Bcl-2 does not require Raf kinase activity for its death-protective function

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It has been widely accepted that the oncogene product bcl-2 protects mammalian cells from programmed cell death (apoptosis). The molecules and signalling pathways upon which bcl-2 acts are, however, still ill-defined. Recently, bcl-2 was shown to interact with c-raf-1 *in vitro*. Furthermore, an active form of c-raf-1 delayed apoptosis induced by trophic factor deprivation and enhanced the death-suppressive function of bcl-2 when co-expressed. This has led to the hypothesis that bcl-2 communicates cell-death protection via a raf-dependent signal transduction pathway. Here we show, by various immunological and biochemical methods, that bcl-2 does not stably associate with c-raf-1 in cellular extracts prepared from fibroblasts before

or after treatment with agents that induce apoptosis. Unexpectedly, bcl-2 function is entirely maintained, if not improved, when raf-dependent signalling is experimentally abrogated. In fact, bcl-2 allows the stable overexpression of a kinase-defective dominant-negative raf mutant that usually interferes with cell viability and/or proliferation. Our results indicate that bcl-2 does not require c-raf-1 kinase activity and an associated mitogen-activated protein kinase signalling pathway for its survival function. This property may be exploited to dissect cellular events that are dependent or independent of c-raf-1 kinase activity.

INTRODUCTION

There is emerging evidence that an amplifiable protease cascade, consisting of multiple interleukin-1 β -converting enzyme (ICE)-like proteases, is responsible for programmed cell death (apoptosis) in mammalian cells [1–3]. Activation of these proteases occurs by the proteolytic cleavage of inactive proforms into their active forms by either ICE-like or other proteases [1,4]. From studies in *Caenorhabditis elegans* [5], and most recently mammalian cells [6–8], it has become clear that bcl-2, a potent inhibitor of apoptosis, blocks the conversion of some pro-ICE-like proteases into their active forms. How bcl-2 achieves this inhibition is unknown. Based on its localization to intracellular membranes (nuclei, endoplasmic reticulum, mitochondria) [9] and its putative structural similarity to pore-forming bacterial toxins [10], bcl-2 may control an ICE-like activator that either is membrane-based or has to cross intracellular membranes.

Numerous laboratories have identified proteins that interacted with bcl-2 in the yeast two hybrid system, in baculovirus-infected insect cells, on glutathione S-transferase (GST)–bcl-2 affinity columns or in anti-bcl-2 immunoprecipitates from cell extracts. These proteins include members of the growing family of bcl-2-related proteins such as Bax [11–14], Bik [15] and Bad [16], the signalling molecules Ras [17], R-Ras [18] and c-raf-1 [19,20], the BAG-1 protein [21], three E1B-interacting proteins called Nip 1-3 [22], a p53 binding protein (53BP2) [23] and the prion protein PrP [24]. Bax is highly similar to bcl-2. It binds to two similar regions (BH1 and BH2) at the C-terminus of bcl-2 [12–14,25] and antagonizes the survival function of the latter [11]. Bax may also exert a killer effector function, either dependent on or independent of bcl-2, via another similar region (BH3) [26]. Bax is the only protein that has reproducibly been seen by several laboratories to interact with bcl-2 in immunoprecipitates [11,15,25], in

the yeast two hybrid system [12–14] and on affinity columns [13–15]. It can therefore be considered to be an avid, physiologically relevant partner of bcl-2.

Physiological significance and reproducible interaction have, however, been difficult to show for the bcl-2-binding proteins R-Ras, c-raf-1 and BAG-1. Nevertheless all three proteins are attractive partners of bcl-2, since they participate in the regulation of apoptosis. Whereas an active form of R-Ras facilitates apoptosis [27], an active form of c-raf-1 (v-raf) inhibits apoptosis induced by growth factor deprivation [28]. In addition, the c-raf-1 protein has been viewed to be essential for proliferation and/or cell survival because stable expressions of dominant-negative raf mutants have been difficult to achieve [29]. Overexpression of the BAG-1 protein enhances the death-sparing activity of bcl-2 [21]. The recent findings that both R-Ras [27] and BAG-1 [30] are capable of binding and activating c-raf-1 indicate that all three proteins may form a membrane-bound complex to which bcl-2 can bind as well. If such a complex is indeed relevant for the regulation of apoptosis, its associated signalling pathway(s) need to be identified. Intracellular signalling elicited by R-Ras or BAG-1 is poorly characterized. By contrast, c-raf-1 activates at least the mitogen-activated protein kinase (MAPK) signalling pathway via phosphorylation and activation of mitogen-activated protein kinase kinase (MEK) [31]. This signalling pathway impinges on the transcriptional activation of genes whose promoters contain, among others, PMA response elements (PREs) [32]. A dynamic balance between the MAPK pathway and a MAPK-related, stress-activated protein kinase pathway [stress-activated protein kinase (SAPK)/Jun N-terminal kinase (JNK) pathway] has recently been suggested to determine whether a cell survives or undergoes apoptosis [33].

Here we show that R6 embryo fibroblasts overexpressing bcl-2 survive apoptotic stresses irrespective of whether the MAPK pathway is functional or down-regulated.

Abbreviations used: MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; ICE, interleukin-1 β -converting enzyme; CAT, chloramphenicol acetyltransferase; PRE, PMA response element; BFA, brefeldin A; GST, glutathione S-transferase; NP40, Nonidet P40.

