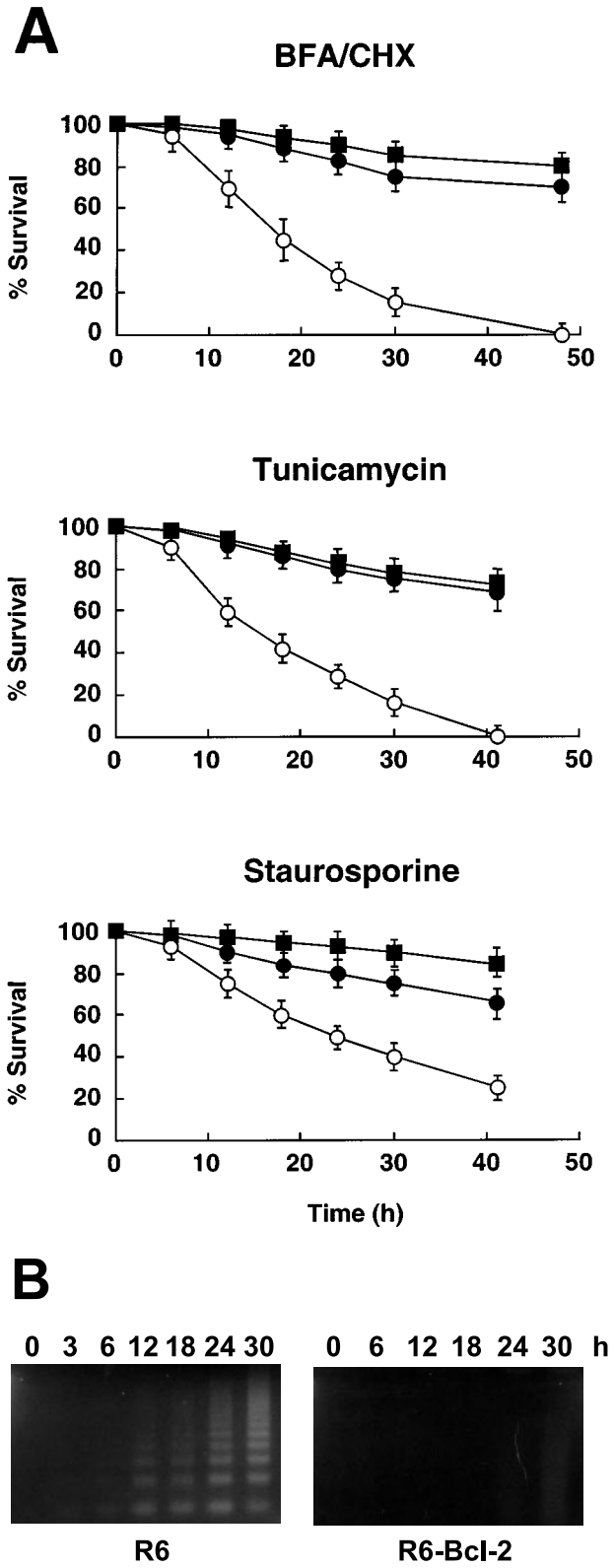

Figure 2 BFA/CHX, tunicamycin and staurosporine induce cell loss and DNA fragmentation blocked by wild-type and ER-targeted Bcl-2. (**a**) Survival of BFA/CHX-, tunicamycin- or staurosporine-treated R6 (open circles), R6-Bcl-2#9 (filled squares) and R6-Bcl-2/cb5#6 cells (filled circles) as determined by the Trypan blue assay. The data represents the microscopic counting of 60 Neubauer fields for each cell line and are the means of three independent experiments ± s.d. (**b**) Agarose gel analysis of genomic DNA from R6 and R6-Bcl-2#9 cells exposed to BFA/CHX for 0–30 h

Western blots and to measure the respective caspase activity with the fluorogenic substrate DEVD-AMC (Ac - Asp - Glu - Val - Asp-[7-amido-4-methylcoumarin]). While there was a low anti-p17 immunofluorescence

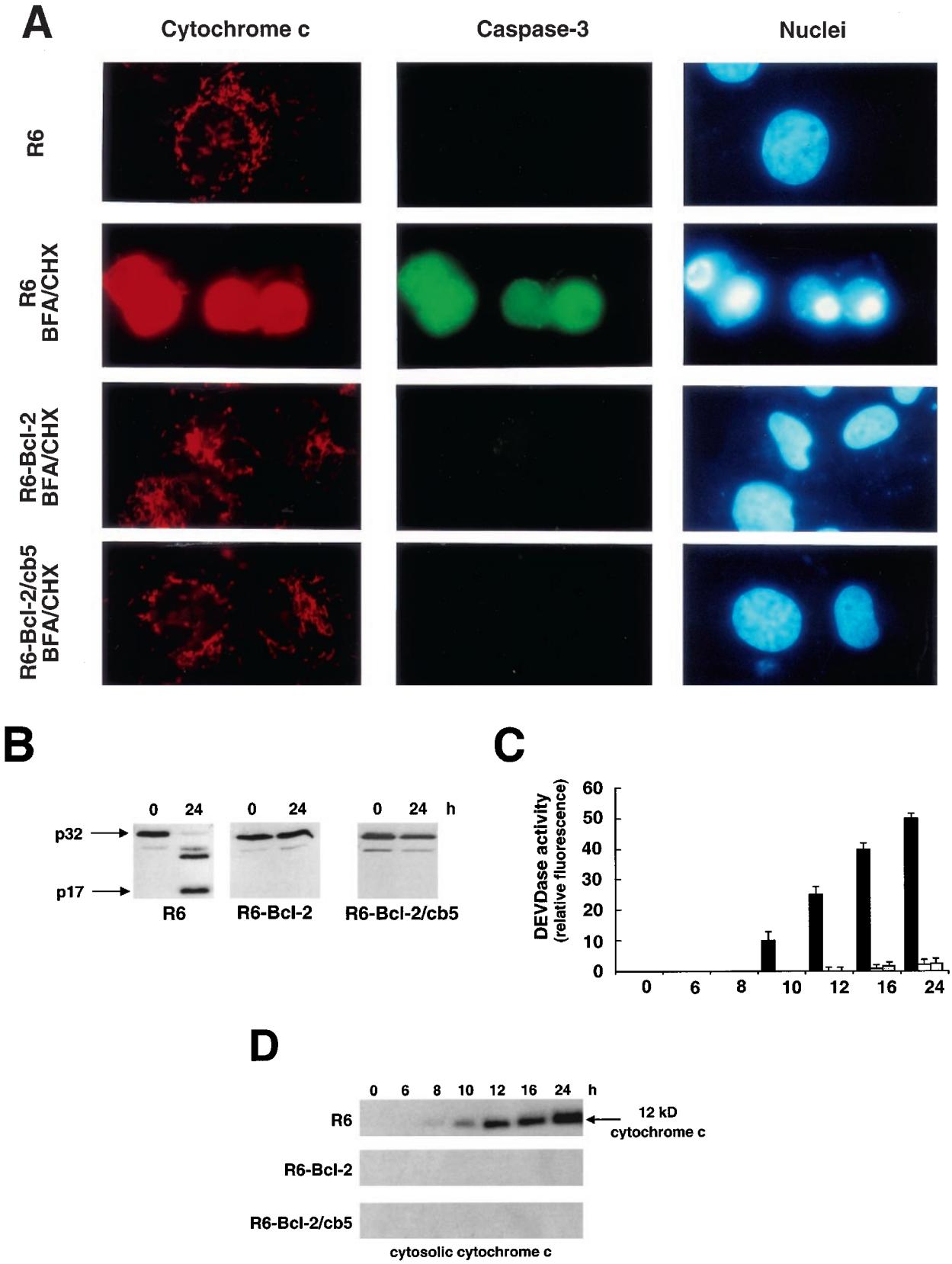

Figure 3 Apoptosis induced by BFA/CHX involves cytochrome c release and caspase-3 activation blocked by wild-type and ER-targeted Bcl-2. (**a**) Nuclear staining (Hoechst 33342, blue), anti-cytochrome c (red) and anti-active p17 caspase-3 (green) immunofluorescence analysis of fixed/permeabilized R6, R6-Bcl-2#9 and R6-Bcl-2/cb5#6 cells, untreated or treated with BFA/CHX for 24 h. (**b**) Anti-caspase-3 Western blot analysis of the cytosols of the cells described in (**a**). (**c**) Caspase-3 (DEVDase) activity in the cytosols of R6 (black bars), R6-Bcl-2#9 (open bars) and R6-Bcl-2/cb5#6 (gray bars) cells treated with BFA/CHX for 0–24 h. The data are the means of three independent experiments ± s.d. (**d**) Anti-cytochrome c (7H8.2C12) Western blot analysis of the cytosols of the various cell types exposed to BFA/CHX for 0–24 h

staining of untreated R6 cells, a 24 h treatment with BFA/CHX led to a strong diffuse staining indicating the presence of active p17 in the cytoplasm and probably the nucleus (Figure 3a). Moreover, the cytosolic extract of BFA/CHX-treated cells had almost all p32 caspase-3 precursor converted into the active p17 form (Figure 3b) and displayed a strong activity towards the DEVD-AMC substrate (Figure 3c). Importantly, both the formation of p17 caspase-3 (data not shown) and its associated DEVDase activity were not significantly detected before 10 h of BFA/CHX treatment (Figure 3c) indicating that they were a consequence rather than a cause of ER dilation. BFA/CHX-treated R6-Bcl-2#9 cells neither showed anti-p17 immunofluorescence staining (Figure 3a), nor the formation of the p17 protein (Figure 3b) or DEVDase activity (Figure 3c) in the cytoplasm at any time of the stress period. The treatment of R6 cells with tunicamycin resulted in a similar degree of caspase-3 activation that was fully blockable by Bcl-2 (Figure 4a). Thus, Bcl-2 protected cells from BFA/CHX- or tunicamycin-induced apoptosis at a step between the ER stress and caspase-3 activation.

We next tested whether BFA/CHX or tunicamycin employed the release of cytochrome c to activate caspase-3. In the absence of drug treatment, cytochrome c was immunodetected in elongated, 'spaghetti'-like-structures typical for mitochondria in adherent R6 cells (Figure 3a). This pattern changed to a strong, diffuse staining when the cells were treated with BFA/CHX indicating that cytochrome c was released to the cytoplasm (Figure 3a). Because cytochrome c release and caspase-3 activation occurred almost simultaneously, it was difficult to determine the exact order of events of apoptotic signaling. Western blot analysis confirmed that, in response to BFA/CHX, cytochrome c appeared in the cytoplasm almost at the same time as the generation of active p17 caspase-3 (data not shown) and its associated DEVDase activity (compare Figure 3c and d). A similar finding was obtained with tunicamycin (Figure 4a,b). These data suggest that cytochrome c release is a signaling event between the damaged ER and caspase-3 activation in BFA/CHX- and tunicamycin-induced apoptosis. As Bcl-2 associates with mitochondrial membranes and blocks the release of cytochrome c by many apoptotic agents, it was not surprising that R6-Bcl-2#9 cells had cytochrome c retained in a 'spaghetti'-like pattern (Figure 3a) and did not show detectable cytosolic cytochrome c on Western blots at any time point of the BFA/CHX or tunicamycin treatments (Figures 3d and 4b).

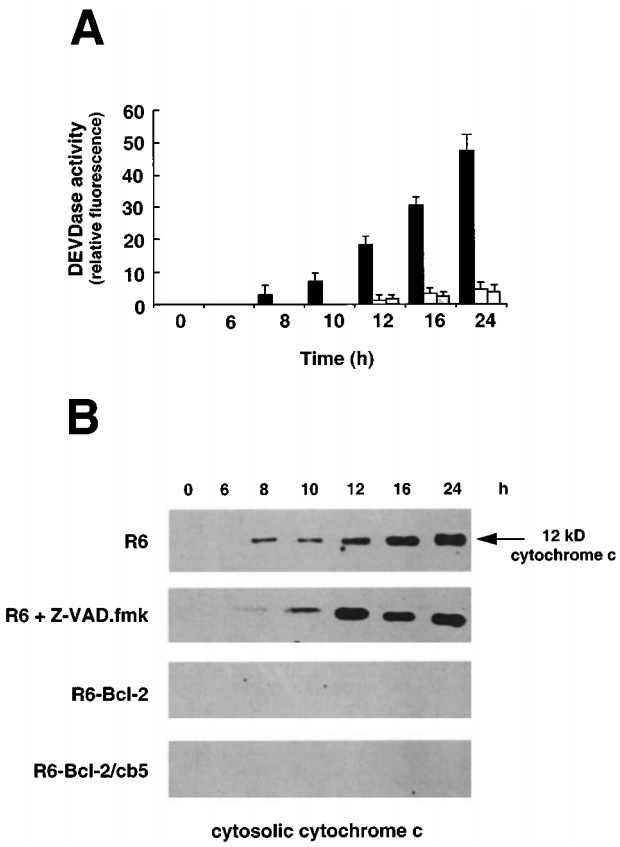

Figure 4 Tunicamycin provokes cytochrome c release and caspase-3 activation blocked by wild-type and ER-targeted Bcl-2. (**a**) Caspase-3 (DEVDase) activity and (**b**) anti-cytochrome c immunoblot analysis of the cytosols of R6 (black bars), R6-Bcl-2#9 (open bars) and R6-Bcl-2/cb5#6 (gray bars) cells treated with tunicamycin for 0–24 h. In certain cases, 100 μM Z-VAD.fmk was added at the same time as tunicamycin (R6+Z-VAD.fmk). The data in (**a**) are the means of three independent experiments ± s.d.

