Bcl-2 phosphorylation is required for inhibition of oxidative stress-induced lysosomal leak and ensuing apoptosis

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Abstract B-cell leukemia/lymphoma 2 (Bcl-2) blocks oxidant-induced apoptosis at least partly by stabilizing lysosomes. Here we report that phosphorylation of Bcl-2 may be required for these protective effects. J774 cells overexpressing wild-type Bcl-2 resist oxidant-induced lysosomal leak as well as apoptosis, and this protection is amplified by pretreatment with phorbol 12-myristate 13-acetate (which promotes protein kinase C (PKC)-dependent phosphorylation of Bcl-2). In contrast, cells overexpressing the Bcl-2 mutant S70A (which cannot be phosphorylated) are not protected in either circumstance. Transfection with Bcl-2(S70E), a constitutively active Bcl-2 mutant which does not require phosphorylation, is protective independent of PKC activation. In contrast, C_2-ceramide, a putative protein phosphatase 2A activator, abolishes the protective effects of wild-type Bcl-2 overexpression but does not diminish protection afforded by Bcl-2(S70E). Additional results suggest that, perhaps as a consequence of lysosomal stabilization, Bcl-2 may prevent activation of phospholipase A2, an event potentially important in the ultimate initiation of apoptosis.

Key words: Apoptosis; B-cell leukemia/lymphoma 2; Lysosome stability; Oxidative stress; Protein kinase C

1. Introduction

A short period of oxidative stress initiates lysosomal rupture catalyzed by intra-lysosomal low molecular weight iron which is normally present in lysosomes [1–5]. This rupture seems to consist of two phases. The early one results in only a limited release of lysosomal hydrolytic enzymes, while the second one, that occurs even in the absence of continued oxidative stress, leads to a much more pronounced lysosomal breach and ensuing apoptosis [6–8]. This lysosomal leakage may help initiate apoptosis; however, the precise mechanisms are presently unknown. Among several possibilities, lysosomal enzymes which are released to the cytosol may predispose to apoptosis by (i) direct activation of pro-caspases [9,10], (ii) attack on mitochondrial and lysosomal membranes resulting in release of pro-apoptotic cytochrome *c* and more lysosomal enzymes [6,11], (iii) activation of pro-apoptotic proteins, such as Bid [12], or (iv) activation of lytic cytosolic enzymes (such as phospholipase A2 (PLA2)) that, in turn, attack mitochondrial and/or lysosomal membranes and further propagate the process [6,13].

Overexpression of B-cell leukemia/lymphoma 2 (Bcl-2) blocks the second, but not the first, phase of lysosomal rupture [6], whereas both phases are prevented by pre-exposure of cells to the potent iron chelator desferrioxamine. The endocytotic uptake of this chelator [14–16], and its almost exclusive localization within the acidic vacuolar compartment, converts the normal lysosomal pool of low molecular weight iron into a redox-inactive form [2–5].

In contrast to desferrioxamine, the way in which Bcl-2 protects against (the second phase of) lysosomal rupture is far from clear. Others have reported that Bcl-2 seems to be active as an anti-apoptotic protein only as a phosphorylated homodimer or when phosphorylated and complexed with Bcl-X_L [17–19]. We have, therefore, tested the importance of Bcl-2 phosphorylation in maintaining lysosomal stability and preventing apoptosis using cells overexpressing a phosphorylation-negative Bcl-2 mutant [Bcl-2(S70A)], a phosphorylation-equivalent mutant [Bcl-2(S70E)], or wild-type Bcl-2 [Bcl-2(wt)]. We also induced phosphorylation/dephosphorylation of Bcl-2(wt) in both parental and overexpressing cells. The results indicate that phosphorylated – but not dephosphorylated – Bcl-2 suppresses both the second phase of lysosomal rupture and prevents ensuing apoptosis. The results further suggest that Bcl-2 may prevent activation of PLA2 by lysosomal enzymes.