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EXPERIMENTAL

Reagents

Hygromycin B was purchased from Juro Supply/Calbiochem and lipofectin from Life Technologies. Protein A-Sepharose, *N*,2',*O*-dibutyryl adenosine 3':5'-cyclic monophosphate sodium salt (dibutyryl cAMP) and peroxidase- or alkaline phosphatase-labelled goat anti-mouse and anti-rabbit antibodies were from Sigma, DNase-free RNase and the chloramphenicol acetyltransferase (CAT) ELISA kit from Boehringer Mannheim, PVDF membranes (Immobilon-P) from Millipore and Enhance was from Dupont-Nemours. [³⁵S]Methionine/cysteine (TRAN-label) was from ICN and the enhanced chemiluminescence detection system from Amersham. Isopropyl β -D-thiogalactoside was purchased from Sigma and brefeldin A (BFA) from Alexis Trading.

cDNAs

The human wild-type and mutant *c-raf-1* cDNAs subcloned in pMT2 were obtained from Dr. Silvia Stabel, Max-Delbrück Laboratory, Köln, Germany. The mutant *c-raf-1* (called *m-raf*) is a dominant-negative kinase-deficient *raf* derivative (*raf*-301) that contains a point mutation in the ATP-binding site (K375W) [32]. Both the *c-raf-1* and *m-raf* cDNAs were released from pMT2 following cleavage with *EcoRI* and ligated into the *EcoRI* site of the retroviral vector pMV7neo, resulting in the expression constructs *c-raf/pMV7* and *m-raf/pMV7*. These constructs were brought into R6-*bcl-2* cells by retroviral transduction as previously described [34]. The FLAG-*bcl-2* cDNA was constructed by inserting the mouse *bcl-2* cDNA in frame into the *EcoRI* site of the pFLAG-1 plasmid (IBI FLAG biosystem; Kodak).

To generate a bacterial GST-*bcl-2* fusion protein, the mouse *bcl-2* cDNA was subcloned into the *EcoRI* site of the pGEX-2T vector (Pharmacia). The resulting *bcl-2/pGEX* plasmid was transformed into bacteria and ampicillin-resistant colonies were tested for isopropyl β -D-thiogalactoside-inducible expression of the GST-*bcl-2* protein. As control, bacteria transformed with the pGEX-2T vector alone (producing GST) were used.

The 3 $\times$ PRE-CAT reporter plasmid was constructed by replacing the β -globin cDNA in the 3 $\times$ PRE- β -globin plasmid (kindly provided by Dr. Sandro Rusconi, Fribourg, Switzerland) by the CAT cDNA.

Antibodies

Polyclonal anti-*bcl-2* antisera against a 14-amino-acid peptide encompassing amino acids 41–54 of murine *bcl-2* (27-6) and against a 17-amino-acid peptide encompassing amino acids 95–111 of murine *bcl-2* (28-5) were used in this study. A full characterization of the antibodies has been previously described [34]. In addition, a hamster monoclonal anti-mouse *bcl-2* antibody (3F11) purchased from Pharmingen was used for Western blotting. For anti-*raf* Western blots we used a mouse monoclonal anti-human *raf* antibody PBB1 [35] kindly provided by Bernd Groner, Tumor Biology Center, Freiburg, Germany. For immunoprecipitations a polyclonal anti-human *raf-1* antibody (166-IV), generously supplied by Silvia Stabel, or a polyclonal anti-*raf-1* antibody (C-12), purchased from Santa Cruz Biotechnology (for immunocomplex *raf* kinase assays), was used. Both the 166-IV and C-12 antibodies are directed against the C-terminus of *c-raf-1*. The monoclonal anti-MEK-1 antibody (clone no. 25) was purchased from Transduction Laboratories and the polyclonal anti-BAG-1 antiserum (N-20) from Santa Cruz Biotechnology. The polyclonal anti-Bax antibody was generously

given by Upstate Biotechnology (Lake Placid, NY, U.S.A.). The mouse monoclonal anti-FLAG antibody (anti-FLAG IgG M2) was purchased from Kodak.

Cells

Vector control (pMV12) and *bcl-2* overexpressing (*bcl-2*) rat embryo fibroblasts (R6) were generated by retroviral transduction of the pMV12hygro plasmid lacking or containing the murine *bcl-2* cDNA as described previously [34]. R6 cells co-overexpressing *bcl-2* and either the wild-type *c-raf-1* (*bcl-2/c-raf*) or the dominant-negative mutant *m-raf* (*bcl-2/m-raf*) were generated by infecting R6-*bcl-2* cells with retroviruses containing the *c-raf/pMV7* or *m-raf/pMV7* plasmids respectively. Construction of the viruses was exactly as described [34]. Following infection, cells were selected on 200 μ g/ml geneticin (G418) for 10 days to obtain a mixed population of cells that overexpress *c-raf-1* or *m-raf*. Co-overexpressing of *bcl-2* and *c-raf-1* or *m-raf* was tested by anti-*bcl-2* and anti-*raf* Western blot analyses of the respective cellular extracts. All R6 cell derivatives were grown in Dulbecco's modified Eagle's medium containing 5% (v/v) fetal calf serum and small amounts (50 μ g/ml) of the respective antibiotics (hygromycin to maintain *bcl-2* expression and geneticin to maintain *c-raf-1* and *m-raf* expression). Where indicated, serum was excluded or specific drugs were directly added to the culture medium to induce apoptosis (see Figure legends).

Cell death analysis

Subconfluent cells were cultured in the presence or absence of serum and treated or not with the apoptosis-inducing agents staurosporine (5 μ M), BFA (5 μ g/ml) and/or dibutyryl cAMP (1 mM) at the times indicated in the Figure legends. Cell viability was determined by a Trypan Blue exclusion assay. To determine the degradation of chromosomal DNA into nucleosome-sized fragments, cells were trypsin treated for 5 min and washed twice in 20 mM Tris, pH 8.0. DNA was immediately extracted by phenol/chloroform/isoamyl alcohol (25:24:1, by vol.), treated with 100 μ g/ml RNase at 37 °C for 30 min to eliminate RNA. The pure DNA fraction was loaded on to a 2% (w/v) agarose gel, electrophoresed and visualized by ethidium bromide staining.

Protein extraction/Western blots

Total cellular extracts were prepared as follows: R6 cell derivatives were washed three times in PBS and then lysed in H8 [20 mM Tris/HCl (pH 7.5)/2 mM EDTA/2 mM EGTA/6 mM β -mercaptoethanol/aprotinin (10 μ g/ml)/leupeptin (2 μ g/ml)] containing 1% (w/v) SDS followed by sonication. Detergent extracts for the *raf* kinase assay and for immunoprecipitations were prepared as described below. Protein was determined by the method of Lowry. Equal amounts of protein were loaded on to 8–12% (w/v) polyacrylamide gels, subjected to SDS/PAGE and transferred to a PVDF or nitrocellulose membrane as described [34]. *c-raf-1* and *m-raf* were immunodetected by the mouse monoclonal anti-*raf-1* antibody PBB1 (1:10000), *bcl-2* by the rabbit polyclonal anti-*bcl-2* antibodies 27-6 or 28-5 (1:5000) or the hamster monoclonal anti-*bcl-2* antibody 3F11 (1:2000), MEK-1 by the anti-MEK antibody no. 25 (1:2000) and BAG-1 by the anti-BAG antibody N-20 (1:1000). Secondary antibodies were horseradish-peroxidase-coupled goat anti-mouse, anti-hamster or anti-rabbit antibodies. The detection system was enhanced chemiluminescence.