As Bcl-2 is widely distributed throughout the cell (Krajewski *et al.*, 1993) we wanted to know which subcellular form was responsible for interfering with cytochrome c release. Immunofluorescence analysis of R6-Bcl-2#9 cells revealed a strong immunostaining of Bcl-2 on the nuclear envelope and its associated endoplasmic network (Figure 5a). This staining was not changed when the cells were treated with BFA/CHX so that Bcl-2 stained around the vacuolarized ER (Figure 5a). To confirm the predominant association of Bcl-2 with the nuclear/ER network, we performed Western blots on subcellular fractions from the R6-Bcl-2#9 cell line. These fractions were up to 90% pure as determined by antibodies against organelle-specific marker proteins (lamin B1 for nuclei, cytochrome c oxidase (COX) for mitochondria, calnexin for ER and Lamp-1 for lysosomes) (Figure 5b). As shown in Figure 5b, most of the Bcl-2 was found in nuclear and microsomal/ER fractions although it was also present on mitochondria and other membranes. Again, the treatment of R6-Bcl-2#9 cells with BFA/CHX did not much change this subcellular distribution of Bcl-2 (Figure 5b). To see whether ER-bound Bcl-2 could interfere with cytochrome c release and caspase-3 activation, we generated a version of Bcl-2 that had its C-terminus exchanged for that of cytochrome b5. Such a Bcl-2/cb5 chimera had previously been shown to target the ER membrane and to block apoptosis in certain, but not all cells (Lee *et al.*, 1999; Zhu *et al.*, 1996). A stable R6 cell line overexpressing Bcl-2/cb5 (R6-Bcl-2/cb5#6) showed the same nuclear/ER immunofluorescence staining as R6-Bcl-2#9 cells (Figure 5a). However, the chimeric protein was absent from mitochondria and exclusively present in nuclear and microsomal/ER fractions (Figure 5b). When compared to wild-type Bcl-2, Bcl-2/cb5 was as effective in interfering with BFA/CHX- or tunicamycin-induced caspase-3 activation (Figures 3a–c and 4a), cell shrinkage and nuclear condensation/fragmentation (Figure 3a) and cell death (Figure 2a). This could be shown for multiple cell lines of the same genotype

(data not shown). Surprisingly, Bcl-2/cb5 did also fully impede BFA/CHX- or tunicamycin-induced cytochrome c release. Neither by immunofluorescence (Figure 3a) nor by Western blotting (Figures 3d and 4b) did we see any appearance of cytosolic cytochrome c in R6-Bcl-2/cb5#6 cells treated with these ER damaging agents. These findings suggest that Bcl-2 can function on the ER to inhibit an ER damage-induced mitochondrial event. Interestingly, the death-protective effect of Bcl-2/cb5 was not limited to ER stress agents but also evident (although less pronounced) for apoptotic stimuli such as staurosporine that are known to act through mitochondrial damage (Figure 2a) (Kluck *et al*., 1997).

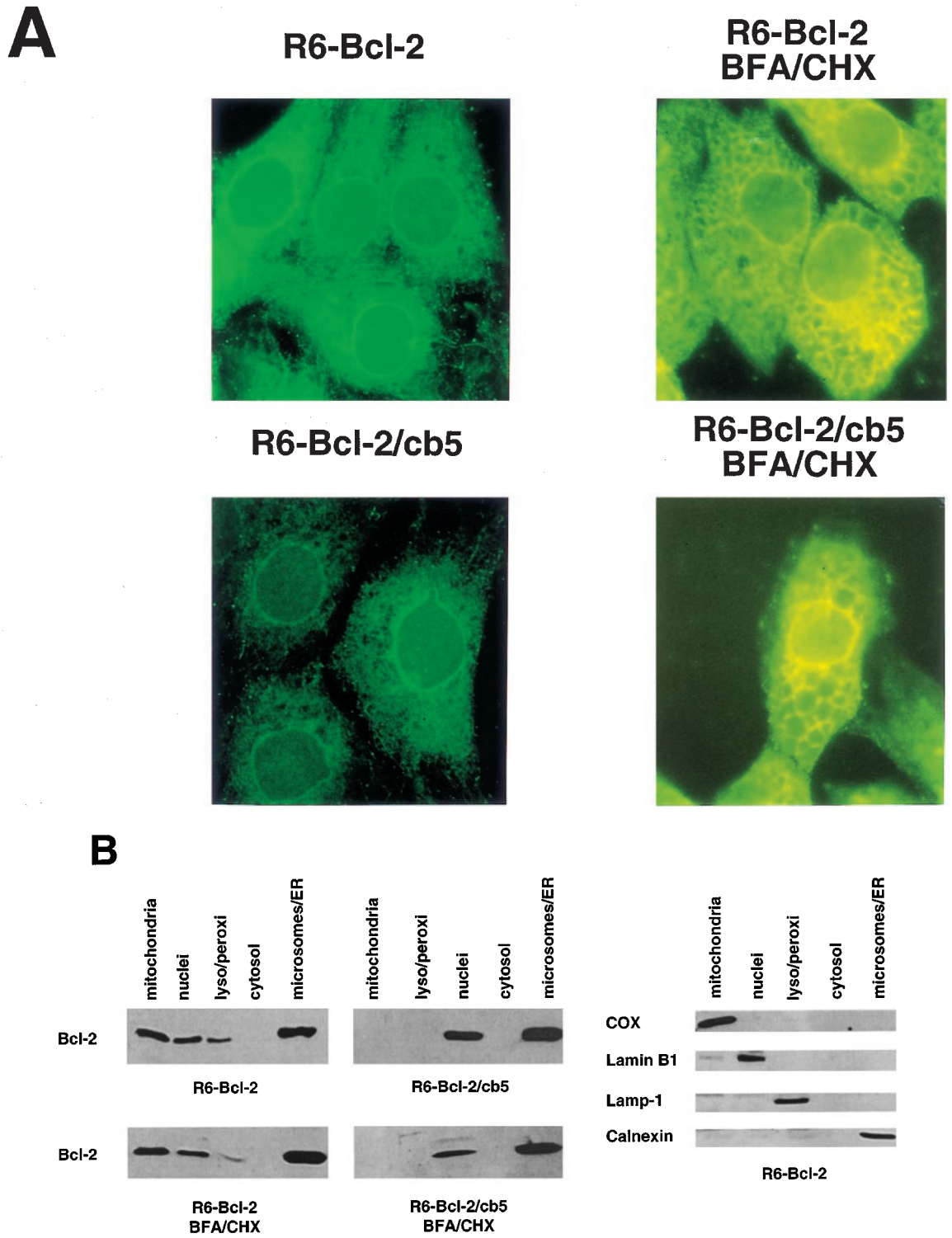

Figure 5 Subcellular localization of wild-type Bcl-2 and ER-targeted Bcl-2/cb5 in untreated and BFA/CHX-treated R6 cells. (**a**) Anti-Bcl-2 (27-6) immunofluorescence analysis of fixed/permeabilized R6-Bcl-2#9 and R6-Bcl-2/cb5#6 cells before and after the treatment with BFA/CHX for 24 h. (**b**) Anti-Bcl-2 (27-6) immunoblot analysis of subcellular fractions from untreated and BFA/CHX-treated R6-Bcl-2#9 and R6-Bcl-2/cb5#6 cells. The fractions were prepared by sucrose gradient centrifugation and tested for their purity using antibodies against COX (mitochondria), lamin B1 (nuclei), lamp-1 (lysosomes) or calnexin (ER/microsomes) as described in Materials and methods

It has been previously shown that cytochrome c release may be triggered by the activation of the initiator caspase-8 which cleaves the Bcl-2 family member Bid to allow its translocation from the cytoplasm to the mitochondrial membrane (Li *et al*., 1998; Luo *et al*., 1998). We therefore examined whether BFA/CHX or tunicamycin used such a pathway to crosstalk with mitochondria. A treatment of JILY B lymphoblasts with anti-CD95 antibodies for 6 h led to a significant increase in cytosolic caspase-8 activity (IETDase activity) (Figure 6a) as well as the cleavage of p24 Bid into its 15 kD fragment and the translocation of both proteins to the mitochondrial membrane (Figure 6b). However, neither BFA/CHX nor tunicamycin provoked any major activation of caspase-8 (Figure 6a) or proteolysis/mitochondrial translocation of Bid (Figure 6b) over a time period of 24 h in R6 cells. Moreover, cytochrome c release induced by both BFA/CHX (data not shown) or tunicamycin (Figure 4b) was not blocked in the presence of the general caspase inhibitor Z-VAD.fmk (Z-Val-Ala-Asp.fluoromethylketone) (data not shown) indicating that none of the known caspases were involved in conveying stress signals from the damaged ER to mitochondria. Thus, the ER-mitochondria crosstalk in response to BFA/CHX or tunicamycin involves a novel, caspase-independent signaling pathway.

Discussion

How can a damage in the ER compartment be translated into an apoptotic response that involves the release of cytochrome c from mitochondria? As the caspase-8/Bid-mediated pathway does not seem to play a role, we propose alternative ways of crosstalks. One possibility is that the ER and mitochondria communicate via membranes as they can be in close contact and easily exchange lipids, calcium and glycoproteins (Chandra *et al*., 1998; Rizzuto *et al*., 1998). A possible mediator may be the sphingolipid ceramide which is generated by sphingomyelin hydrolysis in response to various apoptotic stimuli (Kolesnick and Krönke, 1998), including BFA (Linardic *et al*., 1996) and has recently been shown to directly act on mitochondria to stimulate cytochrome c release (Ghafourifar *et al*., 1999). We have found that BFA/CHX indeed provoked ceramide production in R6 cells that preceded cytochrome c release (data not shown), but due to the lack of appropriate agents that block ceramide signaling we cannot yet say whether this lipid is required for BFA-induced apoptosis. In addition, it has been disputed whether ceramide plays any role in apoptosis induction (Hofmann and Dixit, 1999). Alternatively, an ER-mitochondrial crosstalk could be mediated via yet unidentified proteins that are either released from the damaged ER lumen due to an

