Bcl2-low-expressing MCF7 cells undergo necrosis rather than apoptosis upon staurosporine treatment

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We present a ribozyme-based strategy for studying the effects of Bcl2 down-regulation. The anti-*bcl2* hammerhead ribozyme Rz-*bcl2* was stably transfected into MCF7 cancer cells and the cleavage of Bcl2 mRNA was demonstrated using a new assay for cleavage product detection, while Western blot analysis showed a concomitant depletion of Bcl2 protein. Rz-*bcl2*-expressing cells were more sensitive to staurosporine than control cells. Moreover, both molecular and cellular read-outs indicated that staurosporine-induced cell death was necrosis rather than apoptosis in these cells. The study of the effects of Bcl2 down-regulation was extended to the global MCF7 protein expression profile, exploiting a proteomic approach. Two reference electro-

pherograms of Rz-*bcl2*-transfected cells, one with the ribozyme in a catalytically active form and the other with the ribozyme in a catalytically inactive form, were obtained. When comparing the two-dimensional maps, 53 differentially expressed spots were found, four of which were identified by MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight) MS as calreticulin, nucleophosmin, phosphoglycerate kinase and pyruvate kinase. How the up-regulation of these proteins might help to explain the modification of Bcl2 activity is discussed.

Key words: anti-*bcl2* hammerhead ribozyme, apoptosis, cleavage assay, MS, necrosis, two-dimensional electrophoresis.

INTRODUCTION

Hammerhead ribozymes are RNA molecules that are able to specifically cleave a target RNA. They consist of two short arms that base-pair with the target, and a catalytic core responsible for cleavage activity. Upon cleavage, the integrity of the target mRNA is disrupted and translation of the corresponding protein is prevented [1]. Hammerhead ribozymes are employed in gene function analysis to down-regulate a gene of interest at the mRNA level [2–5].

The 26 kDa integral membrane protein Bcl2 is located mainly at the mitochondrial outer and inner membranes, but also at the ER (endoplasmic reticulum) membrane [6]. It belongs to an ever-growing family of proteins that share four conserved structural motifs known as BH (Bcl2 homology) domains (BH1 to BH4). Bcl2 family members are functionally divided into anti-apoptotic (Bcl2, Bcl-x_L and Mcl1) and pro-apoptotic (Bax, Bak, Mtd/Bok, DIVA, Bid, Bik and Bad) proteins [7]. The BH3 amphipathic domain specifically mediates homo- and hetero-dimerization through which Bcl2 family members titrate one another's activity [8].

When the mitochondria-dependent apoptotic pathway is triggered, efflux of CytC (cytochrome *c*) from the mitochondrial matrix into the cytoplasm occurs [9]. Here, CytC interacts with Apaf-1 (apoptotic protease-activating factor-1) [10] and induces the proteolytic activation of effector caspases (CPP3, CPP9) that are responsible for the biochemical and morphological changes characteristic of apoptotic cell death [11]. CytC efflux is the

result of membrane disruption upon loss of $\Delta\psi_m$ (mitochondrial membrane potential) [12]. $\Delta\psi_m$ is maintained only if the MPT (mitochondrial permeability transition) pore, a megachannel located at the contact site between the mitochondrial outer and the inner membranes [13], remains closed. The MPT pore consists of several proteins, including VDAC (voltage-dependent anion channel), PBR (peripheral benzodiazepine receptor) and ANT (adenine nucleotide translocator).

Bcl2 family members determine the survival or apoptotic death of cells mainly by preventing or favouring the release of CytC from mitochondria [14]. The anti-apoptotic protein Bcl2 competes with VDAC [15] and ANT [16] for Bax: when Bax binds to VDAC or ANT, the MPT pore is open, whereas when Bcl2 heterodimerizes with Bax the pore remains closed and $\Delta\psi_m$ is retained. Through heterodimerization, Bcl2 also prevents Bax from forming a multimeric complex which inserts autonomously into the mitochondrial membrane and, acting as a channel, mediates CytC efflux [17]. Furthermore, Bcl2, acting upstream of CytC efflux, prevents tBid-mediated Bax translocation from the cytoplasm to the mitochondrial membrane [18], while downstream it competes with CytC for binding to Apaf-1 [19].

Most of the studies that have contributed to elucidation of the roles of Bcl2 have been performed using overexpression systems [20]. Nevertheless, it is conceivable that the study of cell behaviour when Bcl2 is down-regulated may contribute further to the understanding of its functions. Based on these concepts, we have obtained a clone derived from the MCF7 cancer cell line in which *bcl2* gene expression is stably down-regulated by

Abbreviations used: ANT, adenine nucleotide translocator; Apaf-1, apoptotic protease-activating factor-1; BH domain, Bcl2 homology domain; CFA, colony-forming ability; CytC, cytochrome *c*; 2D, two-dimensional; DAPI, 4,6-diamidino-2-phenylindole; ER, endoplasmic reticulum; HMGB1, high-mobility group box; $\Delta\psi_m$, mitochondrial membrane potential; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; MPT, mitochondrial permeability transition; RT, reverse transcription; Rz-*bcl2*, anti-*bcl2* hammerhead ribozyme; Rz-*bcl2*_i, catalytically inactive hammerhead ribozyme; STS, staurosporine; VDAC, voltage-dependent anion channel.

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Figure 1 The hammerhead ribozyme *Rz-bcl2*

The pairing arms of *Rz-bcl2* span nucleotides 2365–2378 within the *Bcl2* RNA (3' untranslated region). The cleavage site is located at nucleotide 2372, as indicated by the arrow. Nucleotides necessary for cleavage activity are in bold type. Numbers correspond to the conventional map of the catalytic core, as reported in Hertel et al. [43]. The disabled ribozyme *Rz-bcl2_i* was obtained by a G → A mutation at position 5 within the catalytic core (boxed).

using the anti-*bcl2* hammerhead ribozyme *Rz-bcl2*. We report the molecular and functional characterization, including a proteomic analysis, of this clone and discuss its contributions to the study of the functions of *Bcl2* and *Bcl2*-related proteins.

MATERIALS AND METHODS

Rz-bcl2 and expression vectors

A schematic representation of the anti-*bcl2* hammerhead ribozyme *Rz-bcl2* is shown in Figure 1. It was targeted against nucleotides 2365–2378 within the 3'-untranslated region of the *Bcl2* mRNA (GenBank accession number M13994). The cleavage site is located at nucleotide 2372, after the AUC triplet. *Rz-bcl2_i*, the catalytically inactive hammerhead ribozyme, which is still able to base-pair with *Bcl2* mRNA but is catalytically inactive because of a G⁵ → A⁵ substitution, was used as a negative control of cleavage activity. Both ribozymes were chemically synthesized as described elsewhere [2].