In vitro binding assay

Bacterial extracts containing GST-bcl-2 or GST were prepared according to the manufacturer's protocol (Pharmacia). The recombinant proteins were immobilized on glutathione-Sepharose (Pharmacia), washed extensively with PBS and incubated with [³⁵S]methionine/cysteine-labelled extracts from R6-bcl-2/c-rafmix or vector control cells. After washing in PBS, the radiolabelled protein-GST-bcl-2 complexes were detached from the column matrix by 5 mM glutathione, boiled in Laemmli buffer and analysed on SDS/PAGE (12 % gel) followed by fluorographic detection. Detergent-solubilized [0.2 % (w/v) Nonidet P40 (NP40)] radiolabelled extracts were prepared as described below for immunoprecipitations, cytosolic extracts as previously described [34].

Immunoprecipitations

Confluent 100 mm plates of vector control, *bcl-2* and *bcl-2/c-raf* cells were labelled with 50 μ Ci/ml [³⁵S]methionine/cysteine in methionine/cysteine-free RPMI medium overnight. At 24 h before extraction, cells were treated with 5 μ g/ml BFA or 5 μ M staurosporine. Cells were harvested by centrifugation, washed once in PBS, once in buffer A [10 mM Hepes (pH 7.2)/143 mM KCl/5 mM MgCl₂/1 mM EGTA/aprotinin (10 μ g/ml)/leupeptin (10 μ g/ml)/pepstatin (1 μ g/ml)/0.2 mM PMSF] and lysed in buffer A supplemented with 0.2 % NP40. After leaving on ice for 30 min, the cell lysate was cleared by centrifugation, supplemented with 2.5 mg/ml ovalbumin and subjected to immunoprecipitation using the anti-bcl-2 antibodies 27-6 or 28-5 [34] or the anti-raf-1 antibodies 166-IV [36] or PBB1 [35]. Following antibody incubation for 2 h at 4 °C, 50 μ l of a 50 % (v/v) Protein A-Sepharose suspension was added and immunocomplexes were captured on a end-over-end wheel at 4 °C for 60 min. Immunocomplexes were pelleted by centrifugation, washed three times in buffer A supplemented with 0.2 % NP40 and then boiled in SDS sample buffer for SDS/PAGE analysis. The gels were fixed in 40 % (v/v) methanol/10 % (v/v) acetic acid, treated with Enhance, washed in water, dried and subjected to fluorography.

Raf kinase assay

Cells (3×10^6) were washed with ice-cold PBS and harvested in 500 μ l of Triton lysis buffer [20 mM Tris (pH 8.0)/137 mM NaCl/2 mM EDTA/2 mM sodium pyrophosphate/1 mM sodium orthovanadate/1 mM PMSF/leupeptin (10 μ g/ml)/1 % (v/v) Triton X-100/10 % (v/v) glycerol/25 mM β -glycerophosphate] at 4 °C. A clarified supernatant containing Triton-soluble proteins was prepared by centrifuging the homogenate at 14000 g for 10 min at 4 °C.

Raf protein kinase activity was measured using an immune complex protein kinase assay with recombinant kinase-inactive MEK as an exogenous substrate basically as previously described [37]. The Triton-soluble fraction was incubated for 1 h with 1 μ g of rabbit anti-raf-1 (C-12) antiserum (Santa Cruz Biotechnologies) immobilized on 20 μ l of Protein A-Sepharose (4 °C). The immunoprecipitates were washed three times with lysis buffer and twice with kinase buffer [25 mM Hepes (pH 7.5)/25 mM β -glycerophosphate/1 mM dithiothreitol/5 mM MnCl₂/15 mM MgCl₂]. The washed raf-1 immunoprecipitates were incubated with 50 μ M [γ -³²P]ATP (10 Ci/mmol) and 100 ng of (His)₆MKK(K97M) in a final volume of 50 μ l for 60 min at 30 °C. The reactions were terminated by the addition of 50 μ l of 2 $\times$ sample buffer [250 mM Tris (pH 6.8)/4 % SDS/20 % glycerol/1.4 M β -mercaptoethanol/0.05 % (v/v)

Bromophenol Blue] and then boiled for 5 min before analysis by SDS/PAGE (7 % gel). The gel was dried and the phosphorylation of the substrate MEK was quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

To quantify the c-raf-1 protein, immunoprecipitates were resolved by SDS/PAGE (7 % gel), electrophoretically transferred to PVDF membrane (Immobilon-P) and analysed by Western blotting using the mouse anti-raf-1 antibody PBB1 [35] as described above.

CAT assay

DNA transfections were performed by lipofection in serum-free medium as described by the manufacturer (Life Technologies). Cells (1×10^6), seeded 1 day before transfection, were transfected with 1 μ g of a 3 $\times$ PRE-CAT reporter construct [34] and 19 μ g of genomic R6 fibroblast DNA, such that the final concentration of DNA was 20 μ g/transfection. After an incubation for 8 h the cells were washed, and fresh medium containing 10 % fetal calf serum was added. At 16 h (24 h post-transfection), cells were either kept in serum or depleted of serum and BFA (5 μ g/ml)/1 mM dibutyryl cAMP/100 nM staurosporine or a combination of these drugs were added. Then 24 h later (48 h post transfection) extracts were prepared from living cells, and CAT enzyme levels were determined by a CAT ELISA described by the manufacturer (Boehringer Mannheim). To compare the CAT activity values between non-apoptotic and apoptotic conditions, a specific CAT activity per living cell (determined by Trypan Blue exclusion) was calculated. A 100 % specific CAT activity represents the value measured at 48 h post-transfection in serum-cultured non-stressed vector control cells.