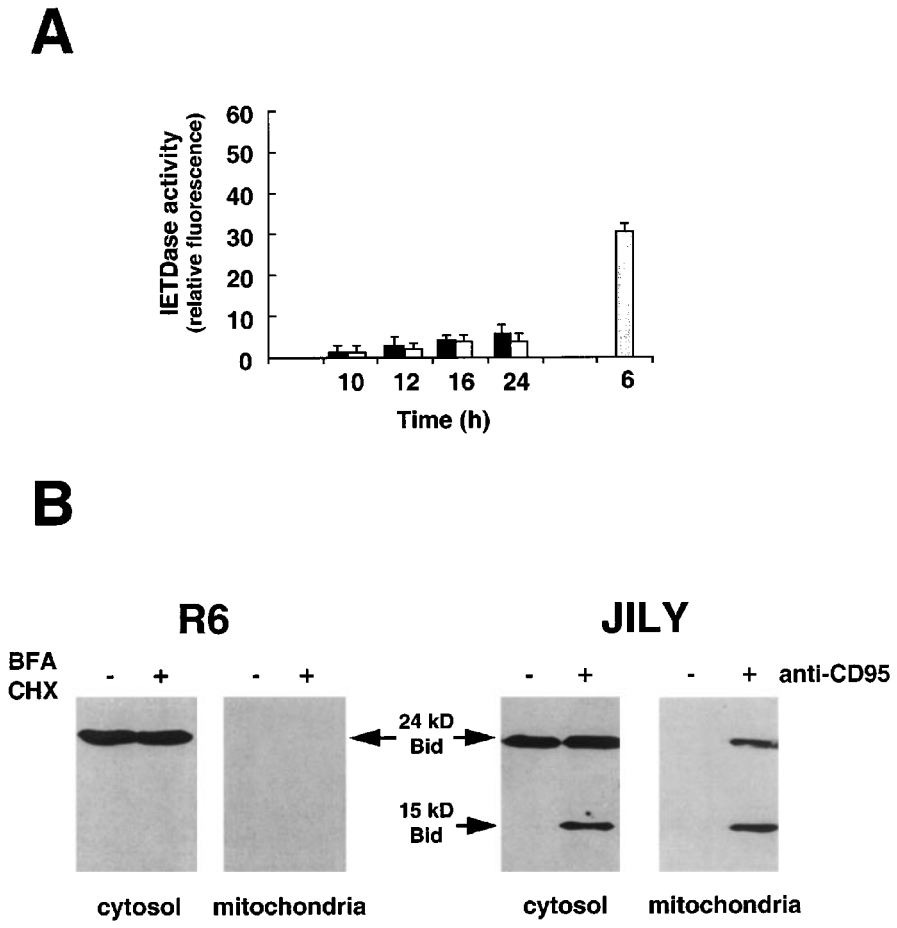

Figure 6 Neither BFA/CHX nor tunicamycin activate caspase-8 and the cleavage and mitochondrial translocation of Bid. (**a**) Caspase-8 (IETDase) activity of the cytosols of R6 cells treated with BFA/CHX (black bars) or tunicamycin (open bars) for 0–24 h or of the cytosol of JILY B lymphoblasts treated with anti-CD95 for 6 h (grey bar). The data are the means of three independent experiments ± S.D. (**b**) Anti-Bid Western blot analysis of cytosolic and mitochondrial fractions of R6 cells treated with BFA/CHX for 24 h and JILY cells treated with anti-CD95 for 6 h. Mitochondria were prepared by sucrose gradient centrifugation as described in Materials and methods

increased membrane permeability or cleaved from the ER membrane by a caspase-independent mechanism. Finally, such a crosstalk may involve components of the unfolded protein (UPR) and ER overload (EOR) response pathways (Pahl and Baeuerle, 1995). The transcription factor CHOP/Gadd153, a component of the UPR pathway, has been shown to mediate apoptosis triggered by tunicamycin (Brenner *et al*., 1997; Olden *et al*., 1982; Zinszner *et al*., 1998). CHOP-regulated genes relevant for apoptosis regulation have not yet been identified but may include pro-apoptotic molecules such as the Bcl-2 family member Bax, a known trigger of cytochrome c release on mitochondria (Rossé *et al*., 1998). On the other hand, it is known that activation of the transcription factor NF-κB via the EOR involves reactive oxygen species (ROS) which are produced from the oxidizing environment of the ER lumen and the action of cytochrome p450 on the ER membrane (Pahl and Baeuerle, 1997; Schreck *et al*., 1992). ROS can mount an oxidative stress that damages membranes, including those of mitochondria (Kowaltowski and Vercesi, 1999). Indeed, preliminary data from our laboratory show that antioxidants such as N-acetylcysteine or pyrrolidine dithiocarbamate (PDTC) can partially protect R6 cells from BFA/CHX-induced cytochrome c release and apoptosis (data not shown).

It has previously been reported that Bcl-2 prevents BFA-induced apoptosis (Guo *et al*., 1998; Olivier *et al*., 1997), but its mode and intracellular site of action have not yet been unveiled. Here we show that BFA or tunicamycin induces mitochondrial cytochrome c release and caspase-3 activation which are both blocked by a version of Bcl-2 targeted to the ER. How could Bcl-2 control mitochondrial events from the ER membrane? Again, the simplest explanation would have been that Bcl-2 sequesters Bid away from its mitochondrial action as the two proteins are known to interact. However, based on our results here, other processes are likely to occur. First, Bcl-2 may bind to and/or neutralize potentially pro-apoptotic ER components such as Bap31 (Ng *et al*., 1997) or yet unknown ceramide targets. Second, Bcl-2 may interfere with the generation or action of ROS as it has been shown to function as an antioxidant (Hockenberry *et al*., 1993). Third, Bcl-2 may modulate ER membrane permeability by either forming an ion channel or regulating ER-based channels or pumps such as the IP_3-receptor calcium channel or the Ca^{2+} ATPase pump (SERCA) (Berridge *et al*., 1998). Bcl-2 can interact with SERCA and either maintain calcium uptake into the ER or reduce calcium efflux of the ER in cells treated with the SERCA inhibitor thapsigargin (Kuo *et al*., 1998; He *et al*., 1997). Finally, we propose that Bcl-2 may act on any intracellular membrane to sequester yet unknown cytoplasmic activators of cytochrome c release. Thus, a Bcl-2 targeted to lysosomal or plasma membranes should retain such an activity, a possibility which we are currently testing. The present study however already shows that Bcl-2 does not need to associate with mitochondria to exert a cytochrome c release inhibiting function. Thus, it cannot be the sole role of Bcl-2 to bind and regulate mitochondria-specific proteins such as VDAC (Shimizu *et al*., 1999) or ANT (Marzo *et al*., 1998) or to directly form ion channels in the mitochondrial membrane as recently proposed (Schendel *et al*., 1997). Our cellular system using BFA/CHX or tunicamycin and ER-targeted Bcl-2 will help us to isolate other targets of Bcl-2 and delineate a novel ER/mitochondrial crosstalk pathway for apoptosis induction.

Materials and methods

Cell lines and treatments

Rat 6 (R6) embryo fibroblasts expressing the retroviral vector pMV12 (controls) or the vector containing mouse Bcl-2 (R6-Bcl-2#9) were generated and cultured as previously described (Borner, 1996). R6 derivatives expressing the ER-targeted Bcl-2/cb5 were produced by transfecting the Bcl-2/cb5/pcDNA3 plasmid in the presence of Superfect (Qiagen) followed by antibiotic selection on 400 μg/ml geneticin (G418) (Life Technologies). A clone expressing Bcl-2/cb5 to similar levels as wild-type Bcl-2 in R6-Bcl-2#9 was used for further studies (R6-Bcl-2/cb5#6). The various cell lines were either treated with 5 μg/ml BFA and 1 μg/ml CHX or 5 μg/ml tunicamycin (all Sigma) or 250 nM staurosporine in the presence or absence of 100 μM of the general caspase inhibitor ZVAD.fmk (Bachem). Human JILY B lymphoblasts were cultured as described (Borner, 1996) and exposed to 100 ng/ml anti-CD95 antibody (anti-APO-1, IgG3, kindly provided by P Krammer, Heidelberg, Germany) where indicated.

Generation of ER-targeted Bcl-2/cb5

To create the Bcl-2/cb5 fusion gene, we amplified by polymerase chain reaction (PCR) the DNA fragment corresponding to the hydrophobic C-terminus of rat cytochrome b5 (aa 100–134) (cDNA kindly provided by Stephen Sligar, Urbana, USA and Nica Borgese, Milano, Italy) by using a 5′ primer with an introduced *Sph*I site (for subcloning into the site corresponding to Met203 of mouse Bcl-2) and a 3′ PCR primer with an introduced *Xba*I site (for subcloning into the multiple cloning site of Bcl-2/pcDNA3). The amplified fragment was purified by Jetsorb (Genomed), cut with *Sph*I and *Xba*I and ligated into the corresponding sites of Bcl-2/pcDNA3 yielding the Bcl-2/cb5/pcDNA3 plasmid. The corresponding ligation product was tested for the correct reading frame by DNA sequencing (Microsynth).

Survival/apoptosis assays

To estimate cell survival, the various cells were trypsinized and mixed with 0.2% (w/v) of the Trypan blue dye. Cells were counted on 20 fields in a Neubauer chamber and the proportion of white (live) to total cells determined. The experiments were repeated at least three times. Genomic DNA fragmentation was tested on 2% agarose gels as previously described (Rossé *et al*., 1998).

Subcellular fractionation

The various R6 cell derivatives were washed twice in PBS and once in extraction buffer STE (10 mM HEPES, pH 7.4, 1 mM EDTA, 0.25 M sucrose, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 1 μg/ml pepstatin) followed by scraping into STE and lysing in a tight-fitting Dounce homogenizer with 20–30 strokes. The extract was inspected under the microscope and only processed further when 90% of the cells were lysed. The homogenate was then applied to a linear gradient of sucrose (0.73–1.9 M) and centrifuged at 20 000 *g* for 1 h. The banded organelles were recovered by syringe, diluted with an equal volume of 10 mM HEPES buffer and sedimented by centrifugation at the speed appropriate for the respective organelles. The purity of the organelles was determined on Western blots using antibodies against specific

organelle markers (lamin B1 for nuclei, COX for mitochondria, lamp-1 for lysosomes/peroxisomes, calnexin for microsomes/ER). For the preparation of cytosols (anti-cytochrome c and anti-caspase-3 immunoblots) the homogenate was precentrifuged at 750 *g* for 10 min to remove nuclei and unbroken cells followed by a centrifugation at 100 000 *g* for 1 h to clear off the membranes.

Western blotting

30–50 μg of protein was subjected to 12–15% SDS–PAGE followed by blotting to a PVDF membrane as previously described (Borner *et al*., 1994). The membrane was blocked in 3% milk and incubated with either mouse monoclonal anti-cytochrome c (1 : 1000) (clone 7H8.2C12, Pharmingen), rabbit polyclonal anti-caspase-3 (1 : 10 000) (generous gift of D Nicholson, Merck-Frosst, Quebec, Canada), rabbit polyclonal anti-Bcl-2 (27-6, 1 : 5000) (Borner *et al*., 1994), mouse monoclonal anti-cytochrome c oxidase subunit VIc (COX) (1 : 1000) (Molecular Probes), mouse monoclonal anti-lamin B1, clone L-5 (1 : 1000) (Zymed), mouse monoclonal anti-rat Lamp-1 (1 : 5000) (generous gift of W Hunziker, University of Lausanne, Switzerland), mouse monoclonal anti-calnexin (1 : 2000) (Transduction Laboratories), or goat polyclonal anti-Bid (1 : 1000) (R&D Systems) followed by peroxidase-coupled anti-mouse, anti-goat or anti-rabbit secondary antibodies (1 : 5000) (Jackson Laboratories) and detection by enhanced chemiluminescence (Socochim, Pierce).

Caspase assays

Caspase-3 and caspase-8 activities were measured in the cytosol using the fluorogenic peptide substrates DEVD-AMC and IETD-AMC, respectively. Briefly, cytosol was prepared from untreated and BFA/CHX- or tunicamycin treated R6 cell derivatives by four cycles of freeze-thawing (−80°/25°C) in buffer A (25 mM HEPES/KOH, pH 7.4, 2 mM EGTA, 2 mM $MgCl_2$, 2 mM DTT, 5 μg/ml cytochalasin B, 100 μM PMSF, 25 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin) followed by ultracentrifugation at 100 000 *g*. 10 μl of cytosol (100–300 μg protein) was mixed with 90 μl of assay buffer (100 mM HEPES, pH 7.5, 2 mM DTT) and 1 μl of a 6 mM solution of the DEVD-AMC or IETD-AMC peptide substrates (Alexis). The specific liberation of AMC from these peptides was monitored during 30 min in black-coated 96 well microtiter plates (Nunc) using a Lambda fluoro FL600 microplate fluorescence reader (MWG Biotech) and 360-nm excitation and 460-nm emission wavelengths. Quantitation was performed using the KC-4 program developed by MWG Biotech.

Immunofluorescence analysis

A modified protocol described by Rossé *et al*. (1998) was used. Briefly, the cells were seeded onto 12 mm glass coverslips at a denstiy of 2×10^4 cells/ml. On the next day, the cells were exposed to 5 μg/ml BFA and 1 μg/ml CHX and kept for 24 h at 37°C. The coverslips were removed from the medium without washing (in order not to lose slightly detached cells) and directly placed in 4% paraformaldehyde for 10 min (fixation), followed by washing in PBS and treatments with 0.05% saponin for 5 min and acetone for 10 min (permeabilization). After washing in PBS the cells were incubated with mouse monoclonal anti-cytochrome c (clone 6H2.B4, 1 : 50), rabbit polyclonal anti-active p17 caspase-3 antibodies (1 : 1000) (both Pharmingen), or polyclonal anti-Bcl-2 (27-6, 1 : 200) for 90 min followed by secondary Texas-Red conjugated goat anti-mouse and fluorescein-conjugated goat anti-rabbit antibodies (both 1 : 75) (Jackson Laboratories). The cells were then postfixed for 10 min in 4% paraformaldehyde containing 2 μg/ml of the Hoechst 33342 stain (Molecular Probes), washed and treated with Slow Fade (Molecular Probes) to prevent fading. The coverslips were viewed under an Axiovert fluorescence microscope (Zeiss) and pictures taken with a Contax 167 MT camera at magnification of 1000×.