The pCWRSPN plasmid (a gift from J. J. Rossi, Beckman Research Institute of the City of Hope, Duarte, CA, U.S.A.) was selected as an expression vector for *Rz-bcl2* because it ensures the cytoplasmic localization of the transcript [21]. By cloning the double-stranded DNA fragments corresponding to *Rz-bcl2* and *Rz-bcl2_i* into pCWRSPN, the p*Rz-bcl2* and p*Rz-bcl2_i* vectors respectively were obtained (Figure 2).

Recipient cells and transfection procedure

The *Bcl2*-high-expressing MCF7 epithelial cell line (IST-CBA, Genoa, Italy), which is derived from a human breast carcinoma, was used as the recipient of transfection. MCF7 cells and clones derived from it were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 units/ml penicillin and 100 mg/ml streptomycin at 37 °C in a humidified atmosphere containing 6% CO₂.

MCF7 cells to be transfected were seeded in a 24-well dish at a cell density of 5 × 10⁴ cells/well. After overnight incubation, p*Rz-bcl2*, p*Rz-bcl2_i* and pCWRSPN vectors were transfected as follows: 300 µl of OptiMem medium containing 0.75 µg of DNA and 1.5 µg of DOTAP (dioleoyltrimethylammonium propane; Roche) was added per well. Transfection was stopped 8 h later by the addition of 1 ml of complete Dulbecco's modified Eagle's

Figure 2 Cloning of *Rz-bcl2* and *Rz-bcl2_i* sequences in plasmid pCWRSPN

Double-stranded DNA sequences corresponding to *Rz-bcl2* and *Rz-bcl2_i* were cloned in the pCWRSPN expression vector using *SalI* and *SpeI* respectively as cloning sites. The sense and antisense strands of the *Rz-bcl2* sequence, with the *SalI* and *SpeI* restriction sites at their 5' and 3' ends respectively, were synthesized, mixed and annealed to form the double-stranded *Rz-bcl2* ds. Both plasmid pCWRSPN and *Rz-bcl2* ds were then digested with *SalI* and *SpeI* and ligated. Colony screening was performed using loss of the internal *XbaI* restriction site that is prognostic of successful cloning. The correct formation of p*Rz-bcl2* recombinant plasmid was tested by sequencing. A forward primer (5' TTA GGA AGG CAA CAG ACA G 3') located 120 bp upstream of the *SalI* restriction site was used. Cloning of the *Rz-bcl2_i* sequence was performed analogously. A *KpnI* restriction site, which is not present within the pCWRSPN plasmid, was used for screening of p*Rz-bcl2_i* recombinant plasmid. MCS, multi-cloning site; ITR, adeno-associated virus inverted terminal repeats, which mediate the stable integration of the pCWRSPN plasmid within mammalian genome; RSV LTR, long terminal repeat promoter from Rous sarcoma virus; SV40 polyA, polyadenylation signal from simian virus 40. The AT base pair which substitutes for GC within the p*Rz-bcl2_i* sequence is boxed.

medium. Cells were collected 48 h later and seeded in a 100 mm-diameter dish containing complete medium and 600 µg/ml G418. After 5–6 weeks, independent G418-resistant (G418^r) colonies were isolated, expanded to clonal populations and maintained in medium containing 600 µg/ml G418 for further analyses.

Detection of p*Rz-bcl2* and p*Rz-bcl2_i* transcripts

Total RNA was extracted from samples of 2 × 10⁶ cells (RNAzolTM; Tel-Test Inc.) and 5 µg of DNA-free RNA was retro-transcribed according to standard protocols using PCR reverse primer r (5' TTG GAC AAA CCA CAA CTA 3'), which is located downstream of the *SpeI* restriction site in the pCWRSPN sequence. To detect the p*Rz-bcl2* transcript, 6 µl of the RT (reverse transcription) mixture was amplified using forward primer f (5' GAC ATA GCT CTG ATG AGG 3'), which starts at a *SalI* restriction site, and reverse primer r. The amplification conditions were: one cycle at 94 °C for 3 min, 44 °C for 45 s and 72 °C for 50 s; 40 cycles at 94 °C for 30 s, 44 °C for 45 s and 72 °C for 50 s; and one cycle at 72 °C for 9 min. The 147 bp PCR fragment obtained is specific for the p*Rz-bcl2* transcript, since the pCWRSPN transcript does not contain the *Rz-bcl2* sequence and therefore is not recognized by the forward primer. Detection of the p*Rz-bcl2_i* transcript was performed similarly: 6 µl of RT mixture was amplified at 45 °C for 35 cycles using the specific forward primer f_i (5' GGT ACC ATA GCT CTA ATG 3'), which spans the *KpnI* restriction site and the 5' end of the

Figure 3 Assay of cleavage of *Bcl2* mRNA

(A) Schematic representation of the cleavage assay. Total RNA was retrotranscribed with primer a, and the mixture of cDNA molecules was expected to also contain one derived from the 3' cleavage fragment. The 3' end of this molecule was specifically extended using primer cb-stop. The b portion of the cb-stop primer base-pairs with cDNAs derived from both cleaved and native mRNAs, but is unable to trigger extension of the complementary strand because its 3' priming end is capped by the 3' *o*-propylamino substituent. The *bcl2*-unrelated c portion of primer cb-stop, acting as template, allows instead extension of the 3' end of the cDNA molecule corresponding to the downstream fragment of the cleavage reaction, so that the c complementary sequence is added. This 3' end extension is selective for the cDNA derived from the cleaved fragment, because the full-length cDNA lacks a free 3'-hydroxy group. The extended cDNA fragment was then amplified by semi-nested PCR, using primer c as a forward primer and primers d and e (which is internal relative to a) as reverse primers. (B) Representation of primers cb-stop and cb. Segment b is the sense sequence downstream of the cleavage site of the ribozyme (the pairing region; solid rectangle); c is the *bcl2*-unrelated sequence. The 3'-hydroxy group of cb-stop, but not cb, is capped by the 3' *o*-propylamino substituent (star). (C) RT-PCR amplification of the 3' cleavage fragment. A 3' end extension was obtained by adding primer cb-stop (lanes 1 and 2, Neo/6 cells; lane 4, Rz/10A cells) or primer cb (lane 3, Neo/6 cells; lane 5, Rz/10A cells), which lacks the 3' cap. Semi-nested PCR amplification was performed with c (lanes 1, 3, 4 and 5) or b (lane 2) as forward primer, and d and e as reverse primers. PCR fragments of 645 and 627 bp were obtained respectively. Lane L, 1 kb DNA ladder.