RESULTS

Bcl-2 does not interact with c-raf-1 in cellular extracts

First, we wanted to know whether our anti-raf and anti-bcl-2 antibodies co-immunoprecipitated known binding partners of c-raf-1 and bcl-2 respectively. For this purpose, we used either vector control R6 fibroblasts expressing a small amount of endogenous c-raf-1 (control) or a mixed population of R6 cell transfectants expressing large amounts of exogenous bcl-2 alone (*bcl-2*) or together with c-raf-1 (*bcl-2/c-rafmix*). As shown in Figure 1(A), a polyclonal anti-raf antibody against the C-terminus of c-raf-1 immunoprecipitated from [³⁵S]methionine/cysteine-labelled vector control (control) or *bcl-2* overexpressing (*bcl-2*) cell extracts, low levels of a p74 protein that corresponded in molecular mass to the endogenous full-length c-raf-1. A five times higher amount of c-raf-1 was noted in the anti-raf immunoprecipitates from R6-bcl-2/c-rafmix cell extracts. In addition, two other proteins of molecular masses 45 kDa and 30 kDa were co-immunoprecipitated (Figure 1A). By Western blotting the immunoprecipitates, we identified them as the known c-raf-1 binding proteins MEK-1 (45 kDa) and BAG-1 (30 kDa) respectively (Figure 1B). No band corresponding to the molecular mass of bcl-2 (26 kDa) was detected in the anti-raf immunoprecipitates from either vector control, R6-bcl-2 or R6-bcl-2/c-rafmix cell extracts (Figure 1A). This was also true for immunoprecipitates using an anti-raf antibody (PBB1) against the ATP binding site of the protein kinase domain [38] (results not shown). An anti-bcl-2 Western blot of the anti-raf immunoprecipitates confirmed the absence of bcl-2 (Figure 1B). In contrast, anti-bcl-2 immunoprecipitates from R6-bcl-2/c-rafmix cell extracts revealed the presence of p26 bcl-2 along with a protein at 21 kDa (Figure 1A). The latter protein was identified

Figure 1 Analysis of proteins associating with bcl-2 and/or c-rac-1

(A) Immunoprecipitations. Lysates from [35 S]methionine/cysteine-labelled vector control (control), bcl-2 overexpressing (bcl-2) and bcl-2/c-rac-1 co-overexpressing (bcl-2/c-rafmix) R6 cells were immunoprecipitated with either the polyclonal anti-raf-1 antibody 166-IV (α -raf IP) or the polyclonal anti-bcl-2 antibodies 27-6 or 28-5 (α -bcl-2 IP). Molecular-mass markers are indicated in kDa. (B) Western blots of immunoprecipitates. Anti-bcl-2 (α -bcl-2 IP, right panel) and anti-raf-1 (α -raf IP; left panel) immunoprecipitates were resolved by SDS/PAGE and transferred to PVDF membranes. The membranes were probed with anti-raf-1 (PBB1, α -raf), anti-MEK-1 (no. 25; α -MEK), anti-BAG-1 (N-20; α -BAG-1), anti-Bax (α -Bax) or anti-bcl-2 (27-6; α -bcl-2) antibodies. A lysate of R6-bcl-2/c-rafmix cells was directly tested on Western blots without prior immunoprecipitation (Extract). (C) In vitro binding assay. Membrane (NP40) or cytosolic (cytosol) extracts from [35 S]methionine/cysteine-labelled vector control (control) or

as Bax on an anti-Bax Western blot (Figure 1B). However, neither BAG-1 nor c-raf-1 was co-immunoprecipitated with anti-bcl-2 antibodies (Figures 1A and 1B). This was the case for at least two anti-bcl-2 antibodies directed against different epitopes: (i) 27-6, an antibody recognizing a putative hinge region in mouse/rat bcl-2 (amino acids 41–54) considered to be irrelevant for the survival activity of bcl-2 [11] and (ii) 28-5, an antibody recognizing part of the BH3 region of bcl-2 (amino acids 95–111; Figure 1A). In addition, we performed immunoprecipitations using an anti-FLAG antibody against an N-terminally FLAG-tagged bcl-2 from R6 cell extracts co-expressing FLAG-bcl-2 and c-raf-1. Although Bax was again co-immunoprecipitated with FLAG-bcl-2, p74 c-raf-1 was not present in these anti-FLAG immunoprecipitates (results not shown). We conclude that the immunoprecipitation conditions used were optimal to co-immunoprecipitate known c-raf-1 (MEK, BAG-1) and bcl-2 (Bax) binding proteins but not a complex of bcl-2 and c-raf-1.

Despite the use of several antibodies recognizing different epitopes on bcl-2 and/or c-raf-1, we were concerned that these antibodies would compete with the interaction site between bcl-2 and c-raf-1. We therefore developed a GST/bcl-2 affinity column to which a [35 S]methionine/cysteine-labelled extract from vector control or R6-bcl-2/c-rafmix cells was applied. Using a detergent-solubilized (0.2% NP40) extract, Bax was the only protein that specifically bound to GST-bcl-2 but not to GST (Figure 1C). No detectable amounts of c-raf-1 could be bound to the GST-bcl-2 matrix, irrespective of whether a detergent-solubilized or a cytosolic extract (containing most of c-raf-1) was applied (Figure 1C). This was also true for radiolabelled extracts previously stressed with apoptotic agents (BFA, staurosporine) and accounted for full-length p74 raf as well as for its putative p35 degradation product (see below) (results not shown).

Since it was possible that bcl-2 interacted with c-raf-1 only when the cells were stressed with apoptotic agents, R6-bcl-2/c-rafmix cells were treated with 5 μ g/ml BFA or 5 μ M staurosporine. BFA is known to block vesicular trafficking by preventing the formation of vesicles from the Golgi apparatus [38]. Staurosporine inhibits protein kinases, in particular protein kinase C [36]. Both drugs induce all the morphologic features of apoptosis when applied to R6 fibroblasts for extended time periods and bcl-2 effectively blocks these events (Figure 5A; results not shown). Surprisingly, a total extract of R6-bcl-2/c-rafmix cells treated with BFA or staurosporine contained a low-molecular-mass form of raf in addition to full-length p74 c-raf-1 (Figure 2A). This was not seen in untreated extracts (Figure 1A; results not shown). The lighter protein corresponded in molecular mass to p35-t-raf-1 (see the Discussion section for t-raf), a truncated, constitutively active version of c-raf-1 previously shown to interact with bcl-2 when co-transfected [19]. Despite their presence in stressed cellular extracts, neither p74 c-raf-1 nor its putative catalytic fragment was co-immunoprecipitated with anti-bcl-2 antibodies (Figure 2A). Conversely, an anti-bcl-2 Western blot did not reveal any bcl-2 in anti-raf immunoprecipitates from BFA- or staurosporine-stressed cell extracts (Figure 2B). This was independent of the duration of the stress.