Electron microscopy

Adherent R6 cells were washed in phosphate buffer (0.1 M phosphate, pH 7.2), fixed in 2.5% glutaraldehyde at 4°C for 30 min and postfixed in 1% OsO_4 at room temperature for 20 min. The cells were then dehydrated with increasing concentrations of alcohol (30, 50, 70% plus 1% uranylacetate, 80, 95, 100%), embedded in a mixture of Epon/alcohol (1 : 3 for 15 min, 1 : 2 for 30 min, 3 : 1 for 30 min and pure Epon for 2×30 min) and polymerized at 60°C for 2 days. The solidified blocks were cut into slices of 50–60 nm thickness. These slices were contrasted in 5% uranylacetate as well as in Reynolds solution (80 mM $Pb(NO_3)_2$, 120 mM sodium citrate, 160 mM NaOH) and then viewed under the electron microscope.

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2.6 ADDITIONAL RESULTS : The cloning of new survival factors.

2.6.1 Materials and Methods

Reagents

Geneticin (G418) was purchased from Life Technologies, hygromycin B from Juro Supply/Calbiochem and DNase-free RNase from Boehringer Mannheim. Staurosporine (dissolved in DMSO) was kindly provided by Novartis and MG132 (dissolved in DMSO) was a gift from Proscript Inc. (formerly Myogenics Inc.).

cDNAs

The cDNA for human Bcl-2 (hBcl-2) was subcloned into the E*coRI* site of the pcDNA3 vector (Invitrogen) as previously described (Borner *et al.*, 1994). The premade cDNA phagemid library from a human Burkitt Lymphoma RNA source (Invitrogen; primary recombinants: 2.9×10^6 colonies; premade amplified titer: 3.3×10^7 colonies/µl) was uni-directionally subcloned between the B*stXI* and N*otI* sites into the multiple cloning site of the pcDNA3 vector (Invitrogen) as described by the manufacturer.

Cells

Vector control (pMV12) and Bcl-2-overexpressing (Bcl-2) rat 6 embryo fibroblasts (R6) were generated by retroviral transduction of the pMV12hygro plasmid lacking or containing the murine Bcl-2 cDNA. Construction of the viruses was exactly as described (Borner *et al.*, 1994). Following infection, cells were selected on 200 µg/ml hygromycin for 10 days. Resistant clones were picked, expanded into cell lines and checked for Bcl-2 expression by anti-Bcl-2 Western blot analyses. R6Bcl-2#9, expressing high level of murine Bcl-2, as well as the vector control cell line R6pMV12, were used for further analyses.
R6pMV12 and R6Bcl-2#9 cells were grown in Dulbecco's modified Eagle medium containing 5% fetal calf serum (FCS) and low amounts (50 µg/ml) of hygromycin to maintain Bcl-2 expression.

Transfection Efficiency Assay by X-Gal Staining

3×10^5 R6pMV12 cells grown on 35-mm plastic plates were transfected with either 10 µg or 5 µg of β-Gal/pcDNA3 using 12.5 µl, 25 µl or 50 µl of Superfect (Qiagen) or Fugene6 (Boehringer Mannheim) as described by the two manufacturers. At 24 h post-transfection, cells were stained with 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (X-Gal). To determine the transfection efficiency, trypsinized blue cells were counted in a Neubauer chamber on 20 randomly selected fields under a Nikon inverted microscope at a magnification of x1000 (phase contrast). The results are the means of three independent experiments.

Cell Viability Assay

Cells (R6pMV12 and R6Bcl-2#9) were seeded at 2×10^5 in triplicate into 35-mm wells. The next day, the cells were treated with 100 nM staurosporine, 500 nM staurosporine or 500 nM MG132 to induce apoptosis. After 24-72 h, viable cells were counted on triplicate plates using the trypan blue exclusion assay. The results are the means of three independent experiments.

Titering of the premade cDNA phagemid library

To verify the titer of the pre-amplified library given by the manufacturer, a small portion of bacteria were scraped from the top of the tube without thawing them. Bacteria were transferred to a sterile eppendorf tube and serial dilutions of 10^3, 10^6, 10^7, $3x10^7$, 10^8 in LB or LB-Amp (75 µg/ml ampicillin) media were prepared. 100 µl of each dilution was plated onto 100 mm agar plates containing or lacking the ampicillin (75 µg/ml) antibiotic and the plates were incubated overnight at 37 °C. The colonies on each plate were counted and the titer was calculated by multiplying the number of colonies by the dilution factor. The titer was adjusted by taking the means of three independent experiments giving $6.3x10^6$ colonies/µl as a new premade amplified titer.

Preparation of pure plasmidial DNA from the premade cDNA phagemid library

To respect a ratio of 1:6 between primary recombinants and total colonies (6 fold security that all recombinants are present), 2 µl of the cDNA phagemid library infected in E. coli was diluted in 20 ml of LB-Amp (75 µg/ml) medium and grown overnight at 37 °C. 20 x 250 µl of this latter solution were plated individually onto 150 mm agar plates containing 75 µg/ml ampicillin as antibiotic and the 20 plates were incubated overnight at 37 °C. The colonies of each plate were scraped, put into 30 ml of LB-Amp (75 µg/ml ampicillin) medium and grown overnight at 37 °C until a constant OD^{599} of about 1.5. DNA was separately isolated and purified following the manufacturer's protocol (Macherey-Nagel) giving 20 pure DNA pools ready to be screened for the presence of putative survival factors.

Transfection, selection and drug treatment into eukaryotic cells

R6pMV12 cells were grown on 100 mm plates until 60-70% confluency and transfected with 10 µg of the pcDNA3 vector, the hBcl-2/pcDNA3 plasmid (positive control) or the 12th purified DNA pool using 25 µl of Superfect (Qiagen) as described by the manufacturer. After 3 h, the Superfect-DNA complex was removed and cells were cultured for another 45 h in fresh medium. At 48 h post-transfection, cells were trypsinized, transferred on 150 mm plates and an antibiotitc selection on 200 µg/ml geneticin (G418) was applied. As negative control, a plate containing non-transfected R6pMV12 cells was used. As soon as this negative control plate showed no more cells, cells on the other plates were trypsinized and grown on another 100 mm plate with fresh DMEM, in the presence of 200 µg/ml geneticin (G418), until 80-90% confluence so as to avoid the formation of clusters. Then, cells were treated either with 500 nM or 100 nM staurosporine until all R6pMV12 cells transfected with the pcDNA3 vector died (3 days). Finally, the stressed medium was removed and cells were grown in DMEM containing 200 µg/ml G418 until 100% confluence.

DNA isolation, PCR amplification and subcloning into pcDNA3

After reaching 100% confluency, cells were washed twice in ice-cold phosphate-buffered saline (PBS) and lysed in 1 ml of a solution containing 0.6% sodium-dodecylsulfate (SDS) and 10mM EDTA with a pH of 7.5. After an incubation of 10 minutes at room temperature (RT), the lysates were scrapped and kept on ice in separate 4 ml polyallomer tubes. 250 µl of 5 M NaCl was added and each tube was mixed by inverting slowly 10 times. These mixtures were incubated overnight at 4 °C on ice and centrifuged at 100'000 g and 4 °C for 30 minutes. Genomic/extrachromosomal DNA was extracted from the supernatant. To rescue the

transfected cDNA encoding a putative survival factor, the insert region of the pcDNA3 DNA was amplified by polymerase chain reaction (PCR) using primers annealing to the 5' and 3' sites of the insert/multiple cloning site (MCS) region. The same primers were used to anneal just before and after the E*coRI* site used to create the hBcl-2/pcDNA3 plasmid which served as the positive control. The amplified DNA fragments were all together purified by JetSorb (Genomed), cut with B*stXI*/N*otI* and ligated into the corresponding sites of the pcDNA3 vector.

2.6.2 Principle of the method

For a better understanding, the text written into the different boxes correlates with the chapters described in the previous "Materials and Methods" section so as to find directly the protocol used for each step (Figure 11).

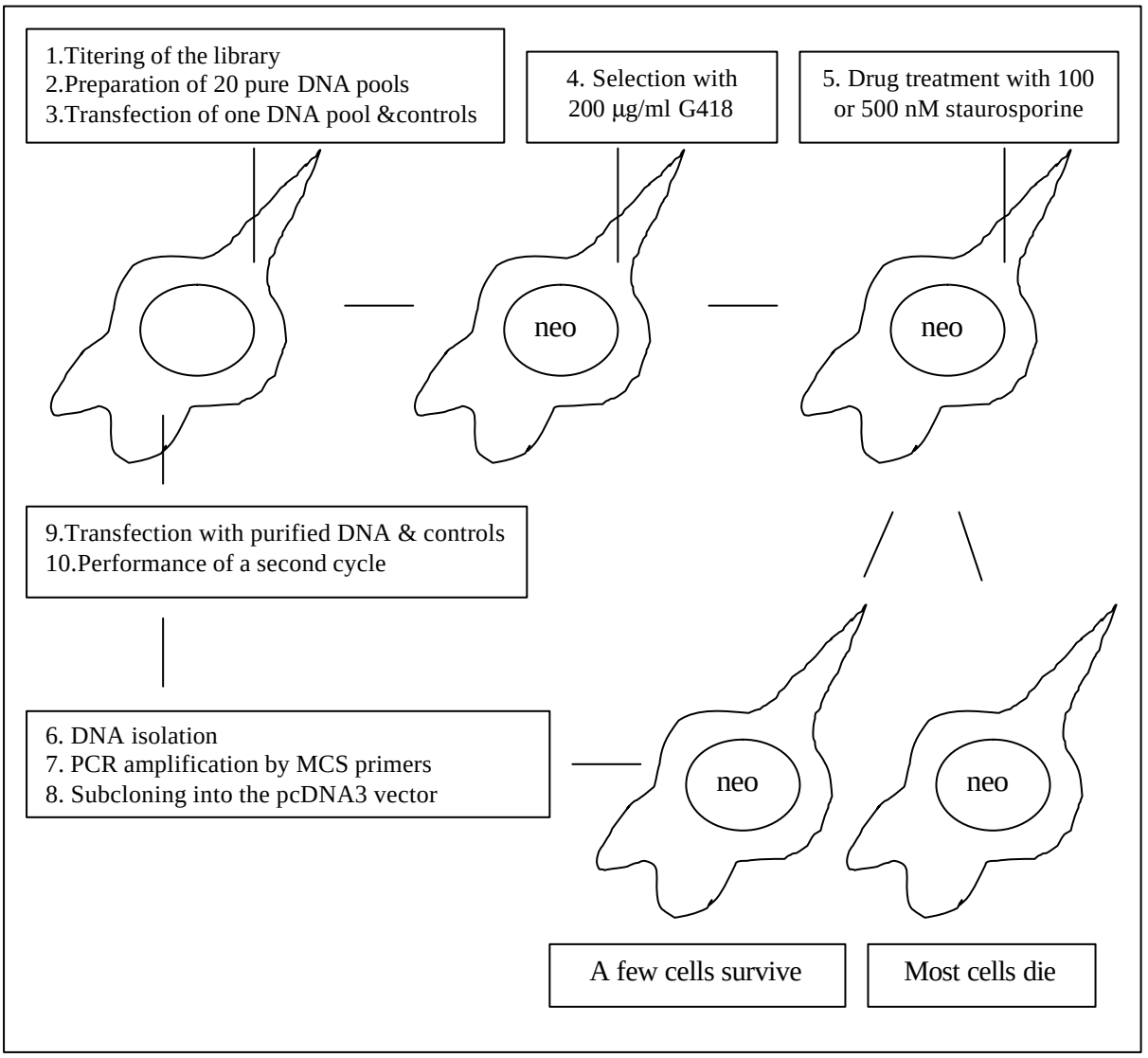

Figure 11 : Schematic presentation of the method to clone new survival factors.

To minimize the risk of amplifying false-positive factors, a second cycle was performed using the same steps (Figure 11, steps 9, 10 and again steps 4 and 5). At the end of this second cycle, genomic/extra-chromosomal DNA was isolated, amplified by PCR using multiple cloning site (MCS) primers, and each amplified DNA fragment was subcloned individually into the pcDNA3 vector. All different constructions were sequenced (Microsynth) and compared with the BLAST database to search for known factors. The sequences were screened manually to find the B*stXI*/N*otI* sites, a start codon in frame with a stop codon, a Kozak consensus sequence and eventually a poly(A) sequence just before the N*otI* site because the cDNA library was prepared from a human Burkitt Lymphoma RNA source by using oligo dT and/or random primers. Finally, the most interesting cloned factors were tested for their survival activity by using the cell viability assay as described above.

2.6.3 Results

Establishing the best conditions for transfection and cell killing in R6 cells.