Rz-*bcl2*_i sequence, and the same reverse primer as used for pRz-*bcl2* transcript detection. A 149 bp PCR fragment was obtained.

Assay of the cleavage of *Bcl2* mRNA

The cleavage assay is depicted in Figure 3(A). Samples of 5 µg of total RNA were retrotranscribed as described above using

reverse primer a (5' CCC CTC TAC TGC TCT TT 3'), which is located downstream of the cleavage site. The cDNA molecule derived from the 3' cleavage fragment was extended at its 3' end using the primer cb-stop (5' GAA GCA AGC CTC CTG AAA AGC TAT TTA CTG CCA AAG GGA 3'; Figure 3B) as template. The cb-stop primer contains, in addition to a 21 nt b sequence complementary to the cDNA fragment, an additional 18 nt c

segment unrelated to the *bcl2* sequence. Furthermore, its 3' terminus is chemically capped by a 3' *o*-propylamino substituent. The cb-stop primer at a final concentration of 0.3 μ M and 2.5 units of *Taq* polymerase (Finnzymes) were added to the RT mixture for incubation at 94 °C for 3 min, 85 °C for 3 min, 80 °C for 2 min and 60 °C for 10 min.

Samples of 5 μ l of the extended cDNA fragment were then amplified by semi-nested PCR. The c forward and d reverse (5' GGT AGG TTC TGC GGA CTT 3') primers, which amplify a 805 bp fragment, were used first. Amplification conditions were: one cycle at 94 °C for 3 min, 50 °C for 45 s and 72 °C for 50 s; 30 cycles at 94 °C for 30 s, 50 °C for 45 s and 72 °C for 50 s; and one cycle at 72 °C for 9 min.

The same conditions were then used to re-amplify 5 μ l of the first PCR mixture with forward primer c and reverse primer e (5' GGG GGT GCG TAT CCA AAA 3'), which is located upstream of primer d. The c and e primers amplify a 645 bp PCR product.

Immunodetection of Bcl2 and Bax proteins

Cell lysates were subjected to Western blot analysis as described previously [22]. Briefly, 30 μ g of protein per sample was separated on SDS/12 %-PAGE and blotted on to a nylon membrane (Hybond-C extra; Amersham Life Science). The blot was then incubated with 0.2 μ g/ml mouse anti-(human Bcl2) monoclonal IgG₁ antibody (Santa Cruz Biotechnology) and 0.4 μ g/ml sheep anti-(mouse IgG) secondary antibody (Santa Cruz Biotechnology). Chemiluminescent detection of antibody binding was accomplished using an ECLTM Detection Kit (Amersham Biosciences). The blot was stripped by incubation in distilled water at 70–80 °C for 30 min and reprobed with 0.2 μ g/ml anti-(human Bax) monoclonal antibody (Santa Cruz Biotechnology). To ensure that equal amounts of proteins were loaded, the blot was reprobed with 0.2 μ g/ml mouse anti-actin monoclonal antibody (Roche).

Cell death analysis

Cell proliferation

Cells were seeded in 96-well plates (10⁴ cells/well). After 24 h the medium was changed and fresh medium containing 1, 3, 6 or 10 μ M STS (staurosporine) was added. The inhibition of cell growth was determined 24 h later by a reduction assay based on tetrazolium salt: 20 μ l of CellTiter 96 AQ_{ueous} One Solution reagent (Promega) was added per well, and absorbance at 490 nm was recorded 2 h later using a 96-well plate reader.

CFA (colony-forming ability)

The ability of a single cell to form a colony in the presence of STS, reported as CFA, was determined by seeding cells at a density of 2 $\times$ 10² per 60 mm dish. After 24 h, 0.3, 1 or 3 nM STS was added, and 14–21 days later colonies were stained with Crystal Violet and counted to determine relative CFA.

Annexin assay

Cells were seeded at a density of 3 $\times$ 10⁵ cells per 35 mm dish, and 24 h later were exposed for 5 h to 300 nM STS. At that time cells were collected, processed according to the Annexin-V-FLUOS test (Roche) and analysed by flow cytometry (FACScalibur; Becton Dickinson). Approx. 10000 cells per sample were used. Apoptotic (lower right quadrant) and necrotic (upper right quadrant) cells were identified and expressed as a percentage of total dead cells.

HMGB1 (high-mobility group box 1) protein detection

Cells were seeded at a density of 3 $\times$ 10⁵ cells per 35 mm dish, and 24 h later were exposed for 5 h to 500 μ l of OPTIMEM (Invitrogen) containing 150 nM STS. Thereafter, the culture medium was collected and concentrated to 50 μ l by using a Microcon (Millipore), while cells were trypsinized and centrifuged (400 g, 10 min). The proteins of both fractions were then extracted and submitted to immunoblotting as described above. The blot was incubated with 0.15 μ g/ml rabbit anti-HMGB1 primary antibody (Pharmingen) and 0.4 μ g/ml goat anti-(rabbit IgG) secondary antibody. Ponceau Red staining was used to ensure that equal amounts of protein were loaded (results not shown). The quantification of signal intensity was performed using Scion image software.

The detection of HMGB1 by immunofluorescence was done as follows. Approx. 2 $\times$ 10⁴ cells were seeded on slides. The following day, cells were exposed to 150 nM STS. After 5 h, cells were fixed with 2% paraformaldehyde in PBS for 15 min at room temperature. The slides were blocked with 0.15% glycine and 0.5% BSA in PBS, and then incubated with 0.1 μ g/ml anti-HMGB1 primary antibody and 5 μ g/ml Alexa Fluor 594 donkey anti-(rabbit IgG) secondary antibody (Molecular Probes). DAPI (4,6-diamidino-2-phenylindole) reagent, which allows visualization of nuclear material, was added to the mounting reagent (Vectashield).