2. Materials and methods

2.1. Materials

Chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated. RPMI 1640 medium, fetal bovine serum, glutamine, penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, NY, USA). The CaspACE FITC-VAD-FMK In Situ Marker® and the serine/threonine phosphatase kit were both from Promega Corporation (Madison, WI, USA), while *N*-[1-(2,3-dioleoyl)propyl]-*N*,*N*,*N*-trimethyl-ammonium salt (DOTAP) was from Boehringer Mannheim (Indianapolis, IN, USA). Monoclonal agarose-conjugated anti-Bcl-2 antibodies (sc-7382 AC) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and monoclonal anti-phosphoserine antibodies (clone PSR-45) were from Sigma. The plasmids (see below) were kind gifts from Dr. W.S. May, University of Texas, Medical Branch, Galveston, TX, USA.

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Abbreviations: AA, arachidonic acid; AO, acridine orange; Bcl-2, B-cell leukemia/lymphoma 2; DOTAP, *N*-[1-(2,3-dioleoyl)propyl]-*N*,*N*,*N*-trimethyl-ammonium salt; OA, okadaic acid; LY, lucifer yellow; PKC, protein kinase C; PLA2, phospholipase A2; PMA, phorbol 12-myristate 13-acetate; PP2A, protein phosphatase 2A

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2.2. Cell cultures

J774 cells (a murine histiocytic lymphoma cell line) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 1% non-essential amino acids. Cells were maintained in plastic flasks and sub-cultivated at confluence twice a week. For experiments, cells were transferred into 35 mm 6-well plates (5×10^5 cells/well) 24 h before use.

2.3. Preparation of transfected cell lines

Parental J774 cells were sub-cultivated into 6-well plates as described above and transfected, using liposomal DOTAP delivery according to the manufacturer's directions, with the expression plasmids (mouse) Bcl-2α(wt)/pMKITNeo, Bcl-2α(S70A)/pMKITNeo, Bcl-2α(S70E)/pMKITNeo, and the empty vector pMKITNeo [17,20]. Each vector contains a neomycin resistance element (Neo). Ten stable transfected clones of each line were selected by neomycin resistance and their expression levels of exogenous Bcl-2 were assayed by Western blot analysis (vide infra). Transfected clones which each had a 5-fold increase of immunoreactive Bcl-2 protein were selected, expanded, and used for experiments. The three different Bcl-2 overexpressing J774 cell lines are hereafter named 'J774-Bcl-2(wt)', 'J774-Bcl-2(S70A)', and 'J774-Bcl-2(S70E)', while the non-overexpressing 'empty vector' control cell line is named 'J774-vector'.

2.4. Oxidative stress

Parental cells, J774-vector cells, and the three lines (wt, S70A and S70E) of Bcl-2 overexpressing cells, were exposed for 1 h at 37°C to a steady-state concentration (40 μM) of H_2O_2 as described previously [6,7,21]. To abruptly obtain this steady-state concentration of H_2O_2, 37 mU/ml glucose oxidase (1.5 μg/ml) was added to complete medium together with an initial bolus of H_2O_2 to bring the starting H_2O_2 concentration to 40 μM. Following 1 h of incubation under otherwise standard culture conditions, catalase was added to clear the H_2O_2. The cells were then quickly rinsed with sterile phosphate-buffered saline (PBS, 0.1 M NaCl, 0.002 M KCl, 0.01 M Na_2HPO_4, 0.001 M KH_2PO_4, pH 7.4), and fresh complete medium was added.

2.5. Lysosomal stability assessment

Cells were assessed for lysosomal stability at different times following oxidative stress using the acridine orange (AO) and lucifer yellow (LY) uptake methods [2–4,6–8,11,15,22].

AO is a lysosomotropic base and a metachromatic fluorochrome exhibiting red fluorescence when highly concentrated (as is the case in intact lysosomes where AO is retained in its charged – AOH^+ – form). Lysosomal intactness was evaluated by assaying red fluorescence by flow cytometry [6,7,23], using a Becton-Dickinson FACScan (Becton-Dickinson, Mountain View, CA, USA) equipped with a 488 nm argon laser. Cells were analyzed following exposure to 5 μg/ml AO for 15 min, under otherwise standard culture conditions, followed by a short rinse (×2) in complete medium. After AO staining, cells were detached by scraping with a rubber policeman and collected for flow cytometric assessment using the FL3 channel for red fluorescence. CellQuest software was used to analyze the data.