In this project, the first step was to establish the best conditions for the transfection/killing process. For that purpose, a transfection efficiency assay was performed by transfecting R6pMV12 cells with β-Gal/pcDNA3 using Superfect (Qiagen) or Fugene6 (Boehringer Mannheim), because these transfection reagents were supposed to be effective for R6 cell lines (Figure 12).

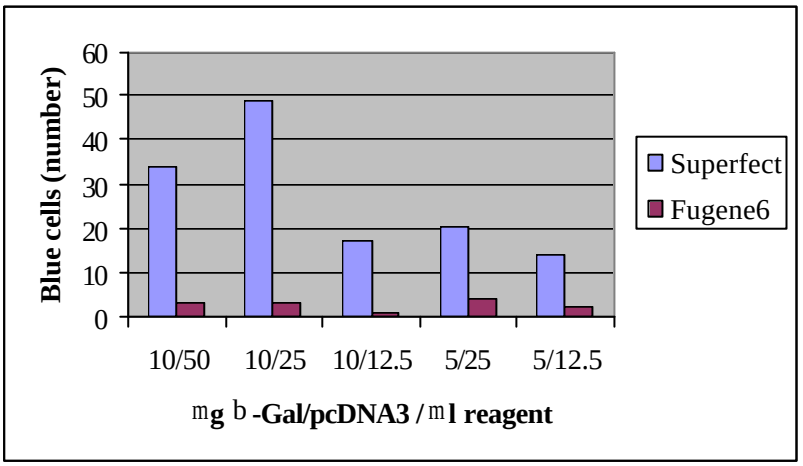

Figure 12 : Transfection efficiency assay by X-Gal staining into R6pMV12 cells.
R6pMV12 cells were transfected with different ratios µg β-Gal/pcDNA3 versus µl Superfect or Fugene6 and stained with X-Gal at 24 h post-transfection. Transfection efficiency was monitored by counting trypsinized blue cells in a Neubauer chamber. The displayed cell counts are the means of three independent experiments.

As seen in Figure 12, the best transfection was obtained with Superfect and with a ratio between µg β-Gal/pcDNA3 versus µl Superfect of 10 to 25. Fugene6 was not as good as predicted by the manufacturer (Figure 12). Therefore, we tested other transfection reagents (like Lipofectamine and Effectene) on R6 cell lines but none was better than Superfect (data not shown). Moreover, we transfected other cell lines like HEK293 or human epithelioid cervical carcinoma (HeLa) cells. They gave better transfection efficiency than R6 cells (about

3 times more blue cells), but were less sensitive to drug treatments as judged by a cell viability assay in response to staurosporine or MG132 (data not shown and Figure 13).

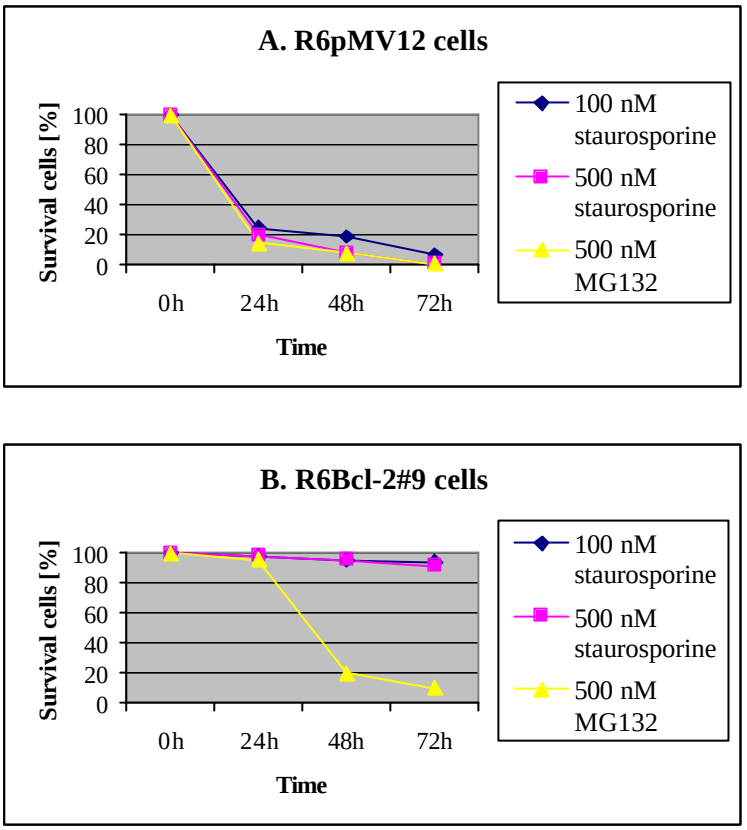

Figure 13 : Cell viability assay after stresses with MG132 and staurosporine into R6 cell lines. Subconfluent R6pMV12 (vector control cells, panel A) or R6Bcl-2#9 (stably survival factor-overexpressing cells, panel B) cells were treated with 100 nM staurosporine, 500 nM staurosporine or 500 nM MG132 for 3 days, and each day the cell viability was determined by trypan blue exclusion. The data are the means of three independent experiments.

Maximal sensitivity to apoptotic stresses is a crucial point to choose the best cell line, because we want cells lacking survival factors to die completely such that cells which obtained survival factors by transfection can grow out. Therefore, we decided to take for our experiments the R6 cell line which permitted a good transfection efficiency using Superfect and was maximally killed (~90%) in response to 100 nM or 500 nM staurosporine (Figure 12 and Figure 13, panel A). Bcl-2 effectively protects these cells from apoptosis. MG132 was too potent as cell killing agent because it also induced apoptosis in R6Bcl-2 cells (Figure 13, panel B).

We also had to make sure that each of the transfected cDNA clone was represented in the transfected R6 cells. For that purpose, our transfection efficiency had to be at least 10% which was indeed the case.

In conclusion, we chose R6pMV12 cells, transfected them with the ratio of 10 µg β-Gal/pcDNA3 and 25 µl Superfect followed by a stress of 100 nM or 500 nM staurosporine for 3 days.

Heavily diluted hBcl-2 cDNA can be recloned by our strategy.

The next step was to test whether we can clone a known survival factor with our strategy. For that purpose, we chose the hBcl-2/pcDNA3 plasmid because we knew that Bcl-2 is well expressed in R6 cell lines and is a potent survival factor (Otter *et al.*, 1998). R6pMV12 cells were either non-transfected or transfected with a DNA solution containing a ratio between hBcl-2/pcDNA3 and pcDNA3 of 1:5'000, 1:10'000 or 1:20'000 so as to be in the same range as a particular cDNA in the library (2 µl in 20 ml of LB-Amp medium giving a dilution of 1:10'000). At 48 h post-transfection, cells were selected with G418 until all non-transfected R6pMV12 cells were killed, stressed with 100 nM staurosporine for 3 days and lysed to extract genomic/extra-chromosomal DNA. Finally, extracts were amplified by PCR and all amplified DNA fragments were analyzed on an agarose gel for the detection of the fragment corresponding to Bcl-2 (Figure 14).

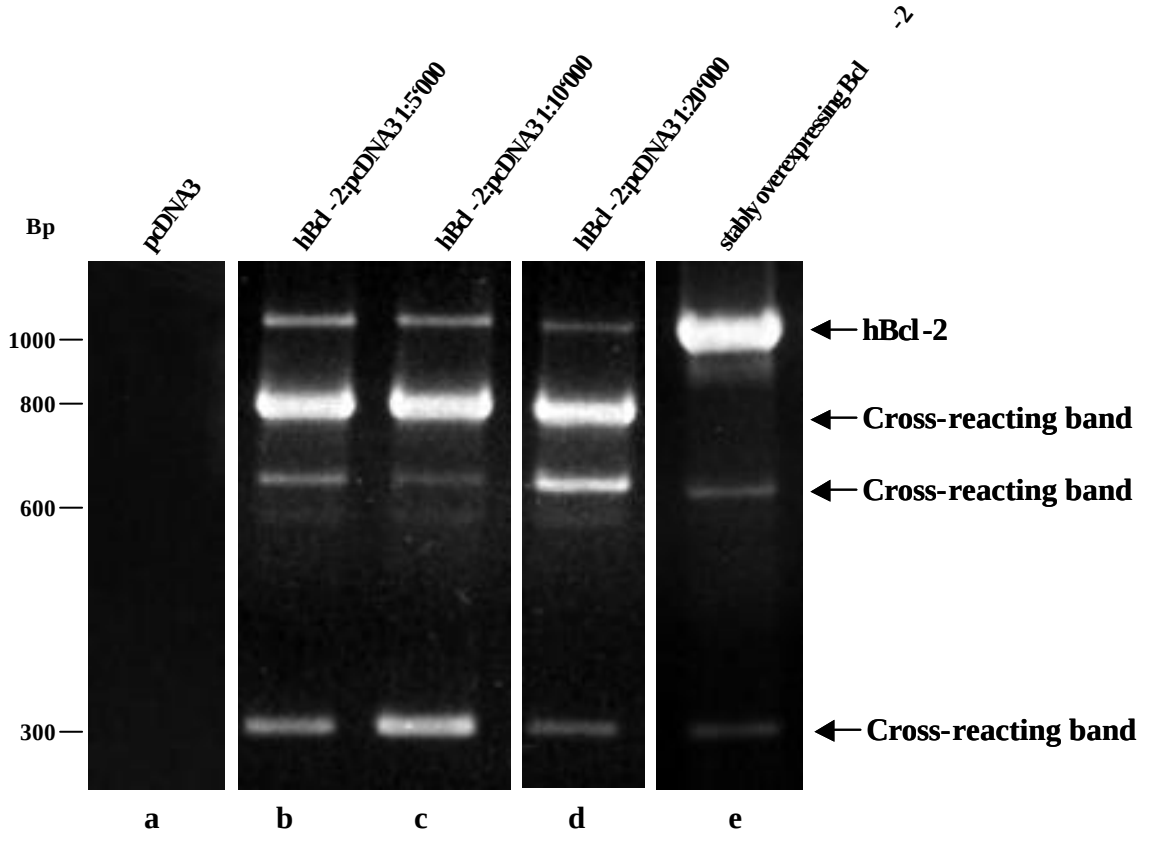

Figure 14 : PCR amplification on genomic/extra-chromosomal DNA extracts from R6pMV12 cells transfected with diluted hBcl-2.
By using MCS primers, a 40 cycles-polymerase chain reaction (PCR) was performed on pcDNA3 alone (lane a, negative control) or on genomic/extra-chromosomal DNA extracts from R6 cells transfected with hBcl-2 diluted 1:5'000 in pcDNA3 (lane b), hBcl-2 diluted 1:10'000 in pcDNA3 (lane c) or hBcl-2 diluted 1:20'000 in pcDNA3 (lane d). As positive control, a genomic/extra-chromosomal DNA extract from R6 cells stably overexpressing Bcl-2 was amplified (lane e). Note that Bcl-2 is present in the lanes b, c and d, while there is no specific amplification in lane a.

As seen by the presence of a DNA fragment corresponding to Bcl-2 (Figure 14, compare lane e with lanes b, c and d), we were able to clone again this survival factor even though hBcl-2 was 20'000 times less abundant than pcDNA3. We obtained three other DNA fragments

(Figure 14, lanes b, c and d), which were cross-reacting bands, because if we performed a PCR on an extract from non-transfected R6pMV12 cells, we detected them as well (Figure 15, lane h). This indicated that our method was suitable to isolate new putative survival factors.

Cloning of new possible survival factors from the library.

After establishing the experimental conditions, we directly screened the library for novel survival factors. We chose the 12th of the 20 DNA pools prepared from the library (pool#12/pcDNA3). R6pMV12 cells were either non-transfected or transfected with pool#12/pcDNA3 or hBcl-2/pcDNA3. Cells were selected with G418 as described in the previous section, stressed with 100 nM or 500 nM staurosporine, lysed to obtain genomic/extra-chromosomal DNA and the DNA was amplified by PCR. The amplified DNA fragments were analyzed on an agarose gel to detect all transfected and selected DNA fragments (Figure 15).