2D (two-dimensional) gel electrophoresis, image acquisition and data analysis

2D electrophoresis was performed as described in [23]. Isoelectric focusing was carried out on preformed immobilized 18 cm gel strips with a non-linear gradient from pH 3 to pH 10 (supplied by Amersham Biosciences). Isoelectrofocusing strips were rehydrated in a medium containing 8 M urea, 4% CHAPS, 1% dithioerythritol, a trace of Bromophenol Blue and supplemented with carrier ampholyte at 0.2% for analytical and at 0.7% for preparative gels. Samples of 60 μ g were loaded (simultaneously with rehydration) in the analytical runs, while 1 mg samples were loaded (after rehydration) in preparative gels. The analytical and preparative gels were run under conditions described previously [23]. After isoelectric focusing runs, strips were equilibrated at room temperature in denaturing solutions: (1) for 12 min in 50 mM Tris/HCl, 6 M urea, 2% SDS, 2% dithioerythritol and 30% glycerol, pH 6.8; and (2) for 5 min in 50 mM Tris/HCl, 6 M urea, 2% SDS, 2.5% iodoacetamide, 30% glycerol, pH 6.8, and a trace of Bromophenol Blue. Then the second dimension (SDS/PAGE) was carried out on 9–16% (w/v) polyacrylamide linear gradient gels (18 cm $\times$ 20 cm $\times$ 1.5 mm), at a constant current of 40 mA/gel and at 10 °C, until the dye front reached the bottom of the gel. Analytical gels were stained with ammoniacal silver nitrate as described previously [24], while preparative gels were stained according to a silver staining protocol compatible with MS [25]. Stained gels were then digitized using a computing densitometer (300S; 4000 $\times$ 5000 pixels, 12 bits/pixel; Molecular Dynamics, Sunnyvale, CA, U.S.A.). To highlight all qualitative and quantitative variations, image analysis was carried out with the MELANIE 3 computer system (GeneBio, Geneva, Switzerland). Reported pI and molecular mass values were determined experimentally by co-migration with human plasma as internal standard [26].

Protein identification of separated spots by MS

Protein identification was carried out by peptide mass fingerprinting on an Ettan MALDI-TOF (matrix-assisted

Figure 4 Analysis of pRz-*bcl2*-transfected cells

(A) Western blot of Bcl2 and Bax in transfected MCF7 cells. Bcl2 and Bax were detected in MCF7 parental cells and in G418^r clones transfected with pCWRSPN empty vector (Neo/6), pRz-*bcl2*, vector recombinant for the disabled ribozyme Rz-*bcl2*_i (Rz/2A), and pRz-*bcl2* vector recombinant for the Rz-*bcl2* active ribozyme (Rz/10A). Actin was immunodetected to control for loading per lane. (B, C) Expression assay of Rz-*bcl2* and Rz-*bcl2*_i in transfected MCF7 cells. The transcripts from pRz-*bcl2* (Rz/10A clone) and pRz-*bcl2*_i (Rz/2A clone) vectors were amplified by RT-PCR using a forward primer specific for the Rz-*bcl2* and Rz-*bcl2*_i sequences respectively. The Neo/6 clone was used as a negative control, since the transcript from pCWRSPN contains no ribozyme sequence and therefore is recognized by neither forward primer. Lanes L, 100 bp DNA ladder; lanes 1, Neo/6 cells; lanes 2, Rz/10A cells (B) or Rz/2A cells (C). The Figures are inverted tone images.

laser-desorption ionization–time-of-flight) Pro instrument (Amersham Biosciences). After visualization by silver staining, electrophoretic spots were excised and destained/dehydrated [27]. Protein digestion was performed as described in [28]. Mass fingerprinting database searching was carried out using ProFound (<http://129.85.19.192/profound.bin/WebProFound.exe>), Pept-Ident (<http://www.expasy.org>) and Mascot (Matrix Science Ltd., London, U.K.; <http://www.matrixscience.com>) software available on-line.

RESULTS

Decreased Bcl2 protein levels in MCF7 G418^r clones

Double-stranded DNA fragments corresponding to the active (Rz-*bcl2*) and disabled (Rz-*bcl2*_i) ribozymes were cloned in the pCWRSPN expression vector, giving pRz-*bcl2* and pRz-*bcl2*_i respectively (Figure 2). A series of independent G418^r clones derived from transfection of the various vectors in MCF7 cells were analysed by Western blotting for Bcl2 protein levels. In eight out of ten pRz-*bcl2*-transfected clones, the Bcl2 protein level was lower than that in MCF7 parental cells or clones transfected with pCWRSPN empty vector (three clones) or pRz-*bcl2*_i vector (three clones) (results not shown). Clone Rz/10A (representative of pRz-*bcl2* transfection) and control clones Neo/6 and Rz/2A (representative of pCWRSPN and pRz-*bcl2*_i transfections respectively) were used in all experiments. Immunodetection of Bcl2 in these clones clearly showed that Bcl2 protein was decreased to almost undetectable levels (Figure 4A).

Detection of Rz-*bcl2* expression

Expression of Rz-*bcl2* and Rz-*bcl2*_i was checked in Rz/10A and Rz/2A cells respectively by RT-PCR using a forward primer specific for each transcript and a reverse primer common for both transcripts. A 147 bp PCR fragment from pRz-*bcl2* (Figure 4B, lane 2) and a 149 bp PCR fragment from pRz-*bcl2*_i (Figure 4C, lane 2) were obtained respectively. The ribozyme was detected in both clones, while no PCR fragment was obtained

Figure 5 Growth inhibition (A) and CFA (B) in the presence of STS

(A) Neo/6 (■), Rz/2A (●) and Rz/10A (▽) cells were treated with 1, 3, 6 or 10 μ M STS for 24 h, and then the percentage of viable cells was determined using the CellTiter 96 AQueous One Solution assay (Promega). Values are means $\pm$ S.D. for three independent experiments. (B) Neo/6 (■), Rz/2A (●) and Rz/10A (▽) cells were treated with 0.3, 1 or 3 nM STS, and 14–21 days later colonies were counted. Values are means $\pm$ S.D. for three independent experiments.

in Neo/6 cells, which contain no ribozyme sequence (Figure 4B, lane 1; Figure 4C, lane 1).

Assay of cleavage by Rz-*bcl2* in clone Rz/10A

In order to relate Bcl2 knock-down to Rz-*bcl2* activity, the 3' cleavage fragment produced from Bcl2 mRNA by the ribozyme was detected using the newly developed cleavage assay depicted in Figure 3(A). The results of the cleavage assay are reported in Figure 3(C). When primer cb-stop (Figure 3B) was used, the 645 bp PCR product was detected in Rz/10A cells, where Rz-*bcl2* exerts its cleavage activity (lane 4), but not in Neo/6 control cells (lane 1). In contrast, when primer cb, containing a 3'-hydroxy group instead of the 3' cap (Figure 3B), was used, the PCR product was obtained from both Rz/10A (lane 5) and Neo/6 (lane 3) cells, since the c sequence is added not only to the cDNA molecule complementary to the 3' cleavage fragment, but also to the full-length cDNA. As a positive control of retrotranscription, semi-nested PCR was performed in Neo/6 cells with b instead of c as forward primer. In this way a 627 bp PCR product was obtained (lane 2) which did not contain the 18 bp section corresponding to the c sequence.