Cells were grown on coverslips and exposed to LY (0.25 mg/ml medium), a bright fluorochrome and a marker of endocytosis, for 1 h at otherwise standard culture conditions. The cells were then kept for another 4 h in complete medium without the marker molecule (to obtain selective labeling of secondary lysosomes) and then exposed to oxidative stress as described above. Following this, the cells were returned to standard culture condition for various periods of time and, finally, analyzed using a Nikon microphot-SA fluorescence microscope (Nikon, Tokyo, Japan) equipped with a Hamamatsu ORCA-100 color digital camera (Hamamatsu, Japan).

2.6. Apoptosis assessment assay

The DNA fragmentation (Nicoletti) assay is based on propidium iodide staining of nuclear DNA and was performed essentially as described elsewhere [24]. Briefly, cell pellets from individual wells were gently resuspended in 1.5 ml of a hypotonic and membrane-disrupting solution of propidium iodide (50 μg/ml in 0.1% sodium citrate with 0.1% Triton X-100) in 12×75 mm polypropylene tubes. The tubes were kept in the dark overnight at 4°C before flow cytometric analyses. The propidium iodide-induced red fluorescence of suspended individual nuclei was measured by flow cytofluorometry, using the FL3 channel. Nuclei with partly degraded DNA were counted and their frequency was expressed as percent of the total number of analyzed nuclei (10 000).

Apoptosis was also evaluated by microscopical analysis of living cells, initially stained according to the manufacturer's directions with a FITC-conjugated broad spectrum inhibitor of caspases, Casp-ACE™ FITC-VAD-FMK In Situ Marker® that irreversibly binds to activated caspases. Briefly, cells were seeded at 5×10^5 cells/ml, exposed to oxidative stress as described above, and returned to standard culture conditions for another 4 h. The marker was added to the medium at a final concentration of 10 μM. The cells were incubated in the dark for 20 min, rinsed (×3, 5 min in all) in PBS, and observed, counted, and photographed using the above Nikon fluorescence microscope.

2.7. Measurement of ^{3}H-arachidonic acid (AA) release (PLA2 activity)

Cells were labeled with 1.5 μCi/ml ^{3}H-AA by incubation in serum-free medium at 37°C for 18 h as previously described [25]. Unincorporated ^{3}H-AA was removed by washing (×3) with PBS. Cells were then resuspended in fresh complete medium and exposed to oxidative stress as described above. The culture medium was then replaced by serum-free medium containing 1% fatty acid-free albumin and cells were returned to standard culture conditions for another 8 h. Cells were then scraped into their medium, and cells and fragments were pelleted by centrifugation at $13\,000\times g$ for 10 min. The supernatant was chromatographed on Cibracron blue 3GA-agarose and retained radioactivity (representing free AA bound to albumin) was eluted using 0.1 M guanidine-HCl. Radioactivity was measured by scintillation counting. Counts from cells+medium, reflecting total incorporation of ^{3}H-AA into cell membrane phospholipids, were given the value *A*, while counts from the eluted supernatant, considered to reflect AA released to the medium and bound to albumin, were given the value *B*. The amount of released AA was expressed as $B/A\times100$.

2.8. Protein phosphatase 2A (PP2A) assay

PP2A activity was determined on lysed cells by measuring the generation of inorganic phosphate (P_i) from the phosphorylated peptide, RRA(pT)VA, using a molybdate-malachite green-phosphate complex assay (serine/threonine phosphatase assay system) as described by the manufacturer. Cell lysates were prepared in a detergent lysis buffer (0.25% Nonidet P-40, 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin). The phosphatase assay was performed in a PP2A-specific reaction buffer (final concentration 50 mM imidazole (pH 7.2), 0.2 mM ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.02% 2-mercaptoethanol, 0.1 mg/ml bovine serum albumin) using 100 μM phosphorylated peptide as a substrate/μg cell lysate protein. After a 30-min incubation at room temperature, molybdate-malachite green was added and P_i was measured by optical density at 600 nM.