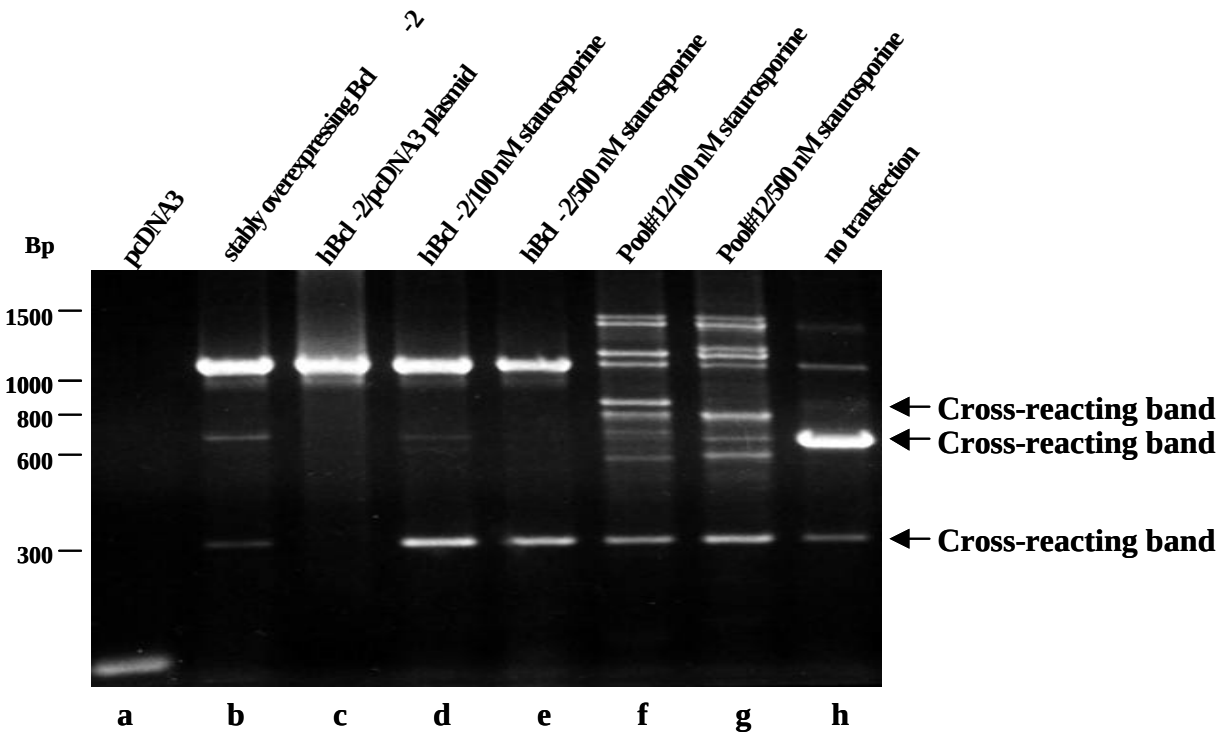

Figure 15 : PCR amplification on genomic DNA extracts from R6 cells transfected with pool#12 after the first cycle

By using MCS primers, a 30 cycles-polymerase chain reaction (PCR) was performed on pcDNA3 plasmid alone (lane a, negative control), on genomic/extra-chromosomal DNA extracts from R6 cells stably overexpressing hBcl-2 (lane b, positive control), on hBcl-2/pcDNA3 plasmid alone (lane c) or on genomic/extra-chromosomal DNA extracts from R6 cells transfected with hBcl-2/pcDNA3 and stressed with 100 nM staurosporine (lane d), hBcl-2/pcDNA3 stressed with 500 nM staurosporine (lane e), pool#12/pcDNA3 stressed with 100 nM staurosporine (lane f) or pool#12/pcDNA3 stressed with 500 nM staurosporine (lane g). A genomic/extra-chromosomal DNA extract from non-transfected R6pMV12 cells was also used as a second negative control (lane h). Note that we obtained a lot of bands in the lanes f and g indicating that putative survival factors were amplified.

As shown in Figure 15, we faithfully amplified hBcl-2 from stably transfected R6 cells as well as different DNAs with sizes up to 1.5 kb from R6 cells transfected with pool#12 (Figure 15, compare lane b with lanes f and g). Some DNA fragments appeared to be unspecific because they were also present when we amplified genomic/ extra-chromosomal DNA extracts from non-transfected R6pMV12 cells (Figure 15, compare lane h with lanes f and g). To increase the probability of cloning only survival factors, we cut out all amplified pool#12 DNA fragments up to 1.5 kb and subcloned them into pcDNA3. We then performed the same transfection/selection process for a second time. At the end, we amplified the genomic/extra-chromosomal DNA by PCR and analyzed it on an agarose gel (Figure 16).

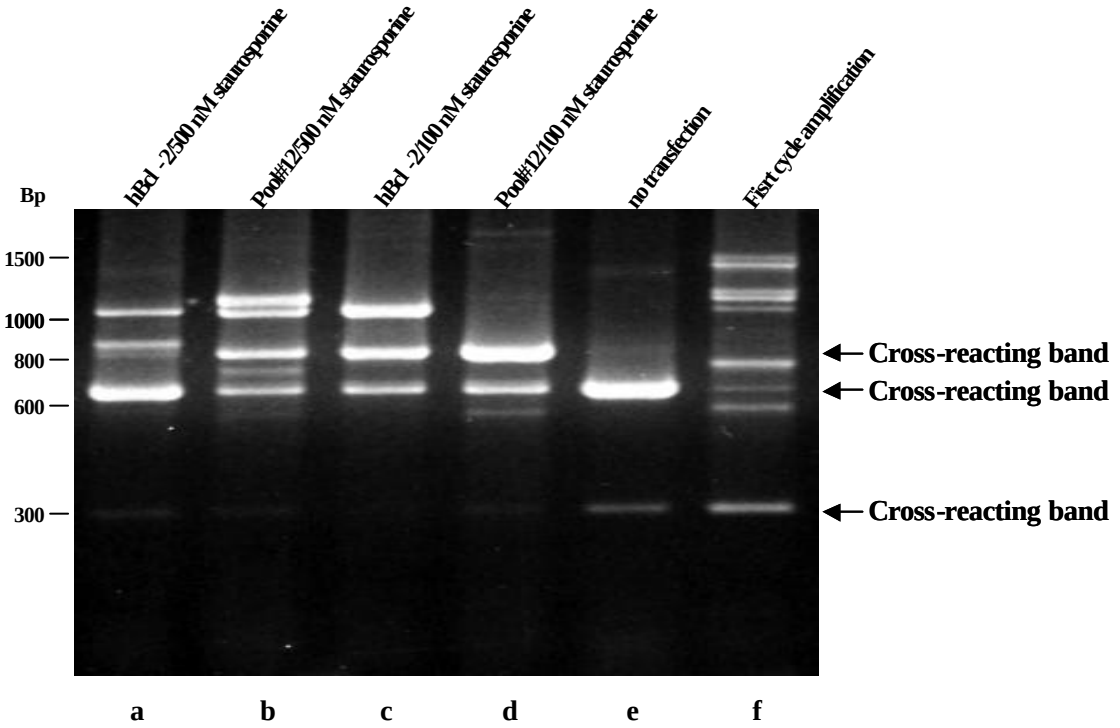

Figure 16 : PCR amplification on genomic DNA extracts from R6 cells transfected with pool#12 after the second cycle.

A 30 cycles-polymerase chain reaction (PCR) was performed on genomic/extra-chromosomal DNA extracts from R6 cells either non-transfected (lane e, negative control) or transfected with hBcl-2/pcDNA3 and stressed with 500 nM staurosporine (lane a), pool#12/pcDNA3 stressed with 500 nM staurosporine (lane b), hBcl-2/pcDNA3 stressed with 100 nM staurosporine (lane c) or pool#12/pcDNA3 stressed with 100 nM staurosporine (lane d). In the lane f, we loaded an amplified genomic/extra-chromosomal extract from R6 cells transfected with pool#12 after the first cycle. Note that some upper bands disappeared and that others were stronger if we compared the lane f with the lanes b and d.

As before, we amplified our positive Bcl-2 controls (Figure 16, lanes a and c, the upper band) and cross-reacting bands (Figure 16, compare lane e with lanes b and d). In the pool#12 sample, DNA bands which were amplified the second time seem to correspond to the sizes of the first cycle (Figure 16, compare lane f with lanes b and d). However, two upper bands were lost which could be false positives or weak survival factors which did not “survive” a second selection. Due to the enrichment during the second cycle, we obtained some stronger DNA

bands. These DNA fragments were interesting because they should correspond to survival factors. To characterize these factors, we individually purified each DNA band (Figure 16, lanes b and d), subcloned them one by one into the pcDNA3 vector and sequenced them at Microsynth (CH). All sequences were screened for the presence of B*stXI*/N*otI* sites, a start codon in frame with a stop codon, a Kozak consensus sequence and eventually a poly(A) sequence just before the N*otI* site indicating that these cloned factors were from the library. We then used the BLAST database to see whether these cDNAs were already known (Table 2).

BLAST database name (human proteins)	**Identity (%)**	**Number of similar amino acids versus full-length protein**	**B*stXI*/N*otI* sites**	**Kozak sequence**	**PolyA sequence**
Small nuclear ribonucleoprotein U2	89 (227/255)	227/255	Yes	Yes	Yes
Ribosomal protein L13 (60S subunit)	87 (183/210)	183/210	Yes	Yes	Yes
New protein FIS-Clone	87 (89/102)	89/250 (middle part)	Yes	Yes	Yes
Ribosomal protein L18 (60S subunit)	100 (187/187)	187/187	Yes	Yes	Yes
Ribosomal protein S2 (40S subunit)	100 (79/79)	79/293 (only the C-term)	Yes	Yes	Yes
Truncated hBcl-2 (BH4 + loop region)	100 (72/72)	72/239	Yes	Yes	Yes

Table 2 : Presentation of all cloned factors after two cycles.

Six known human cDNAs have been found in the BLAST database (Table 2). In fact, we obtained more than 6 DNA fragments after the second cycle (Figure 16, lanes b and d), but all others were contaminations as determined by sequencing. Interestingly, we amplified a new cDNA encoding a protein (FIS) which is not yet well characterized. Otherwise, we obtained a ribonucleoprotein, 3 ribosomal proteins and a truncated form of human Bcl-2 (hBcl-2). More importantly, some publications mentioned human ribosomal proteins, such as L13, as cell proliferating regulators which either induce apoptosis or maintain cell survival (Chen and Ioannou, 1999). In addition, a publication showed that the BH4 domain of anti-apoptotic Bcl-2 family members inhibits apoptotic mitochondrial changes and cell death (Shimizu *et al.*, 2000).

We therefore verified whether these factors were able to protect cells from apoptosis. For that purpose, R6pMV12 cells were either non-transfected or transfected with hBcl-2/pcDNA3, each selected cDNA, or pcDNA3 alone for 24 h. Cells were then stressed with 500 nM staurosporine for 3 days. Each day, viable cells were counted on triplicate plates using the trypan blue exclusion assay (Table 3).

Transiently transfected factors	Stress time (24h)	Stress time (48h)	Stress time (72h)
No transfection	+	-	-
hBcl-2	+++++	++++	++++
Small nuclear ribonucleoprotein U2	+(+)	+	+
Ribosomal protein L13 (60S subunit)	+++	+++	++
New protein FIS-Clone	++++	++++	++++
Ribosomal protein L18 (60S subunit)	++(+)	+(+)	+(+)
Ribosomal protein S2 (40S subunit)	+++(+)	+++	++(+)
Truncated hBcl-2 (BH4 + loop region)	+(+)	+(+)	+
pcDNA3	(+)	-	-

Table 3 : Cell viability assay into R6 cell lines.

As shown in Table 3, while pcDNA3 transfectants readily underwent apoptosis, the cells transfected with the various cDNAs were all more or less protected. Best protection was achieved with the **FIS clone** which had almost the same survival potency as Bcl-2. The least protection was attained with the BH4 truncated Bcl-2. This finding is inconsistent with the hypothesis of Shimizu *et al.* indicating that the BH4 domain could be an anti-apoptotic factor. In their disfavor, they performed an *in vitro* study by using reconstituted liposomes which did not reflect the physiological conditions. Therefore, their results may not represent the reality, because it has also been published and widely accepted that the BH1, BH2 and BH4 domains are all required for death protection (Adams and Cory, 1997). The ribosomal and ribonuclear proteins were weak death protectors but their effect was significant. Ribosomal proteins are often overexpressed in human tumors (Loging and Reisman, 1999), explaining why we probably obtained a lot of such factors at the end of our process.

In conclusion, we isolated one very interesting factor : the new protein FIS. Further investigations will be necessary to characterize this factor. For example, it would be interesting to test its protective function against other physiological stresses, such as IL-3 or serum withdrawal, in cell lines other than R6 and to perform a clonogenicity assay to see if it is capable of protecting and rescuing cells in a more generalized way. Moreover, 19 other pools remain to be screened in which we could find new anti-apoptotic factors.

3. DISCUSSION

Life requires the death of cells at correct places and times. Multicellular organisms eliminate redundant, damaged, or infected cells by a stereotypic program of cell suicide termed apoptosis (Kerr *et al.*, 1972). Interest in the control of apoptosis has grown exponentially with the recognition of its vital roles in normal development, tissue homeostasis, and defense against pathogens (Raff, 1992; Vaux *et al.*, 1994), and the realization that disturbed apoptosis may contribute to diseases (Adams and Cory, 1998).