Functional assays

The ability of Bcl2 to protect against cell death was challenged using STS. STS was selected because its apoptotic pathway is blocked by Bcl2 at the mitochondrial level (see Discussion), so that a lower Bcl2 level should result in higher sensitivity to the drug. Data on growth inhibition and survival (Figure 5) agree

Figure 6 FACS analysis of cells after staining with annexin V-FLUOS (FL1) and propidium iodide (FL2)

(A) Rz/10A control cells; (B) STS-exposed Rz/10A cells; (C) Rz/2A control cells; (D) STS-exposed Rz/2A cells. LL (lower left), living cells; UR (upper right), necrotic cells; LR (lower right), apoptotic cells.

with this antagonistic relationship between Bcl2 and STS. STS at 1 μ M was sufficient to decrease the growth of the Rz-*bcl2*-expressing clone by up to 60% compared with control (untreated) cells. In contrast, cells transfected with the inactive ribozyme or with control plasmid needed 10-fold higher doses of STS to reach the same level of growth inhibition (Figure 5A). To relate growth inhibition to cell death, we determined the ability of single cells to form colonies in the presence of STS (Figure 5B). STS decreased the CFA of both Rz/10A and control cells, but at each concentration tested the CFA of Rz/10A cells was lower than that of controls, indicating a greater induction of cell death.

To investigate whether necrosis or apoptosis was responsible for cell death, Rz/10A and Rz/2A cells were exposed to 300 nM STS for 5 h, stained with annexin V and subjected to FACS analysis (Figure 6). In untreated Rz/10A (Figure 6A) and Rz/2A (Figure 6C) cells, necrosis (upper right quadrant) and apoptosis (lower right quadrant) were represented equally. Following exposure to STS, the percentage of necrotic Rz/10A cells (26%; Figure 6B, upper right quadrant) was 2.6-fold higher than that of Rz/2A necrotic cells (10%; Figure 6D, upper right quadrant). In contrast, the percentage of apoptotic Rz/10A cells (74%; Figure 6B, lower right quadrant) was 0.78-fold lower than that of Rz/2A apoptotic cells (90%; Figure 6D, lower right quadrant). These results indicate that more necrosis than apoptosis was at the basis of cell death induced by STS in Rz/10A cells.

The results of the annexin V assay were confirmed by staining of HMGB1 protein. HMGB1 binds to the chromatin of interphase cells, with an association/dissociation equilibrium [29]. With apoptosis, association prevails over dissociation (Figure 7A, a), whereas with necrosis dissociation prevails over association (Figure 7A, n), so that HMGB1 is released into the cytoplasm and thereafter into the culture medium, where it can be detected. Upon treatment with STS, the frequency of DAPI-stained nuclei

Figure 7 Chromatin association of HMGB1 protein

(A) Immunofluorescence. Apoptotic cells (labelled a) show nuclear material condensed into spots and positivity to anti-HMGB1 antibody, while in necrotic cells (n) the condensation of nuclear material is not associated with HMGB1 staining. Interphase healthy cells (i) are positive to anti-HMGB1 antibody, like apoptotic cells, but show diffuse nuclear staining. (B) STS-induced damage. Cell death analysis (right panel) of fragmented nuclei (left panel) of Rz/10A (Δ) and Rz/2A ($\bullet$) cells exposed to STS for 5 h. Each point represents the mean of two independent experiments. (C) Immunoblotting. Proteins in the pellet (p) and in the culture medium (s) of Rz/10A and Rz/2A cells exposed to 150 nM STS for 5 h were analysed by SDS/PAGE and immunodetected with anti-HMGB1 antibody.

presenting chromatin-condensed bodies (fragmented nuclei) was enhanced (Figure 7B, left panel). Those that were HMGB1 positive (necrotic) and those that were HMGB1 negative (apoptotic) were distinguished and their ratio at each STS concentration tested was calculated (Figure 7B, right panel). It can be seen that the necrosis/apoptosis ratio of Rz/10A cells was higher than that of Rz/2A cells at all STS concentrations tested, even if a tendency to decrease was observed. The binding of HMGB1 to chromatin was also measured by Western blot (Figure 7C). In the absence of STS treatment, the HMGB1 signal of both Rz/2A and Rz/10A cells was in the pellet (87.4% and 78.6% respectively) rather than in the supernatant (12.6% and 21.3% respectively). Following STS treatment, the HMGB1 signal in Rz/2A cells was detected both in the pellet (43.3%) and in the supernatant (56.7%), whereas in Rz/10A cells it was mostly in the supernatant (87.1%) rather than in the pellet (12.9%).

Figure 8 Computer-processed images of silver-stained 2D polyacrylamide gels of the RZ/2A clone (left) and the RZ/10A clone (right)

Arrows and circles indicate spots that differ qualitatively and quantitatively between the two electroblotograms. Numbers from 1 to 19 indicate spots increased in the inactive ribozyme gel, while numbers from 20 to 41 indicate those augmented in the active ribozyme gel. Letters from A to G indicate spots present only in the RZ-*bcl2* gel, and those from H to L indicate those present exclusively in the RZ-*bcl2* gel.

Table 1 MALDI-TOF peptide mass fingerprinting identification parameters

The Z-score, as defined by the ProFound search tool, was higher than 1.66 for all identified spots. Mean Δ mass indicates the discrepancy between experimental and theoretical peptide mass values.

Spot no.	Protein name	SwissProt accession no.	No. of matching peptides	Mean Δ mass (p.p.m.)	Sequence coverage (%)
26	Phosphoglycerate kinase 1	P00558	8	61	24
27	Nucleophosmin (fragment)	P13084	5	29	26
33	Pyruvate kinase M1/M2 (fragment)	P14618	8	67	23
36	Calreticulin (fragment)	P27797	8	45	16

Evaluation of the effects of pRZ-*bcl2* on the proteome of transfected MCF7 cells

Cellular extracts from clone RZ/10A (expressing the catalytically active form of RZ-*bcl2*) and from clone RZ/2A (expressing the catalytically inactive form) were separated by high-resolution 2D electrophoresis. In order to rule out experimental variations not due to the presence of RZ-*bcl2*, at least three gels were analysed for each clone and two synthetic images were built by computer matching. Each synthetic image represents all spots constantly present in all gels from the same clone. All 2D silver-stained electroblotograms contained more than 3000 spots, as computed using MELANIE 3 software (Figure 8).