2.9. Western blot analysis

Approximately 10^6 J774-Bcl-2 cells were lysed by addition of 0.5 ml of a solution containing 20 mM Tris–HCl, 120 mM NaCl, 10% glycerol, 1 mM Na_3VO_4, 2 mM ethylenediaminotetraacetic acid (EDTA), 1% Triton X-100, 1 mM PMSF, pH 7.5. The lysate was incubated with agarose-conjugated monoclonal anti-Bcl-2 antibodies overnight and the agarose-conjugated immuno-complex was spun down ($500\times g$, 5′). Bcl-2 and antibodies were separated on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels (2 h, 50 mA, room temperature) and transferred onto an Immobilon® membrane (2 h, 200 mA, 4°C). Filters were then incubated for 1 h at room temperature in blocking buffer (5% low-fat milk powder in Tris-buffered saline (TBS)) and then for another 2 h in dilution buffer (0.5% low-fat milk powder in TBS) containing the monoclonal anti-phosphoserine antibody (1:500). After washing (TBS with 0.06% Tween 20), the membranes were incubated (1 h, room temperature) in the above dilution buffer with peroxidase-conjugated secondary antibodies (1:1500). After washing, peroxidase-dependent chemiluminescence was measured employing enhanced chem-

iluminescence Western blotting reagents and hyperfilm according to the manufacturer's instructions.

2.10. Statistical analysis

All experiments were repeated at least three times. Values are given as arithmetic means ± S.D. Significance of differences between groups was determined using Student's two-tailed *t*-test.

3. Results

Cells overexpressing Bcl-2(wt) were protected against oxidant stress-induced lysosomal rupture assayed as numbers of 'pale cells' following AO labeling (Fig. 1A) or cells with reduced number of LY-positive granules (Fig. 1B). In