Significant parallels exist between the prototypic system *C. elegans* and mammalian systems. Developmentally programmed cell death in the nematode has been shown to be controlled by small number of genes, all of which have counterparts that are conserved across evolution (Ellis and Horvitz, 1986). The ced-3 gene encodes a protein that corresponds to the caspases family. It is activated by association with the product of ced-4, which shares homology with Apaf-1 (Zou *et al.*, 1997), Nod1/CARD4 (Bertin *et al.*,1999; Inohara *et al.*, 1999), Nod2 (Ogura *et al.*, 2001), DEFCAP (Hlaing *et al.*, 2001) and NAC (Chu *et al.*, 2001). Cell death is prevented by the expression of ced-9, which is homologous to the Bcl-2 family members. Based on overexpression studies in heterologous systems, it has been concluded that CED-9, which is localized to the mitochondrial membrane, binds CED-4 and prevents its interaction with CED-3 (Chinnaiyan *et al.*, 1997). Expression of EGL-1 (mammalian homologue: Bax) displaces CED-4, permitting apoptosis to proceed (Del Peso *et al.*, 2000; Gottlieb, 2000).

Vertebrate apoptotic machinery is substantially homologous to that of invertebrates, although it is more elaborate and degenerate: caspases, Bcl-2 and IAP family proteins, and survival signaling pathways all exist in bewildering multiplicity, consistent with the more sophisticated needs for control of apoptosis in vertebrate tissues (Meier *et al.*, 2000). As in invertebrates, a variety of transcriptional mechanisms are important in the regulation of developmental apoptosis in vertebrates such as the classical apoptotic paradigm of interdigital cell death which is determined by the transcriptional readout of the transforming growth factor-β signaling pathway (reviewed in Merino *et al.*, 1999). Nonetheless, mammalian apoptosis is also significantly regulated, in both development and throughout life, by two non-transcriptional signaling systems whose exact counterparts are either absent or have proven elusive in the worm.

First, mammals possess a family of death receptors whose ligation can directly trigger activation of specific initiator caspases (like caspase-8, -10 and –2) through induced assembly of discrete apoptosome complexes. No equivalent of death receptors has yet been identified in *C. elegans*.

Second, in mammals, many pro-apoptotic insults seem to impact directly upon mitochondria to induce their leakiness and the release of various pro-apoptotic polypeptides. However, there is no evidence for any involvement of mitochondria in cell death in the worm. Thus, it remains unclear whether vertebrates have evolved a more complex elaboration of the apoptotic machinery that incorporates the mitochondrion or whether *C. elegans* represents a stripped down version of a more evolutionarily ancient mechanism (Meier *et al.*, 2000).

Mitochondria as central players of apoptosis

Nevertheless, for mammals, it is now clear that mitochondria have an important function in at least some apoptotic signaling cascades (Newmeyer *et al.*, 1994; Kroemer, 1997b; Green and Reed, 1998). Following a death stimulus, many proteins from the mitochondrial intramembrane space, including cytochrome c, adenylate kinase, Smac/DIABLO, pro-caspases, and apoptosis-inducing factor (AIF), have been reported to be released into the cytosol (Bossy-Wetzel *et al.*, 1998; Single *et al.*, 1998; Kohler *et al.*, 1999; Lorenzo *et al.*,

1999; Du *et al.*, 2000; Verhagen *et al.*, 2000). Cytochrome c exit is an almost universal feature of apoptotic cell death. However, in some cases, it is a late event. For example, apoptosis induced by death receptors often bypasses the mitochondrial pathway (Scaffidi *et al.*, 1998). As might be expected, such deaths are relatively insensitive to protection by Bcl-2 (Scaffidi *et al.*, 1998), and cytochrome c release into the cytosol is likely to be the result of caspase activation, rather than its cause (Hengartner, 2000).

But is release of pro-death factors from mitochondria really the point of no return ? Several lines of evidence suggest that cells can occasionally still be rescued at this stage, at least for a while. First, pharmacological inhibitor of caspases will often, but not always, rescue cells from apoptosis or simply prolonged the survival of cells which, for example, overexpress Bax (Nicholson, 1996; Rossé *et al.*, 1998; Robertson *et al.*, 2000). Second, caspase-3- and caspase-9-knockout mice show reduced neuronal apoptosis during development and a significant defect in apoptosis following insult (Earnshaw *et al.*, 1999; Zheng *et al.*, 1999; Wang and Lenardo, 2000). Third, mammals, as well as the fruitfly *Drosophila* and some viruses, carry a family of genes that encode potent caspase inhibitors, known as the inhibitor-of-apoptosis proteins (IAPs) (Budihardjo *et al.*, 1999; Miller, 1999). There would be little reason for such proteins to exist if they could not influence the apoptotic process (Hengartner, 2000).

Therefore, it might seem that cells suffer from a terminal case of indecisiveness when it comes to apoptotic cell death, letting apoptotic signaling go down endless trails but never fully committing. But, quite to the contrary, the apoptotic pathway contains a number of amplification steps and positive feedback loops that insure that a cell will either fully commit to death or completely abstain from it (Hengartner, 2000). For example, the fact that pro-caspases are caspase substrates insures rapid and complete conversion of a pool of pro-enzymes even if only a few molecules were initially activated (Thornberry and Lazebnik, 1998). Similarly, there is likely to be positive feedback between caspase activation and cytochrome c exit from mitochondria (Green and Kroemer, 1998; Green and Reed, 1998).

But positive feedback loops do require the presence of buffers and/or dampeners, or even the smallest perturbation would eventually lead to full activation and apoptotic death of the cell. The IAP proteins act as such dampeners. These proteins function by directly binding to and inhibiting caspase function (Deveraux and Reed, 1999b), because the tight regulation of these enzymes is critical to the normal development and survival of an organism (Goyal, 2001). Thus, they serve either to inhibit both initiator and effector caspases, laying roadblocks in both the mitochondrial and the death receptor-mediated apoptotic pathways (Goyal, 2001), or also to squelch spurious spontaneous caspase activation. This is further supported by the recent identification of a mammalian IAP inhibitor, known as Smac (Du *et al.*, 2000) or DIABLO (Verhagen *et al.*, 2000), which binds to IAP family members and neutralizes their anti-apoptotic activity. Thus, if a cell is committed to apoptotic death such that it releases its mitochondrial contents, then Smac/DIABLO will sequester the IAP proteins and insure that they do not attempt to stop the program in its track (Hengartner, 2000).

Bcl-2 family members as regulators of apoptosis

By analogy, anti-apoptotic Bcl-2 family members can be thought as buffers. In mammals, the principal action of Bcl-2 proteins, and of the survival signaling pathways that impact on them, seems to be the stabilization of mitochondrial integrity and prevention of release of these pro-apoptotic polypeptides (Meier *et al.*, 2000).

This differs from the role of nematode CED-9, which acts by directly interfering with the ability of CED-4 to activate CED-3 (Meier *et al.*, 2000). Such an interaction between mammalian CED-9 and CED-4 homologues, based also on the findings that Bcl-2 could act

after Bax-induced cytochrome c release in cells stably overexpressing both Bcl-2/Bax (Rossé *et al.*, 1998) and that apoptosis induced by microinjection of cytochrome c is strongly reduced by Bcl-2 overexpression (Zhivotovsky *et al.*, 1998), has even been tested in mammals. Unfortunately, Bcl-xL or nine other Bcl-2 family members, whose Bcl-2 and Bax, do not co-immunoprecipitate (Moriishi *et al.*, 1999; Conus *et al.*, 2000a) or co-localize (Hausmann *et al.*, 2000) with Apaf-1 inside cells. This was clearly confirmed in this thesis. Moreover, *in vitro* assays and cells deficient in Apaf-1 show that Bcl-2 and Bcl-xL do not require Apaf-1 for their caspase-inhibiting and death protective function (Haraguchi *et al.*, 2000; Newmeyer *et al.*, 2000). Thus, Apaf-1 seems to constitutively reside in the cytoplasm to await the release of cytochrome c for the activation of caspase-9. Nevertheless, it would be interesting to test if Bcl-2 family members could interact with one of the four newly identified CED-4 homologues, Nod1/CARD4 (Bertin *et al.*, 1999; Inohara *et al.*, 1999), Nod2 (Ogura *et al.*, 2001), DEFCAP (Hlaing *et al.*, 2001) and NAC (Chu *et al.*, 2001), although they are majorly involved in NF-κB activation than caspase activation, because it is still possible that a CED-4 homologue functions directly or indirectly upstream of mitochondria to initiate the release of cytochrome c and other apoptogenic factors.

So, how Bcl-2 could prevent caspase-3 activation and DNA degradation even when significant levels of cytochrome c remained in the cytoplasm (Rossé *et al.*, 1998)? The explanation could provide from the formation of the apoptosome. In fact, it has been shown that the apoptosome contains, cytochrome c, dATP/ATP, oligomerized Apaf-1, active caspase-9, active caspase-3 and XIAP (Bratton *et al.*, 2001). In such a scenario, apoptosome-bound IAPs would serve to immediately inhibit caspase-9 and –3 and/or prevent release of caspase-3 from the apoptosome. After a modest stress (corresponding to a slight excess of Bax molecules compared to that of Bcl-2 in cells stably overexpressing Bax and Bcl-2), in which only a small portion of cytochrome c is released and caspases are activated, these IAPs, and not Bcl-2 itself, would probably prevent apoptosis (Bratton *et al.*, 2001). When cytochrome c is microinjected, the activation of too many apoptosome complexes and active caspases would overcome the protective effect of IAPs. In addition, the result of caspase activation would be the release of cytochrome c, and other apoptogenic factors like Smac/DIABLO which also counteracts the protective effect of IAPs, serving as an amplification loop and resulting in a very efficient apoptosis. Thus, in this case, the overexpression of Bcl-2 would not allow the release of pro-apoptotic factors from the intermembrane space of mitochondria impeding the amplification loop to proceed and delaying considerably cell death.

However despite intensive research, it is still unknown how the Bcl-2 family really act on mitochondria. Recently, the three dimensional solution structure of the full-length Bax protein has been solved by NMR (Suzuki *et al.*, 2000). The Bax structure shows a high similarity to the overall conformation of the two other Bcl-2 family proteins for which structural information is available, the anti-apoptotic protein Bcl-xL and the BH3 domain-only protein Bid (Muchmore *et al.*, 1996; Chou *et al.*, 1999; McDonnell *et al.*, 1999). The proteins contain central hydrophobic helices (α5 and α6) surrounded by amphipathic helices. Of the three proteins, the Bax structure is the only one that contains the C-terminal hydrophobic domain. This domain forms a helix (α9) that protects the BH3-containing hydrophobic cleft on the protein. The structures of the Bcl-2 proteins are reminiscent of diphteria toxin and the colicins A and E1. These toxins are pore-forming proteins that function as membrane channels allowing passage of ions or small polypeptides. Bax and other Bcl-2 family proteins have been shown to possess channel–forming activity in artificial membranes (Antonsson *et al.*, 1997; Minn *et al.*, 1997; Schendel *et al.*, 1997; Schlesinger *et al.*, 1997). In this way of reasoning, it has been shown in cells exposed to the apoptosis inducer staurosporine or UV irradiation, that Bax forms oligomers with itself and probably also with yet unidentified

mitochondrial membrane proteins (Antonsson *et al.*, 2001). In addition, the formation of these Bax oligomers involves a series of events, including a change in Bax conformation triggered by Bid or other "BH3-only" proteins (Desagher *et al.*, 1999) and insertion into the outer mitochondrial membrane (Eskes *et al.*, 2000). Therefore, Bax oligomerization and insertion in the outer mitochondrial membrane resulting in a Bax-only channel or hybrid channels formed by Bax binding to lipid, VDAC or ANT represent the possible mechanism to permit the release of apoptogenic factors like cytochrome c (Antonsson *et al.*, 2001). But the exact protein composition of these oligomers remains to be determined, although we already know that the core structure is composed of several Bax subunits. Moreover, it remains to be determined whether these large Bax oligomers really constitute the cytochrome c-conducting channel in mitochondria (Antonsson *et al.*, 2001).