Quantitative differences were considered significant that showed a variation of at least 2 times in spot relative volume (volume is integration of absorbance over spot area; relative volume is the volume of a single spot divided by the total spot volume over the whole image). By comparison of RZ-*bcl2* gels (Figure 8, left panel) with RZ-*bcl2* gels (Figure 8, right panel), we observed that, in the RZ-*bcl2* gels, 19 spots decreased and 22 spots increased in intensity, seven spots disappeared and five new spots appeared. The observed variable spots are indicated in Figure 8 by numbers and letters. Numbers from 1 to 19 indicate

the spots that were increased in the inactive ribozyme gels, while numbers from 20 to 41 indicate those that were increased in the active ribozyme gels. Letters from A to G mark the spots present only in the RZ-*bcl2* gels, and those from H to L mark spots present exclusively in the RZ-*bcl2* gels. Some of these protein spots were then digested with trypsin and identified by mass fingerprinting analyses followed by database searching. Peptide masses of 2D-separated proteins derived by trypsinization were compared with theoretical peptide masses calculated for all proteins in the NCBI and SWISS PROT databases using specific algorithms (see the Materials and methods section). Proteins were identified on the basis of corresponding experimental and theoretical peptide fingerprinting patterns, as limited by the following searching criteria: a difference of < 75 p.p.m. in mass between experimental and theoretical peptide masses and a Z-score higher than 1.66, as defined by the ProFound search tool. In this way, unambiguous protein identification was achieved for four proteins, corresponding to spot numbers 26, 27, 33 and 36 (Table 1).

Figure 9 shows the peptide mass fingerprinting spectra and peptide coverage maps obtained for the four proteins, which were identified as phosphoglycerate kinase, nucleophosmin, pyruvate

Figure 9 Peptide mass fingerprinting spectra obtained by MALDI-TOF MS for spot numbers 26, 27, 33 and 36

The distributions of matching peptides in the complete protein sequences are underlined (see Table 1 for identification parameters).

kinase and calreticulin. It is worth noting that three out of four identified spots represented fragments of the complete sequences. Further analyses are in progress to identify low-abundance proteins.

DISCUSSION

Down-regulation of protein levels using a hammerhead ribozyme represents a valuable tool for investigating the role of a gene of interest. In the present work, we report the use of the anti-*bcl2* hammerhead ribozyme Rz-*bcl2* (Figure 1) for the study of Bcl2 functions. The Rz-*bcl2* sequence was cloned in the pCWRSPN expression vector (Figure 2) and transfected into MCF7 tumour cells. In this way the Rz/10A clone, which stably expresses the ribozyme (Figure 4B, lane 2), was obtained. The marked decrease in Bcl2 protein levels observed in this clone relative to the MCF7 parental cell line and to control clones (Figure 4A) indicates that Rz-*bcl2* is able to affect

the expression level of *bcl2*. Furthermore, the fact that the inactive-ribozyme-expressing Rz-/2A clone did not show any significant variation in Bcl2 protein levels indicates that Rz-*bcl2* acts by cleaving the Bcl2 mRNA rather than through an antisense effect. The evidence that the decrease in Bcl2 protein was due to Rz-*bcl2* was supported by molecular analysis of the cleavage activity inside living cells. Using a newly developed cleavage assay, we were able to identify the cleaved fragment of Bcl2 mRNA produced by Rz-*bcl2* in Rz/10A cells (Figure 3C, lane 4). The novelty and the simplicity of this cleavage assay, relative to others reported previously, are of note. Selective amplification of the 3' fragment by cleavage is obtained by RT-PCR, exploiting the ability of *Taq* polymerase to extend the cDNA fragment using the tagging primer (cb-stop in Figure 3B) as template. The advantages relative to terminal deoxynucleotidyl transferase-mediated RT-PCR [30] and reverse ligation-mediated RT-PCR [31] are that neither polydeoxynucleotidyl tailing of cDNA nor 5' end phosphorylation of the 3' cleavage fragment

and subsequent ligation of an RNA tag are required. A Northern blot-based method to detect ribozyme cleavage fragments in living cells has also been reported [32], but our method offers higher sensitivity, since PCR ensures the amplification of even few molecules of cleavage fragment.

Taken together, the molecular results reported so far indicate that the hammerhead ribozyme Rz-*bcl2* efficiently lowers *bcl2* expression. Thus Rz/10A cells can be considered a Bcl2-low-expressing clonal variant of MCF7 cells, and offer a suitable model in which to study Bcl2 in the context of down-regulation, where new aspects of its functioning may be disclosed.

In Rz/10A cells, the anti-apoptotic activity of Bcl2, which was investigated by comparing the sensitivity of the Rz/10A and control clones to STS, showed some unexpected features (Figures 5–7). STS, a protein kinase inhibitor that induces apoptosis, was selected as an ideal apoptotic drug since it is known to induce a decrease in *bcl2* gene expression at the transcriptional [33], post-transcriptional [34] and post-translational [35] levels. Conversely, Bcl2 is known to counteract the apoptotic pathway triggered by STS, preventing CytC efflux from mitochondria [20]. On the basis of this antagonism, a higher sensitivity to STS is expected where Bcl2 protein levels are lower. Data on growth inhibition (Figure 5A) and CFA (Figure 5B) confirmed our expectations, since Bcl2-low-expressing Rz/10A cells were much more sensitive to STS than control cells. However, when we used two independent assays to investigate the type of STS-induced cell death, in both cases we found that Rz/2A cells underwent apoptosis, whereas Rz/10A cells underwent necrosis (Figures 6 and 7). An at least partial explanation for such a result, which is in agreement with previously reported data [36,37], comes from the analysis of Bax expression levels. Pro- and anti-apoptotic members of Bcl2 family are known to titrate one another's activity by heterodimerization, so that when the expression level of one of the members is altered, compensatory changes occur to restore the balance [8]. In agreement with these findings, we observed that in Rz/10A cells the pro-apoptotic protein Bax, which Bcl2 antagonizes at various levels (see Introduction), was down-regulated along with Bcl2 (Figure 4A). Thus we can hypothesize that Rz/10A cells, which lack Bcl2 and could in theory undergo Bax-dependent apoptosis, shift towards Bax-independent necrosis because they also have a diminished content of Bax.

Rz/10A cells are useful for the systematic analysis of those proteins which, like Bax, are already known to be related to Bcl2, but also offer the unique possibility of identifying new proteins that modify their expression levels in response to Bcl2 down-regulation and may contribute to our understanding of the Bcl2-controlled network. Such proteins are the 53 identified by 2D electrophoresis as being differentially expressed in Rz/10A cells relative to Rz/2A cells (Figure 8). Four of the overexpressed proteins were sequenced by MALDI-TOF MS, and were identified as calreticulin, nucleophosmin, phosphoglycerate kinase and pyruvate kinase (Table 1).