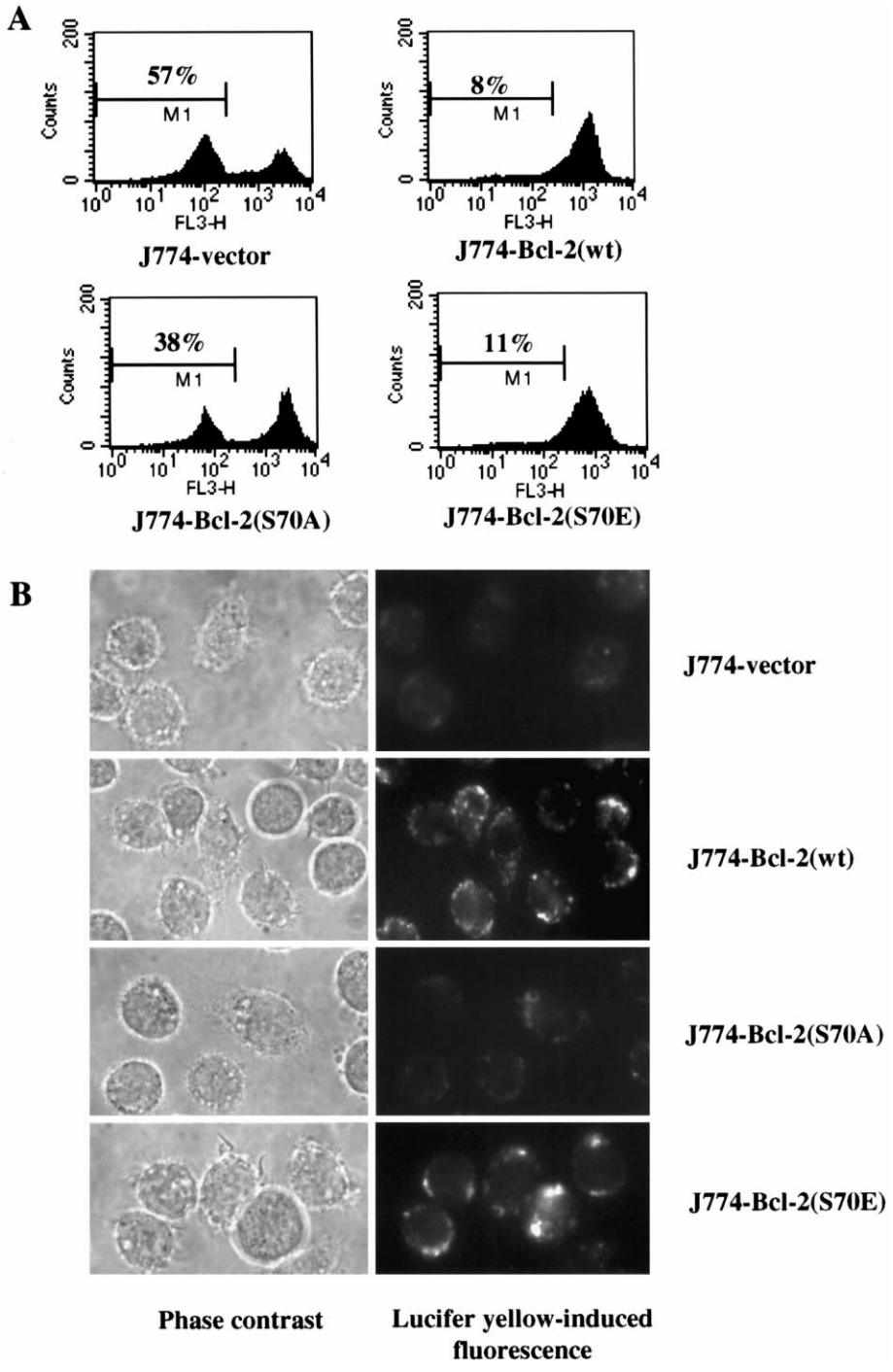

Fig. 1. Phosphorylation of Bcl-2 is required for protection against oxidative stress-induced lysosomal rupture. J774-vector cells and cells from the Bcl-2-overexpressing cell lines, Bcl-2(wt), Bcl-2(S70A) and Bcl-2(S70E), were exposed to oxidative stress, as described in Section 2. After exposure to oxidative stress for 1 h, cells were kept for another 8 h (A) under standard conditions before being exposed to AO and analyzed by cytofluorometry. M1 = 'pale cells' with reduced numbers of intact lysosomes. The J774-vector and J774-Bcl-2(S70A) groups did not differ ($P > 0.05$), while they were both significantly different from the J774-Bcl-2(wt) and J774-Bcl-2(S70E) ($P < 0.01$; $n = 3$ in each group). As an independent assessment of lysosomal intactness, other cells were initially stained with LY, as described in Section 2, then exposed to oxidative stress for 1 h, returned to standard culture conditions for another 2 h and, finally, analyzed by fluorescence microscopy (Fig. 2B). Results shown are representative of three independent experiments.