Surprinsingly, despite their great sequence homology, anti-apoptotic proteins like Bcl-2 and Bcl-xL behave differently from Bax. First of all, it has been reported that the crystal structure of soluble, anchorless Bcl-xL is a monomer (Muchmore *et al.*, 1996). Then, as we show here Bcl-2 does not require homodimerization for its survival activity (Conus *et al.*, 2000b) and unlike Bax, Bcl-2 and Bcl-xL monomers did not oligomerize in the presence of various detergents (Antonsson *et al.*, 2000; Conus *et al.*, 2000b). In fact, the quaternary structures of Bcl-2 and Bcl-xL do not change significantly during apoptosis (Antonsson *et al.*, 2001), allowing them to retain their globular structure so as to bind crucial death-regulatory proteins (including BH3-containing proteins), instead of changing their conformation to expose their BH3 domains for an oligomerization. Interestingly, it has also been found that Bax oligomerization does not occur in Bcl-2-overexpressing cells that are resistant to staurosporine-induced apoptosis (Estoppey *et al.*, 1997; Antonsson *et al.*, 2001). This result is consistent with previous data showing that Bcl-2 prevents the conformational change of Bax, which precedes its activation and membrane insertion (Desagher *et al.*, 1999; Eskes *et al.*, 2000). Therefore, it seems that one of the main function of anti-apoptotic proteins is not to form channels on mitochondria, but simply to impede the homo-oligomerization of pro-apoptotic proteins such as Bax by heterodimerization. This hypothesis is reinforced by our findings that no other cellular protein associates stably with Bcl-2, Bax or the Bcl-2-Bax heterodimer, and that Bcl-2/Bax co-localize to the same subcellular compartment and form strong, equimolar heterodimers in both normal and exposed to apoptotic stimuli cells (Otter *et al.*, 1998).

Even more amazingly, Bcl-2 and Bcl-xL seem to possess additional activities inside cells. Although they have a direct action on the mitochondrial membrane due to their subcellular localization, they also reside in the cytosol (Desagher and Martinou, 2000; personal data) and on other membranes such as the nuclear envelope and the endoplasmic reticulum (ER). Indeed, as we show here some Bcl-2 mutants that are not addressed to the mitochondrial membrane retain significant ability to inhibit apoptosis induced by agents like Brefeldin A (BFA) or tunicamycin which both provoke the release of cytochrome c into normal cells (Oltvai *et al.*, 1993; Zhu *et al.*, 1996; Lee *et al.*, 1999; Häcki *et al.*, 2000). Therefore, how could Bcl-2 control mitochondrial events from the ER membrane? The easiest explanation is that Bcl-2 sequester yet unknown cytoplasmic activators of cytochrome c which may stem from any parts of the cell. This factor is not necessarily Bid, because Bid is not involved in apoptosis induced by ER stresses (Häcki *et al.*, 2000).

The increasing evidence for a role of the ER-targeted Bcl-2 in apoptosis, and also the fact that anti-apoptotic proteins have been shown to protect the mitochondrial integrity by reducing, for example, the ROS levels (Vander Heiden *et al.*, 1999b), suggests that the regulation of apoptosis by Bcl-2 family proteins is more complex than previously thought and needs further investigations to understand how it really works. It seems that these anti-apoptotic proteins, which mainly sequester pro-apoptotic proteins, including BH3-only proteins, act at several

sites within the cell, like the cytosol, the mitochondrial membrane, the ER membrane and perhaps other membranes. Nevertheless, for the moment, no other protein than Bcl-2 has been shown to control apoptosis from the ER or other membranes. Moreover, the presence of anti-apoptotic proteins in the cytosol is a mystery, as nobody has ever shown whether this pool can prevent the conformational change that Bax undergoes during apoptosis (Desagher and Martinou, 2000) or sequester BH3-only proteins. Despite that, cytoplasmic Bcl-2 has been shown to be still partially active to protect cells from apoptosis (Borner *et al.*, 1994).

Therefore, the identification of new anti-apoptotic proteins could answer some questions about how apoptosis in general, and Bcl-2 family proteins in particular, are regulated. That is why it is interesting to clone new survival factors as has been started in other groups and also done in the present work. However, such studies are not easy because they require a lot of tests, like a series of different stresses on different cell lines followed by a clonogenicity assay to confirm that the cloned factors really act by protecting and rescuing cells from apoptosis in a wide range of situations. As we have now at least one or even more putative survival factors in hand, we next have to examine under which conditions and how they work.

For all of these reasons, cell death will continue to be a lively field for the foreseeable future !

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Diploma thesis in Physical Chemistry ; Supervisor: Prof. Dr. C. Rohrbasser, EIF school Dean, Fribourg and Dr. F. Sugnaux, director of Devex S.A., Chatel-St-Denis (FR), Switzerland.
Topic : Study of different surface treatments on metallic nickel allowing a reduction of its corrosion.
Mark: 5.5 (scale from 1.0 to 6.0, best mark 6.0).

1990-1987 **Chemical lab technician apprenticeship**, Ciba-Geigy S.A. (Novartis, Basel), Marly, Switzerland.

Positions

1998-2002	**Teaching assistant** of practice courses for third year biochemistry students.
1997-1998	**Teaching assistant** of practice courses for second year medical students and biochemistry students.

Work experience

Chemistry	inorganic and organic synthesis (pigments, dyes, additives, ...), gravimetry, titration, distillation/rectification, extraction, nuclear magnetic resonance (^{1}H-NMR, ^{13}C-NMR) , mass spectrometry (MS), spectroscopy (IR and UV), gas chromatography (GC), high performance liquid chromatography (HPLC), thin layer chromatography (TLC), ion exchange chromatography, fast performance liquid chromatography (FPLC), functional groups research, work with gas, fusing/solidification and boiling temperature determination, determination of density, refraction, sublimation, crystallization, physico-chemical separation, analytical techniques like ions research, organic disintegration.
Biochemistry	subcellular fractionation (glucose gradient), phosphorylation and dephosphorylation reactions in vitro and in vivo, extraction of lipids and proteins, FPLC, cell-free system, affinity column, in vitro transcription/translation.
Immunology	Generation of polyclonal/monoclonal antibodies, ELISA, Western Blot and silver staining, immunoprecipitation, immunohistochemistry on tissue specimens and immunocytochemsitry on cell lines, flow cytometry.
Cell Biology	Cell labeling, growth experiment, assay of apoptosis, biological assays (cytotoxicity), freezing and thawing of cells, radiolabelling of cells with ^{32}P, ^{35}S, and ^{3}H.
Molecular Biology	cDNA subcloning, purification of RNA, DNA and plasmids (CsCl, maxi/midipreparation…), stable and transient transfection, analysis by restriction enzymes, site-directed mutagenesis, PCR, expression of recombinant proteins in bacteria, DNA repair, sticky/blunt ligation, precipitation of DNA, electroporation, infection of bacteria with phages, screening, determination of library titer.
Microbiology	bacterial culture, microscopy techniques, coloration methods, protein extraction, transformation (electroporation, chemical methods).

Seminars, congresses, special courses

Chemistry:

1995 **Chemistry course** on "The organic synthesis of additives for the hardening by radiation" at Ciba-Geigy S.A. (Novartis) into the Additives Research Service, July 3-September 15, Marly, Switzerland

1994 **Chemistry course** on "The determination of pharmaceutical and parasiticidal substances into animal tissues by analytical methods like HPLC and GC-MSD" at Ciba-Geigy S.A. (Novartis) into the Animal Health Division of the Scientific Services Group, August 1-September 30, St-Aubin, Switzerland.

1992 **Chemistry course** on "The organic synthesis of essences" at Firmenich, July 15-August 26, Geneva, Switzerland.

Biochemistry:

2000 **ELSO's meeting (together with USGEB),** September 2-6, Geneva, Switzerland.

Poster Presentation of data at the Swiss Apoptosis Meeting on "Apoptosis", August 24-25, Bern, Switzerland.

Poster Presentation of data at the 3rd symposium of the International Cell Death Society on "Mechanisms of cell death 2000", May 6-9, Madrid, Spain.

Cosmital's symposium on "redox regulation", April 6-7, Fribourg, Switzerland.

1999 **Poster Presentation** of data at the Cold Spring Harbor Conference on "Apoptosis", September 29-October 3, New York, USA.

Oral Presentation of data at the 1st retreat on "Apoptosis", August 28-31, Toscany, Italy.

Poster Presentation of data at the ISREC Conference on "Cancer and the Cell Cycle", January 26-30, Lausanne, Switzerland.

1998 **STCS's symposium** on "Transfection", June 3, Basel, Switzerland.

Symposium on "Intracellular protein transport" at the 3ème cycle romand, September 1, Neuchatel, Switzerland.

Poster Presentation of data at the 3rd Annual Swiss Tissue Culture Society (STCS) meeting on "Life and Death on a Cell", September 17-18, Fribourg, Switzerland.

List of publications

Papers

1. **Conus, S.**, Kaufmann, T., Fellay, I., Otter, I., Rossé, T., Wartmann, W., and Borner, C. (2000) Bcl-2 is a monomeric protein : prevention of dimerization by structural constraints.
   Embo **19**(7), 1534-1544.

2. **Conus, S.**, Rossé, T., and Borner, C. (2000) Failure of Bcl-2 family members to interact with Apaf-1 in normal and apoptotic cells.
   Cell Death & Differentiation **7**(10), 947-954.

3. Häcki, J., Egger, L., Monney, L., **Conus, S.**, Rossé, T., Fellay, I., and Borner, C. (2000) Apoptotic crosstalk between the endoplasmic reticulum and mitochondria controlled by Bcl-2.
   Oncogene **19**(19), 2286-2295.

4. Otter, I., **Conus, S.**, Ravn, U., Rager, M., Olivier, R., Monney, L., and Borner, C. (1998) The binding properties and biological activities of Bcl-2 and Bax in cells exposed to apoptosis stimuli.
   J. Biol. Chem. **273**(11), 6110-6120.

5. Rossé, T., Olivier, R., Monney, L., Rager, M., **Conus, S.**, Jansen, B., and Borner, C. (1998) Bcl-2 prolongs cell survival after Bax-induced release of cytochrome c.
   Nature **391**(6666), 496-499.

Reviews

1. Borner, C., Monney, L., Olivier, R., Rossé, T., Häcki, J., and **Conus, S.** (1999) Life and death in a medieval atmosphere.
   Cell Death and Differentiation **6**(2), 201-206.

Abstracts

1. Borner, C., Monney, L., Olivier, R., Rossé, T., **Conus, S.**, and Fellay, I. (1998) Bcl-2 and Bax : the "good" and the "bad" for cell survival.
   On behalf of the international STCS meeting on "Life and Death of a Cell", September 17-18, 1998, Fribourg, Switzerland.
   Anticancer Research **18**, 4521.

2. **Conus, S.**, Fellay, I., Otter, I., Rossé, T., Wartmann, M., and Borner, C. (1998) Bcl-2 does not readily form homodimers.
   On behalf of the international STCS meeting on "Life and Death of a Cell", September 17-18, 1998, Fribourg, Switzerland.
   Anticancer Research **18**, 4524.

3. Häcki, J., Monney, L., **Conus, S.**, Rossé, T., Olivier, R., Baggiolini, M., and Borner, C. (1998) Loss of pro-caspase-3 in response to the caspase inhibitor z-VAD-fmk.
On behalf of the international STCS meeting on "Life and Death of a Cell", September 17-18, 1998, Fribourg, Switzerland.
Anticancer Research **18**, 4533.

Memberships

Swiss Society Union of Experimental Biology (USGEB) since 1998.
Swiss Society for Cell Biology, Molecular Biology and Genetics (ZMG) since 1998.
Swiss Society of Biochemistry since 1998.

Hobbies

Sport :	Soccer, squash, tennis, swimming.
Music :	All sorts.
Literature :	Specially detective novels.

References

Professional

1. Prof. Dr. Christoph Borner, Thesis Director, Institute of Molecular Medicine and Cell Research, Central Clinical Research, University of Albert-Ludwigs, Freiburg, Germany (Tel. : ++49-761-270-62-17, e-mail: borner@uniklinik-freiburg.de).
2. Prof. Dr. Andreas Conzelmann, Director, Institute of Biochemistry, University of Fribourg, Rue du Musée 5, Pérolles, CH-1700 Fribourg (Tel.:++41-26-300-86-30, e-mail: andreas.conzelmann@unifr.ch)
3. Dr. Claude Rohrbasser, Dean of Fribourg Engineer School (EIF), Bd de Pérolles 80, CH-1700 Fribourg (Tel.:++41-26-429-66-11, e-mail:claude.rohrbasser@eif.ch)

Private

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2. Roulin Jean-Luc, Chimiste ETS, Institut de Chimie Physique, Université de Fribourg, Pérolles/Rue du Musée 5, CH-1700 Fribourg (Tel.:++41-26-300-87-09, e-mail :Jean-Luc.Roulin@unifr.ch)
3. Jeannet Cyril, Chemistry Engineer, Analytical Research, Philip Morris Product SA, Neuchâtel, Switzerland (Tel. : 032-888-76-78 ; e-mail : cyril.jeannet@pmintl.com)