This effect on calreticulin, a Ca^{2+} binding protein which resides within the lumen of the ER, may help to explain the behaviour of Rz/10A cells upon STS treatment. Calreticulin is one of the most important proteins devoted to the storage of Ca^{2+} in the ER [38], and controls the folding of newly synthesized (glyco)proteins [39]. The calreticulin expression level has also been associated with cell sensitivity to apoptosis, with calreticulin-overexpressing/deficient cells showing a higher/lower sensitivity to STS-induced apoptosis [40]. Bcl2, which also localizes to the ER [6], has been shown to lower the ER Ca^{2+} concentration when overexpressed [41,42]. The up-regulation of calreticulin in Bcl2-low-expressing Rz/10A cells could therefore be considered to be a consequence of an increased ER Ca^{2+} concentration, which would

require a higher level of calreticulin to be stored. Furthermore, the overexpression of calreticulin with the concomitant depletion of Bcl2 and down-regulation of Bax (Figure 4A) could render Rz/10A cells highly prone to STS-induced damage, explaining why necrosis instead of apoptosis is preferentially induced (Figures 6 and 7).

In conclusion, combining the results of cellular read-outs with information from proteome analysis, we can affirm that, in MCF7 cells, a new expression profile is installed in response to Bcl2 knock-down that may help to clarify aspects of Bcl2 functioning that are still relatively obscure. We also believe that the identification of the other differentially expressed proteins will contribute to further understanding of the processes in which Bcl2 is involved.

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REFERENCES

- 1 Takagi, Y., Warashina, M., Stec, W. J., Yoshinari, K. and Taira, K. (2001) Survey and summary: recent advances in the elucidation of the mechanisms of action of ribozymes. *Nucleic Acids Res.* **29**, 1815–1834
- 2 Probst, J. C. (2000) Antisense oligodeoxynucleotide and ribozyme design. *Methods* **22**, 271–281
- 3 Tang, C. K., Concepcion, X. Z., Milan, M., Gong, X., Montgomery, E. and Lippman, M. E. (1999) Ribozyme-mediated down-regulation of ErbB-4 in estrogen receptor-positive breast cancer cells inhibits proliferation both *in vitro* and *in vivo*. *Cancer Res.* **59**, 5315–5322
- 4 List, H. J., Lauritsen, K. J., Reiter, R., Powers, C., Wellstein, A. and Riegel, A. T. (2001) Ribozyme targeting demonstrates that the nuclear receptor coactivator aib1 is a rate-limiting factor for estrogen-dependent growth of human mcf-7 breast cancer cells. *J. Biol. Chem.* **276**, 23763–23768
- 5 Kondo, H., Mori, S., Takino, H., Kijima, H., Yamasaki, H., Ozaki, M., Tetsu, I., Urata, Y., Abe, T., Sera, Y. et al. (2000) Attenuation of expression of gamma-glutamylcysteine synthetase by ribozyme transfection enhance insulin secretion by pancreatic beta cell line MIN6. *Biochem. Biophys. Res. Commun.* **278**, 236–240
- 6 Gotow, T., Shibata, M., Kanamori, S., Tokuno, O., Ohsawa, Y., Sato, N., Isahara, K., Yayoi, Y., Watanabe, T., Leterrier, J. F. et al. (2000) Selective localization of Bcl-2 to the inner mitochondrial and smooth endoplasmic reticulum membranes in mammalian cells. *Cell Death Differ.* **7**, 666–764
- 7 Adams, J. M. and Cory, S. (2001) Life-or-death decision by the Bcl-2 protein family. *Trends Biochem. Sci.* **26**, 61–66
- 8 Kelekar, A. and Thompson, C. B. (1998) Bcl-2-family proteins: the role of the BH3 domain in apoptosis. *Trends Cell Biol.* **8**, 324–330
- 9 Gottlieb, R. A. (2000) Programmed cell death. *Drug News Perspect.* **13**, 471–476
- 10 Zou, H., Henzel, W. J., Liu, X., Lutschg, A. and Wang, X. (1997) Apaf-1, a human protein homologous to C elegans CED-4, participates in cytochrome C dependent activation of caspase-3. *Cell* **90**, 405–413
- 11 Earnshaw, W. C., Martins, L. M. and Kaufmann, S. H. (1999) Mammalian caspases: structure, activation, substrates and functions during apoptosis. *Annu. Rev. Biochem.* **68**, 383–424
- 12 Petit, P. X., Goubert, M., Dirole, P., Susin, S. A., Zamzami, N. and Kroemer, G. (1998) Disruption of the outer mitochondrial membrane as a result of large amplitude swelling: the impact of irreversible permeability transition. *FEBS Lett.* **426**, 111–116
- 13 Cassarino, D. S. and Bennett, Jr, J. P. (1999) An evaluation of the role of mitochondria in neurodegenerative diseases: mitochondrial mutations and oxidative pathology, protective nuclear responses, and cell death in neurodegeneration. *Brain Res. Rev.* **29**, 1–25
- 14 Jurgensmeier, J. M., Xie, Z., Deveraux, Q., Ellerby, L., Bredesen, D. and Reed, J. C. (1998) Bax directly induces release of cytochrome c from isolated mitochondria. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 4997–5002
- 15 Shimizu, S., Matsuoka, Y., Shinohara, Y., Yoneda, Y. and Tsujimoto, Y. (2001) Essential role of voltage-dependent anion channel in various forms of apoptosis in mammalian cells. *J. Cell Biol.* **152**, 237–250
- 16 Vieira, H. L., Haouzi, D., El Hamel, C., Jacotot, E., Belzacq, A. S., Brenner, C. and Kroemer, G. (2000) Permeabilization of the mitochondrial inner membrane during apoptosis: impact of the adenine nucleotide translocator. *Cell Death Differ.* **7**, 1146–1154