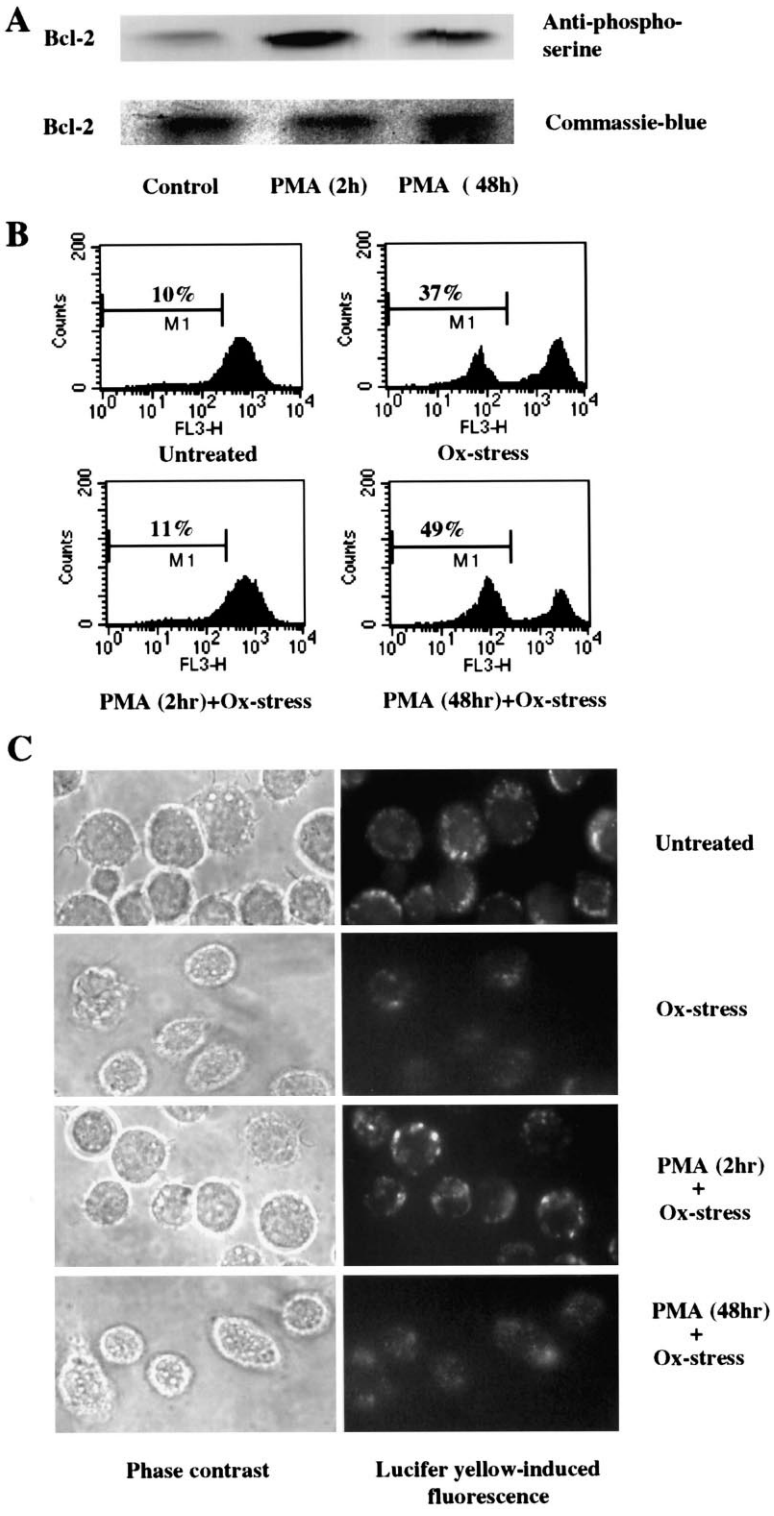

Fig. 2. PKC activity is crucial for stabilizing lysosomal membranes against oxidative stress. Parental J774 cells were pre-incubated with 100 nM PMA for 2 h or 500 nM PMA for 48 h and Bcl-2 phosphorylation was assessed using a monoclonal anti-phosphoserine antibody (A). In additional experiments, the cells were incubated with or without PMA under the same conditions and then exposed to oxidative stress for 1 h and then kept under standard culture conditions for another 8 h and exposed to AO (B). M1 = 'pale cells' with reduced numbers of intact lysosomes. The Ox-stress and the PMA (48 h)+Ox-stress groups were not significantly different ($P > 0.05$), while both of these groups were significantly different from the untreated and the PMA (2 h)+Ox-stress groups ($P < 0.01$; $n = 3$ in each group). Alternatively, following 1 h oxidant exposure the cells pre-incubated with LY were incubated an additional 2 h (C) as described in the legend to Fig. 1. Results presented in B and C are representative of three independent experiments.