To ensure further that no bcl-2/raf complexes were present in our extracts, we varied the pH (5.0–8.5) and salt concentrations (0–150 mM) during anti-bcl-2 immunoprecipitations and subsequent washes, and/or included phosphatase inhibitors (okadaic

R6-bcl-2/c-rafmix (bcl-2/c-rafmix) cells were applied to a GST-bcl-2 or GST affinity column respectively. Bound proteins were eluted by 5 mM glutathione and resolved on SDS/PAGE (12% gel) followed by fluorography. Molecular-mass markers are indicated in kDa.

Figure 2 Analysis of a possible association between bcl-2 and c-raf-1 under apoptotic conditions

Lysates from R6-bcl-2/c-rafmix cells non-treated (—) or treated (+) with 5 μ g/ml BFA or 5 μ M staurosporine (stauro) for 24 h were immunoprecipitated with either the anti-bcl-2 antibody 27-6 (α -bcl-2 IP) or the anti-raf antibody 166-IV (α -raf IP). Immunoprecipitates were resolved by SDS/PAGE and transferred to PVDF membranes. The membranes were probed with the monoclonal anti-raf antibody PBB1 (**A**) or the hamster monoclonal anti-bcl-2 antibody 3F11 (**B**). In parallel, lysates were directly tested on Western blots without prior immunoprecipitation (Ex). The raf fragment represents a putatively proteolysed 35 kDa form of p74 c-raf-1. Molecular-mass markers are indicated in kDa.

Table 1 Raf protein and activity in non-stressed and stressed R6 cells

Lysates from vector control (Control) and bcl-2 overexpressing cells (bcl-2) treated with 5 μ g/ml BFA or 5 μ M staurosporine (stauro) in the absence or presence of serum for 24 h were immunoprecipitated using the polyclonal anti-Raf antiserum C-12. The immunoprecipitates were assayed for p74 Raf protein by anti-Raf Western blotting and for Raf kinase activity using a recombinant kinase-inactive MEK mutant as substrate, (His)₆MKK(K97M). p74 raf protein was quantified by densitometric scanning of the autoradiograph of the Western blot. raf kinase activity was determined by quantifying the phosphorylated MEK peptide in the anti-raf immunoprecipitates using a PhosphorImager; arbitrary units are 32 P transferred from [γ - 32 P]ATP to the MEK substrate. Values are means $\pm$ S.D. of three independent experiments. nd, not determined.

Treatment	10 ⁴ $\times$ raf kinase activity (arbitrary units)		p74 raf protein (densitometric units)	
	Control	bcl-2	Control	bcl-2
+ Serum	11.9 $\pm$ 1.8	4.2 $\pm$ 0.6	34.6 $\pm$ 2.1	26.1 $\pm$ 1.6
— Serum	1.9 $\pm$ 0.3	1.4 $\pm$ 0.2	20.0 $\pm$ 2.8	5.7 $\pm$ 1.7
— Serum + BFA	nd	1.3 $\pm$ 0.2	nd	9.8 $\pm$ 2.9
— Serum + stauro	nd	1.0 $\pm$ 0.1	nd	1.1 $\pm$ 0.1
— Serum + BFA + stauro	nd	1.2 $\pm$ 0.2	nd	0.8 $\pm$ 0.1

acid, sodium orthovanadate, β -glycerophosphate, NaF) to account for a possible phosphorylation-dependent interaction. The latter modification was introduced because it had been shown that both c-raf-1 and bcl-2 were phosphorylated in response to the apoptotic drug taxol [20]. In none of these cases did we detect any binding activity between bcl-2 and c-raf-1 (results not shown). In summary, bcl-2 does not stably interact with c-raf-1 in immunoprecipitates or on GST-bcl-2 affinity columns even when cell extracts have been previously stressed with apoptotic agents and/or both proteins are highly overexpressed.

Inhibition of raf activity and raf-dependent signalling by serum removal, and treatment with cAMP or staurosporine does not ablate the survival activity of bcl-2

Despite a lack of direct interaction between bcl-2 and c-raf-1, it

Table 2 CAT activity in the presence of raf kinase inhibiting agents

A reporter CAT assay was used to measure signalling downstream of the raf protein kinase. Vector control (control) and bcl-2 overexpressing R6 cells (bcl-2) were transfected with a 3 $\times$ TRE-CAT construct and subsequently treated (or not) with 1 mM dibutyl cAMP or 5 μ M staurosporine (stauro) or 5 μ g/ml BFA in the absence or presence of serum for 24 h. Cells were extracted and subjected to a CAT assay. CAT activity of vector control cells was taken as 100%. Values are means $\pm$ S.D. of three independent experiments. nd, not determined.

Treatment	Relative CAT activity (%)	
	Control	bcl-2
+ Serum	100 $\pm$ 15.0	75 $\pm$ 11.2
+ Serum + cAMP	50 $\pm$ 7.5	38 $\pm$ 5.7
+ Serum + stauro	45 $\pm$ 6.7	33 $\pm$ 4.9
— Serum	35 $\pm$ 5.2	27 $\pm$ 4.0
— Serum + cAMP	16 $\pm$ 2.4	12 $\pm$ 1.8
— Serum + cAMP + BFA	nd	6 $\pm$ 0.9
— Serum + stauro	9 $\pm$ 1.3	6 $\pm$ 0.9
— Serum + stauro + BFA	nd	4 $\pm$ 0.6

was still possible that bcl-2 uses a raf-dependent signalling pathway to confer cell survival. To investigate this we measured endogenous raf kinase activity in anti-raf immunoprecipitates and the transcriptional activation of a transfected CAT reporter gene driven by three PMA-responsive DNA consensus elements (3 $\times$ PRE). The latter construct can be used to measure raf-dependent signalling because its transcriptional activation by serum or growth factors is impaired by the co-expression of a dominant-negative raf [32]. To interfere with raf signalling, we decreased the endogenous raf kinase activity by serum removal and/or cellular treatments with cAMP or staurosporine.

