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 homodimer 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 G1 phase of the cell cycle (19–21), enhances gene amplification and recombination (22), and prevents activation of a subclass of cysteine proteases called caspasases (formerly 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-xL 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-xL, 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 caspasases.

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 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 homodimer or multimer, or through the interaction with non-proteaceous targets (lipids, ions).

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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 Raffmitogen-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-XL, Bcl-w, Bcl-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 eukaryotic 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 (BHI 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. Lithofectin 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, Velsketswil, Switzerland. Enhance was from DuPont-Nemours, Geneva, Switzerland. [35S]Methionine/cysteine (Tran35S-label) was bought from ICN, Zürich, Switzerland, and the enhanced chemiluminescence (ECL) detection system was from Amer sham, Buckinghamshire, U.K. Brefeldin A (BFA) (dissolved in methanol) was from Alexis Corp., Laufelfingen, Switzerland, and dithiothreitol (dissolved in dimethyl sulfoxide) was from Pierce, Rockford, IL. MG132 (dissolved in Me2SO) was a gift from Proscript Inc., Boston (formerly known as Myogenics Inc.). Staurosporine (dissolved in Me2SO), okadaic acid (dissolved in Me2SO), 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. 

**eDNAs—**The Flag-Fluc probe was cloned into the mouse Bcl-2 cDNA to the EcoRI 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 eDNAs were inserted into the EcoRI 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 NH2-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 13869E, 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 13868E, 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 embryonic 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-Fluc/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 pcDNA3 vector containing the murine 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 epimorphic pMEphy gro 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% CO2 and 37 °C.

**Drug Treatments and Cell Viability Assay—**Cells were seeded at 2 × 104 in triplicate in 35-mm wells. The next day, the cells were treated with the solvent Me2SO (0.1%), 1 μM staurosporine, or 1 μM MG132 to induce apoptosis. After 24 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 immunoprecipitated Bcl-2 and Bax proteins (27-6) immunoprecipitates from unlabelled cell extracts of R6-Bcl-2#9 were analyzed by anti-Flag Ab 06-499 immunoblotting at a titers 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—**Cells were labeled with 50 μCi/ml [35S]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 (Me2SO 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 MgCl2, 1 mM EGTA, 100 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml peptatin, 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 preclared 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-Bcl-2 antibody 27-6 or 25-5, 3 μl of anti-human Bax antibody Ab 13866E, 3 μl of anti-rat/mouse rat Bax antibody Ab 13866E, 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,
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 H2O, 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 mM Na3VO4, 25 mM β-glycerophosphate), an ATP regeneration system (1 mM DTT, 2 mM ATP, 10 mM phosphocreatine, and 50 μM/ml creatine kinase), 1 mM CaCl2 in the absence of EGTA, or various amounts of KCl (0–50 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, RIPPA buffer (buffer A plus 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) was used for cell disruption, immunoprecipitations, 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/pDNA3 and human Bcl-2/pDNA3 was combined with 3 μl (3 μg) of Lipofectin in Dulbecco’s modified Eagle’s medium (without serum and antibiotics) and transfection into the cells as described by the manufacturer (Life Technologies). After 6 h, the Lipofectin-DNA complexes were replaced by fresh Dulbecco’s modified Eagle’s medium plus 10% fetal cell serum in the absence or presence of 5 μl/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 antisera, clone 100 (1:10) and the polyclonal anti-human Bax antisera 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 anti fluorophaged 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 × 106 R6-Bcl-289 cells were transfected with a 1 μm concentration of the antisense phosphorothioate oligonucleotide 5’-TTGTCCTCCGAC/CGGTAT3’ 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’-ATGGACGGGTCCGGAGACA/CCGTCTAT3’ was transfected. After each day, a total cell extract was determined by amido black staining.

Interactions between Bcl-2 and Bax during Apoptosis

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 (66). First, we raised an antibody (27-6) against an epitope shown to be irrelevant for the surmounting of the Bcl-2 according to mutagenesis data (amino acids 41–60) (26, 57). This antibody immunoprecipitated a high amount of Bcl-2 from [35S]methionine/cysteine-labeled extracts of the Bcl-2-overexpressing R6 cell line R6-Bcl-289 (Fig. 1A, 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-289 cell extracts (Fig. 1A, middle panel). Similar findings were obtained with anti-27-6 immunoprecipitates from extracts of Bcl-2-overexpressing JILLY B lymphoblasts and L929 fibroblasts (Fig. 1B) 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. 1A, 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. 1A, 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. 1D). 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, JILLY-Bcl-2mix, and R6-Bcl-289 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. 2C), 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 NH2-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. 1C).

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. 4B 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-289 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. 2A, 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-289 cell extracts (Fig. 2A, 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. 2A, compare lanes c and d). These data indicate...
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. 2A) 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. 2A). These findings were confirmed by another anti-Bax antibody directed against an NH2-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.
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. 2B). 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. 2B, lane a). This is best illustrated with anti-Bcl-2 immunoprecipitates from R6-Bcl-2-Bax#9 cells (Fig. 2B, 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. 2C). Similarly, when comparing anti-human Bax (Fig. 2B, lane a) and anti-Bcl-2 (Fig. 2B, 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. 2B, compare lanes a and b) or anti-Bcl-2 immunoprecipitates (Fig. 2B, 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. 2B, compare lanes a and c). 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. 3A) 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,
whereas R6-Bcl-2#9 cells were protected efficiently for up to 48–72 h (see Figs. 7C and 8C, 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. 4A). 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 or absence of phosphatase inhibitors in the extraction and immunoprecipitation buffers (Fig. 4B). 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 phosphatase inhibitors. 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-treated cells (Fig. 5). These proteins were 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. 3B).

**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 [35S]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).
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. 6A). This was complete after 3 days and persisted for another 2 days (Fig. 6A and data not shown). A sense Bax oligonucleotide encompassing the same sequence did not affect endogenous Bax expression (Fig. 6A). 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. 6B). 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. 7A). 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. 8A).

Similar amounts of endogenous Bax were co-immunoprecipitated from cells exposed to staurosporine or BFA. R6-pMV12 and R6-Bcl-2#9 cells were labeled with [35S]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 copurify 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.
As judged from anti-Bcl-2 immunoprecipitates (Fig. 7B), the level of free Bcl-2 correlated positively with the degree of death protection against apoptosis induced by MG132 (Fig. 7C). 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-Flag-Bcl-2-#7 cells (Fig. 7C). 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. 8C). 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-#9 and R6-Flag-Bcl-2-#7 cells (Fig. 7C). 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. 8C). 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-#9 and R6-Flag-Bcl-2-#7 cells (Fig. 7C).

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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
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 non-stressed 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-immunoprecipitate. 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 studies of the activities of Bcl-2 and Bax in and/or Bcl-2 knockout mice (65). We also find that 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 non-proteaceous molecules, if any, which mediate the biologic activities of Bcl-2 and Bax.
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