- 17 Antonsson, B., Montessuit, S., Sanchez, B. and Martinou, J. C. (2001) Bax is present as a high molecular weight oligomer complex in the mitochondrial membrane of apoptotic cells. *J. Biol. Chem.* **276**, 11615–11623
- 18 Eskes, R., Desagher, S., Antonsson, B. and Martinou, J. C. (2000) Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. *Mol. Cell. Biol.* **20**, 929–935
- 19 Rossé, T., Olivier, R., Monney, L., Rager, M., Conus, S., Fellay, I., Jansen, B. and Borner, C. (1998) Bcl-2 prolongs cell survival after Bax-induced release of cytochrome c. *Nature (London)* **391**, 496–499
- 20 Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P. and Wang, X. (1997) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* **275**, 1129–1132
- 21 Bertrand, E., Castanotto, D., Zhou, C., Carbonnelle, C., Lee, N. S., Good, P., Chatterjee, S., Grange, T., Pictet, R., Kohn, D. et al. (1997) The expression cassette determines the functional activity of ribozymes in mammalian cells by controlling their intracellular localization. *RNA* **3**, 75–88
- 22 Citti, L., Mariani, L., Capecchi, B., Piras, A., Leuzzi, R. and Rainaldi, G. (1998) The sensitization of cells treated with O⁶-methylguanine to alkylation damage is affected by the number of O⁶-methylguanine-DNA-methyltransferase molecules escaped from inactivation. *Mutat. Res.* **14**, 173–179
- 23 Bjellqvist, B., Hughes, G. J., Pasquali, C., Paquet, N., Ravier, F., Sanchez, J. C., Frutiger, S. and Hochstrasser, D. F. (1993) A non linear wide-range immobilized pH gradient for two dimensional electrophoresis and its definition in a relevant pH scale. *Electrophoresis* **14**, 1357–1365
- 24 Oakley, B. R., Kirsch, D. R. and Morris, R. (1981) A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* **105**, 361–363
- 25 Sinha, P., Poland, J., Schnolzer, M. and Rabilloud, T. (2001) A new silver staining apparatus and procedure for matrix-assisted laser desorption/ionization-time of flight analysis of proteins after two-dimensional electrophoresis. *Proteomics* **1**, 835–840
- 26 Bjellqvist, B., Hughes, G. J., Pasquali, C., Paquet, N., Ravier, F., Sanchez, J. C., Frutiger, S. and Hochstrasser, D. F. (1993) The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences. *Electrophoresis* **14**, 1023–1031
- 27 Gharahdaghi, F., Weinberg, C. R., Meagher, D. A., Imai, B. S. and Mische, S. M. (1999) Mass spectrometric identification of proteins from silver-stained polyacrylamide gel: a method for the removal of silver ions to enhance sensitivity. *Electrophoresis* **20**, 601–605
- 28 Hellman, U., Wernstedt, C., Gonen, J. and Heldin, C. H. (1995) Improvement of an "In-Gel" digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing. *Anal. Biochem.* **224**, 451–455
- 29 Scaffidi, P., Misteli, T. and Bianchi, M. E. (2002) Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature (London)* **418**, 191–195
- 30 Albuquerque-Silva, J., Houard, S. and Bollen, A. (1998) Tailing cDNAs with terminal deoxynucleotidyl transferase in RT-PCR assays to identify ribozyme cleavage products. *Nucleic Acids Res.* **26**, 3314–3316
- 31 Wang, J. P., Enjoji, M., Tiebel, M., Ochsner, S., Chan, L. and Teng, B. B. (1999) Hammerhead ribozyme cleavage of apolipoprotein B mRNA generates a truncated protein. *J. Biol. Chem.* **274**, 24161–24170
- 32 Suzuki, T., Anderegg, B., Ohkwa, T., Irie, A., Engebraaten, O., Halks-Miller, M., Holm, P. S., Curiel, D. T., Kashani-Sabet, M. and Scanlon, K. J. (2000) Adenovirus-mediated ribozyme targeting of HER-2/neu inhibits in vivo growth of breast cancer cells. *Gene Ther.* **7**, 241–248
- 33 Peet, G. W. and Li, J. (1999) IkappaB kinases alpha and beta show a random sequential kinetic mechanism and are inhibited by staurosporine and quercetin. *J. Biol. Chem.* **274**, 32655–32661
- 34 Gubina, E., Rinaudo, M. S., Szallasi, Z., Blumberg, P. M. and Mufson, R. A. (1998) Overexpression of protein kinase C isoform epsilon but not delta in human interleukin-3-dependent cells suppresses apoptosis and induces bcl-2 expression. *Blood* **91**, 823–829
- 35 Deng, X., Ruvolo, P., Carr, B. and May, Jr, W. S. (2000) Survival function of ERK1/2 as IL-3-activated, staurosporine-resistant Bcl2 kinases. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 1578–1583
- 36 Saeki, K., You, A., Okuma, E., Yazaki, Y., Susin, S. A., Kroemer, G. and Takaku, F. (2000) Bcl-2 down-regulation causes autophagy in a caspase-independent manner in human leukemic HL60 cells. *Cell Death Differ.* **7**, 1263–1269
- 37 Polisen, L., Mariani, L., Collecchi, P., Piras, A., Zaccaro, L. and Rainaldi, G. (2002) Bcl-2-negative MCF7 cells over-express p53: implications for cell cycle and sensitivity to cytotoxic drugs. *Cancer Chemother. Pharmacol.* **50**, 127–130
- 38 Michalak, M., Robert Parker, J. M. and Opas, M. (2002) Ca²⁺ signaling and calcium binding chaperones of the endoplasmic reticulum. *Cell Calcium* **32**, 269–278
- 39 Ellgaard, L. and Helenius, A. (2003) Quality control in the endoplasmic reticulum. *Nat. Rev. Mol. Cell Biol.* **4**, 181–191
- 40 Nakamura, K., Bossy-Wetzel, E., Burns, K., Fadel, M. P., Lozyk, M., Goping, I. S., Opas, M., Bleackley, R. C., Green, D. R. and Michalak, M. (2000) Changes in endoplasmic reticulum luminal environment affect cell sensitivity to apoptosis. *J. Cell Biol.* **150**, 731–740
- 41 Pinton, P., Ferrari, D., Magalhaes, P., Schulze-Osthoff, K., Di Virgilio, F., Pozzan, T. and Rizzuto, R. (2000) Reduced loading of intracellular Ca²⁺ stores and downregulation of capacitative Ca²⁺ influx in Bcl-2-overexpressing cells. *J. Cell Biol.* **148**, 857–862
- 42 Foyouzi-Youssefi, R., Arnaudeau, S., Borner, C., Kelley, W. L., Tschopp, J., Lew, D. P., Demareux, N. and Krause, K. H. (2000) Bcl-2 decreases the free Ca²⁺ concentration within the endoplasmic reticulum. *Proc. Natl. Acad. Sci. U.S.A.* **23**, 5723–5728
- 43 Hertel, K. J., Pardi, A., Uhlenbeck, O. C., Koizumi, M., Ohtsuka, E., Uesugi, S., Cedergren, R., Ekstein, F., Gerlach, W. L. and Hodgson, R. (1992) Numbering system for the hammerhead. *Nucleic Acids Res.* **20**, 3252

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