As expected, the removal of serum decreased raf kinase and CAT activities of vector control R6 cells to 16 % and 35 % of the values measured in the presence of serum respectively (Tables 1 and 2). Next, we wanted to know whether overexpression of bcl-2 altered raf kinase activity and associated signalling (CAT assay) under both serum-containing and -lacking conditions.

Figure 3 Cell survival in the presence of raf kinase-inhibiting agents

Vector control (control) and bcl-2 overexpressing R6 cells (bcl-2) were monitored for survival following serum removal (top) or serum removal combined with cellular treatments with 1 mM cAMP (middle) or 5 μ M staurosporine (bottom). Serum-deprived R6-bcl-2 cells were also treated with cAMP plus BFA and staurosporine plus BFA (solid circles; bcl-2 + BFA). Data are means $\pm$ S.D. of three independent experiments. For the sake of clarity error bars (approx. $\pm$ 15%) are not shown.

Surprisingly, R6-bcl-2 cells consistently displayed lower raf kinase and CAT activities than vector control R6/pMV12 cells, irrespective of the presence of serum (Tables 1 and 2). To determine the basis for this down-regulation, we quantified the c-raf-1 protein in the anti-raf immunoprecipitates used for the kinase assay. Whereas bcl-2 overexpression only slightly diminished the c-raf-1 protein level in the presence of serum, a marked diminution was seen in the absence of serum. This indicates that bcl-2 may regulate c-raf-1 on two levels: (i) a post-translational level in the presence of serum leading to decreased raf kinase activity while only slightly altering raf protein expression and (ii) a transcriptional/post-transcriptional level in the absence of serum mainly to diminish the production and/or stability of the c-raf-1 protein. The diminution of c-raf-1 protein and kinase activity levels in R6-bcl-2 cells did not affect basic cell survival, since these cells survived as well, and possibly better than vector control cells (see below).

To investigate further the role of c-raf-1 in the survival signalling of bcl-2, we treated serum-deprived vector control and

Figure 4 Protein levels of overexpressed c-raf-1 and m-raf

Total extracts of vector control (control), bcl-2 overexpressing (bcl-2), bcl-2/wild-type c-raf-1 co-overexpressing (bcl-2/c-rafmix) and bcl-2/mutant raf-1 co-overexpressing (bcl-2/m-rafmix) R6 cells were resolved by SDS/PAGE and transferred to PVDF membranes. The membranes were probed with either the polyclonal anti-raf antibody 166-IV (α -raf; top panel) or the anti-bcl-2 antibody 27-6 (α -bcl-2; bottom panel). Molecular-mass markers are indicated in kDa.

R6-bcl-2 cells with the protein kinase inhibitor staurosporine (5 μ M) or the cell-permeable cAMP analogue dibutyryl cAMP (1 mM). This further decreased raf kinase and CAT activities below 10% of control values in both vector control and R6-bcl-2 cells (Table 1; results not shown). c-raf-1 protein levels were also further lowered in R6-bcl-2 cells (Table 1). cAMP is thought to decrease raf kinase activity by a cAMP-dependent protein kinase-mediated phosphorylation of raf at Ser-43, causing a reduction of c-raf-1 interaction with Ras [39–41]. Staurosporine may directly inhibit the raf kinase in the micromolar range [30] and/or protein kinase C, an upstream activator of c-raf-1 [36], in the nanomolar range. Despite a marked reduction of raf-dependent signalling, 95% of bcl-2 overexpressing cells survived a stress with staurosporine or cAMP in the absence of serum for up to 3 days (Figure 3). By contrast, only 10% of vector control cells resisted apoptosis after a 24 h treatment with staurosporine in serum-free medium (Figure 3). These data were corroborated by an additional cellular treatment with the apoptosis-inducing drug BFA. In the absence of serum, the treatment of vector control cells with staurosporine plus BFA or cAMP plus BFA led to complete apoptosis within 24 h, making cellular measurements of raf kinase and CAT activities impossible (Tables 1 and 2). However, 85–95% of bcl-2 overexpressing cells were protected from apoptosis induced by these agents for the same time period (Figure 3). Most importantly, the

Table 3 CAT activity in the presence of a dominant-negative raf mutant

Vector control (control), bcl-2 overexpressing (bcl-2), bcl-2/c-raf-1 co-overexpressing (bcl-2/c-raf) and bcl-2/m-raf-1 co-overexpressing (bcl-2/m-raf) R6 cells were transfected with a 3 × TRE-CAT construct and subsequently treated (or not) with 5 µg/ml BFA or 5 µM staurosporine (stauro) or 5 µg/ml BFA in the absence or presence of serum for 24 h. Cells were extracted and subjected to a CAT assay. CAT activity of vector control cells was taken as 100%. Values are means ± S.D. of three independent experiments.

Treatment	Relative CAT activity (%)			
	Control	bcl-2	bcl-2/c-raf	bcl-2/m-raf
+ Serum	100 ± 15.0	75 ± 11.2	423 ± 63.4	38 ± 5.7
+ Serum + BFA	75 ± 11.2	68 ± 10.2	187 ± 28.0	16 ± 2.4
+ Serum + stauro	50 ± 7.5	38 ± 5.7	163 ± 24.4	3 ± 0.4
– Serum	35 ± 5.2	27 ± 4.0	67 ± 10.1	5 ± 0.7
– Serum + BFA	16 ± 2.4	13 ± 1.9	37 ± 5.5	2 ± 0.3
– Serum + stauro	12 ± 1.8	9 ± 1.3	29 ± 4.3	< 1

latter cells displayed raf kinase and CAT activity levels that were below 10% of those measured in non-stressed serum-cultured vector control cells (Tables 1 and 2), and the c-raf-1 protein was barely detectable in anti-raf immunoprecipitates (Table 1). These findings indicate that bcl-2 is still capable of conferring cell-death protection under conditions of low raf kinase activity and raf-dependent signalling via the classical MAPK pathway.