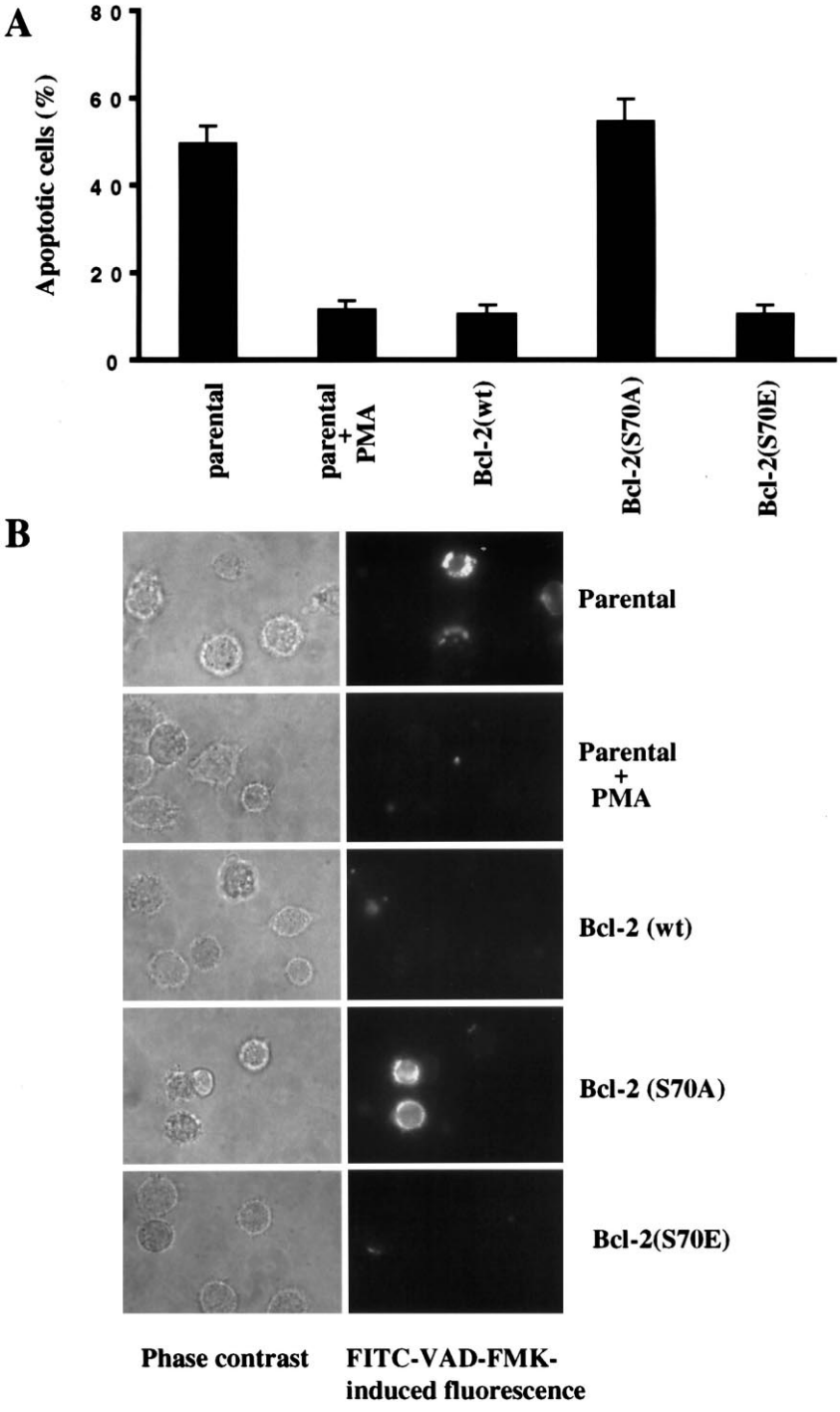

Fig. 3. Bcl-2 phosphorylation is associated with resistance to oxidative stress-induced apoptosis. Five groups of cells were tested: parental cells ± 100 nM PMA for 2 h, cells overexpressing Bcl-2(wt), Bcl-2(S70A) or Bcl-2(S70E). All cells were exposed for 1 h to oxidative stress, as described in Section 2, and then incubated under standard culture conditions for another 8 h (A). Finally, cells were assayed for apoptosis by the Nicoletti method (A). The parental and Bcl-2(S70A) groups did not differ from each other ($P > 0.05$), while each of them was significantly different from each one of the other three groups ($P < 0.01$; $n = 3$ in each group). Alternatively, the cells were incubated 2 h following the 1 h oxidant exposure and analyzed for caspase activation employing the CaspACE™ FITC-VAD-FMK In Situ Marker (B) as described in Section 2. Results shown in B are representative of three independent experiments. The number of FITC-VAD-FMK-positive cells corresponded almost exactly with the frequencies of apoptotic cells shown in A.

contrast to the protective effect of Bcl-2(wt), overexpression of the mutant, site-specific phosphorylation-negative Bcl-2 (Bcl-2(S70A)) did not stabilize lysosomal membranes against oxidative stress. On the other hand, overexpression of Bcl-2(S70E), which is active regardless of phosphorylation status, was at least as effective as Bcl-2(wt) in conferring protection against oxidant-induced lysosomal leak (Fig. 1).