Bcl-2 still protects cells from apoptosis when raf-dependent signalling is blocked by a dominant-negative raf mutant

To substantiate further our finding that bcl-2 acts independently of the raf kinase activity, we introduced into bcl-2 overexpressing R6 embryo fibroblasts the cDNAs encoding either wild-type c-raf-1 or a dominant-negative kinase-deficient human c-raf-1 mutant (raf301, K375W) [32] by retroviral transduction. The raf mutant (m-raf) has been previously shown to block raf activation and raf-dependent signalling towards PRE-CAT [32] in transient transfection assays, presumably by titrating out crucial activators of the endogenous raf kinase. However, it has so far been difficult to stably overexpress m-raf due to its potential interference with cell survival [29]. Indeed, we were unable to maintain stable clones of vector control R6 cells that had been infected with retroviruses carrying the dominant-negative raf construct (results not shown). Based on transient infection assays, cells expressing the m-raf protein either are growth-inhibited or die by apoptosis. Infection of R6–bcl-2 cells with the retroviruses carrying m-raf, however, yielded stable, viable clones that co-expressed high levels of m-raf together with bcl-2 (Figure 4). As a control, cells co-expressing the wild-type c-raf-1 (c-raf) and bcl-2 were generated (Figure 4). To avoid clonal variations, we used mixed cell populations of the over-expressors for further analyses (R6–bcl-2/c-rafmix or R6–bcl-2/m-rafmix).

First we determined whether the overexpression of dominant-negative raf interferes with endogenous raf signalling. In the presence of serum, R6 cells overexpressing wild-type c-raf and bcl-2 (bcl-2/c-raf) exhibited a CAT activity that was 4–5-fold higher (400–500%) than in vector control cells (control) (Table 3). By contrast, overexpression of the dominant-negative raf mutant reduced the basal CAT activity to 38% (bcl-2/m-raf) indicating that the mutant interferes with signalling towards PRE-CAT (Table 3). The effect of the mutant raf was accentuated in the absence of serum. Under the latter conditions, CAT activity was expectedly low in control cells (35%), slightly increased in cells that overexpress c-raf (67%), but negligible in the cells that

Figure 5 Cell survival in the presence of a dominant-negative raf mutant

(A) Vector control (control), bcl-2 overexpressing (bcl-2), bcl-2/c-raf-1 co-overexpressing (bcl-2/c-raf) and bcl-2/m-raf-1 co-overexpressing (bcl-2/m-raf) R6 cells were monitored for survival following serum removal (top graph) or serum removal plus cellular treatments with 5 µg/ml BFA (middle) or 5 µM staurosporine (bottom). Data are means ± S.D. of three independent experiments. For the sake of clarity error bars (approx. ± 15%) are not shown. (B) Genomic DNA from cells treated with 5 µg/ml BFA for 24 h was resolved on a 2% agarose gel. Fragmentation of the DNA into multiples of 200 bp was visualized by ethidium bromide staining.

overexpress m-raf (5%) (Table 3). CAT activity dropped to almost undetectable levels when R6–bcl-2/m-raf cells were additionally stressed with staurosporine or BFA (Table 3). These

results indicate that the mutant raf construct successfully impairs a raf-dependent signalling pathway, especially in the absence of serum and/or in the presence of an apoptosis-inducing agent.

To investigate whether bcl-2 was still functional in a cellular background containing a dominant-negative raf, we measured the survival of the various cell derivatives under normal and apoptosis-inducing conditions. A similar extent of survival was noted for vector control, bcl-2 overexpressing and bcl-2/c-raf or bcl-2/m-raf co-expressing R6 cells in the presence of serum (results not shown). The same was true when the serum was removed for 2 days (Figure 5A). At later time points of serum deprivation, control cells slowly died by apoptosis but all other cells, including R6-bcl-2/m-raf cells, remained fully viable for up to 10 days (Figure 5A). Two apoptotic stresses, staurosporine or BFA, were applied to further examine the survival capacity of bcl-2. In the absence of serum the two agents completely killed vector control cells (control) within 3 days or 5 days respectively. Cell death was due to apoptosis, as evidenced by the degradation of DNA into nucleosome-sized fragments (Figure 5B). In contrast, R6-bcl-2, R6-bcl-2/c-raf and R6-bcl-2/m-raf cells all exhibited marked delays in DNA fragmentation (Figure 5B; results not shown) and cell death in response to staurosporine or BFA (Figure 5A). Surprisingly, R6-bcl-2/m-raf cells survived both staurosporine- and BFA-induced stresses as well, possibly slightly better than cells that overexpressed bcl-2 alone or bcl-2 together with c-raf (Figure 5A; for the sake of clarity, error bars were omitted from the Figures). Under the latter conditions, CAT activities were undetectable (Table 3), indicating that the raf signalling was down-regulated. Thus the death-protective function of bcl-2 is entirely maintained in the absence of an intact raf kinase-dependent signalling pathway. It is even capable of neutralizing the negative impact of a dominant-negative raf mutant on cell survival [29].

DISCUSSION

The present study demonstrates that bcl-2 does not stably associate with c-raf-1 *in vitro* and confers cell-death protection independently of the raf kinase activity. Two previous reports claimed an association of bcl-2 with c-raf-1 in immunoprecipitates [19,20]. However, in the first, a normal rabbit serum immunoprecipitated overexpressed recombinant bcl-2 to a similar extent as a serum containing anti-raf antibodies (Figure 3C in ref. [19]). In the second, the finding was based on an anti-bcl-2 Western blot of an anti-raf immunoprecipitate only and the specificity of the raf antibody used was unclear [20]. In addition, neither report provided any clues to the physiological significance of an interaction between bcl-2 and c-raf-1 in unstressed or apoptotically stressed cells.

Our failure to detect a direct interaction between c-raf-1 and bcl-2 was not due to unfavourable experimental conditions since bindings to established partners of both proteins were observed. MEK and BAG-1 are known to interact with the catalytic domain of c-raf-1 [30,31] and were faithfully co-immunoprecipitated with an anti-raf antibody directed against the C-terminal tail of c-raf-1. Because bcl-2 has been claimed to bind to the catalytic site of c-raf-1 as well [19], we were surprised that it was not present in the very same anti-raf immunoprecipitates. Similarly, Bax was identified as a consistent partner of bcl-2 throughout our experiments. If c-raf-1 bound to bcl-2 with a similar affinity to Bax, it would have been detected in anti-bcl-2 immunoprecipitates. Alternatively, the interaction between bcl-2 and c-raf-1 may have been impeded by the presence of the anti-bcl-2 antibodies. In this case, anti-FLAG antibodies should have co-immunoprecipitated c-raf-1 with N-terminally tagged

FLAG-bcl-2 because these antibodies do not directly interact with bcl-2. It should be noted that overexpression of FLAG-bcl-2 confers a similar cell-death protection to bcl-2 (results not shown), suggesting that physiological binding proteins should be retained. At least we expected a binding of detergent-solubilized or cytosolic c-raf-1 to GST-bcl-2, since this assay was antibody-independent and Bax was again specifically retained.

To account for the possibility that bcl-2/c-raf-1 interactions were destroyed under the extraction/immunoprecipitation conditions used, we modified the salt concentrations and pH of the buffers, included phosphatase inhibitors to allow a phosphorylation-dependent binding and used chemical cross-linkers before the cells were lysed in detergent (results not shown). None of these modifications helped to detect an interaction between c-raf-1 and bcl-2. Furthermore, the detergent conditions used (0.2% NP40) have been considered to preserve protein-protein interactions [30] compared with harsh conditions (RIPA buffer) where Bax did not bind to bcl-2 ([11]; I. Otter and C. Borner, unpublished work). Finally, even in the yeast two hybrid system, where weak protein-protein interactions are usually detected, no association between bcl-2 and c-raf-1 was found [12].

For the first time, putative bcl-2 and/or c-raf-1 binding proteins were investigated under conditions where cells had been stressed with apoptotic agents. Interestingly, c-raf-1 appears to be proteolysed into a 35 kDa form upon cellular treatments with staurosporine or BFA. This may explain why the level of immunoprecipitable p74 c-raf-1 was lower in R6-bcl-2 cells deprived of serum or stressed with apoptotic agents (Table 1). We currently do not know whether c-raf-1 proteolysis has any regulatory role in apoptosis. Given the C-terminal specificity of the anti-raf antibody, the 35 kDa protein may be similar to t-raf-1, a truncated, constitutively active form of c-raf-1 consisting of the catalytic domain only. t-raf-1 has been reported to interact with bcl-2 when co-transfected into haemopoietic 32D.3 cells [19]. This was not seen with the 35 kDa form of c-raf-1 reported here, although this protein may not be identical with t-raf-1.

Irrespective of a direct interaction between bcl-2 and c-raf-1, we wanted to uncover a physiological link between the two proteins. We envisaged that c-raf-1 kinase activity would be required for the survival activity of bcl-2. This was based on a recent report showing that BAG-1 not only interacted with c-raf-1 but also stimulated its protein kinase activity [30]. Given the previous identification of BAG-1 as a bcl-2-binding protein [21], it was proposed that BAG, bcl-2 and c-raf-1 form a trimeric complex that signals for death protection. c-raf-1 would thus be pulled away from the plasma membrane, where it participates in growth-factor signalling, to an intracellular site where bcl-2 and BAG-1 reside (mitochondrial, nuclear, endoplasmic reticulum membranes) [21]. In this complex, c-raf-1 would either use the classical MAPK signalling pathway or a novel, MAPK-unrelated signalling system to suppress apoptosis. We have recently performed immunocytochemical analysis of R6-bcl-2/c-rafmix cells and found that c-raf-1 does not redistribute from its usual cytosolic localization to nuclear/endoplasmic reticulum or mitochondrial envelopes where bcl-2 resides (results not shown). In addition, our results here show that the death-protection function of bcl-2 was fully maintained irrespective of whether the raf kinase activity was reduced by the removal of serum, by the cellular treatments with cAMP or staurosporine or by the co-expression of a dominant-negative kinase-deficient raf-mutant. It has recently been discussed that c-raf-1 may mediate the protective effect of bcl-2 against staurosporine-induced apoptosis because it is a staurosporine-insensitive protein kinase and is inhibited by this drug only at IC₅₀ 5 µM [30]. Although residual

raf kinase activity may have been present, bcl-2 was effectively death-protective on R6 cells at doses up to 20 μ M of staurosporine. In addition, at least one raf-activating protein kinase (protein kinase C) was fully inhibited under these conditions [36], and signalling via the MAPK signalling pathway (towards PRE-CAT) was markedly impeded.

Of course, our data here do not exclude the possibility that bcl-2 and c-raf-1 interact at levels undetectable by immunological and biochemical methods. This would explain why the proteins could be made to associate when highly expressed as recombinant proteins [19], although this approach may also detect protein-protein associations that are non-specific and/or do not reflect those of native proteins. It has been suggested that only 1% of total cellular c-raf-1 and/or bcl-2 may be implicated in a trimeric complex formation with BAG-1 [30]. Whereas such a low level of c-raf-1 protein and kinase activity may suffice to mediate a survival signal, it is difficult to reconcile that 1% of total bcl-2 can provoke a death-protective effect. It has been repeatedly shown that the extent of death protection is directly correlated with the amount of bcl-2 expressed and that a large overexpression of bcl-2 is required to effectively save cells from apoptosis [11,42,43]. Finally, we cannot exclude that bcl-2 uses as-yet unidentified protein kinase-independent properties of c-raf-1 or another form of raf (raf-B) for its survival activity.

To our knowledge this is the first time that a large overexpression of dominant-negative c-raf-1 could be achieved without grossly affecting cell growth and/or survival. This indicates that the negative impact of such an expression can be successfully circumvented by bcl-2 overexpression and reinforces our view that bcl-2 functions independently of the raf kinase activity. We therefore think that bcl-2 uses other molecules than c-raf-1 to block the activation of ICE-like proteases during apoptotic stresses. A gratifying aspect of the present study is that it provides us with a stable, viable cellular system in which signal transduction can be studied in the virtual absence of c-raf-1 protein kinase activity.

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