In indirect support of the importance of Bcl-2 phosphorylation, we found that short-term exposure of the parental J774

cells to the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA) enhanced Bcl-2 phosphorylation while long-term PMA exposure, known to deplete active PKC, suppressed Bcl-2 phosphorylation (Fig. 2A). Coinciding with these effects on Bcl-2 phosphorylation, short-term PMA exposure made parental J774 cells resistant to oxidant-induced lysosomal rupture, whether AO uptake or LY retention was used as an indicator. In contrast, long-term PMA exposure did not confer protection (Fig. 2B/C). Overall, these results support the idea that phosphorylated – but not dephosphorylated – Bcl-2 enhances lysosomal membrane stability in cells challenged with an oxidant stress. Importantly, in all cases, these Bcl-2-dependent differences in lysosomal stability are paralleled by changes in susceptibility to subsequent apoptosis.

The importance of Bcl-2 phosphorylation/activation is further supported by investigations employing cells expressing Bcl-2(S70A) (which cannot be phosphorylated and is inactive) and S70E (which cannot be phosphorylated but is constitutively active). As shown in Fig. 3, cells overexpressing Bcl-2(S70A) show no protection against oxidant challenge whereas those expressing Bcl-2(S70E) are as resistant as cells overexpressing wild-type Bcl-2.

If, as the above results suggest, Bcl-2 phosphorylation is required for both lysosomal membrane stabilization and suppression of apoptosis, then activation of PP2A (with consequent reduction in the level of Bcl-2 phosphorylation) should sensitize to both lysosomal rupture and apoptosis. Indeed, we find that, as reported previously [18], pretreatment of target cells with C_2-ceramide enhances PP2A activity and that okadaic acid (OA), a known PP2A inhibitor, partly abrogates PP2A activity (Fig. 4A). C_2-ceramide-mediated activation of PP2A abolished the protective effect of Bcl-2(wt) overexpression on lysosomal integrity while inhibition of PP2A by OA blocked the C_2-ceramide effect (Fig. 4B). Importantly, whereas ceramide sensitized Bcl-2(wt) cells to lysosomal rupture and apoptosis, it had no effect on the phosphorylation-independent (and constitutively active) Bcl-2(S70E) expressing cells (Fig. 4C).

While the foregoing results support the idea that phosphorylated Bcl-2 does enhance lysosomal stability and suppress apoptosis in oxidant-challenged cells, the mechanism(s) underlying these protective effects are by no means clear. As previously mentioned, early and minor lysosomal rupture due to oxidative stress is followed by a second, more extensive rupture, even in the absence of continued oxidant exposure, suggesting that the later rupture is a consequence of events other than continuing oxidative stress [6–8]. In this regard, we recently reported that activation of PLA2 may play an important role in both lysosomal destabilization and ensuing apoptosis, and that Bcl-2 may somehow prevent PLA2 activation [6,8]. In further support of this, we find that PLA2 is not activated in PMA-pretreated parental J774 cells, in Bcl-2(wt) overexpressing cells or in cells overexpressing the constitutively active (phosphorylation-independent) Bcl-2(S70E) (Fig. 5). Moreover, activation of PP2A by C_2-ceramide in Bcl-2(wt) overexpressing cells – earlier shown to block Bcl-2-dependent protection against lysosomal rupture and apoptosis – restored the activation of PLA2 normally seen in wild-type cells undergoing lysosomal rupture and apoptosis. Importantly, C_2-ceramide had no such influence on AA release by cells expressing the constitutively activated (phosphorylation-independent) Bcl-2(S70E) (Fig. 5). Together, these data suggest that phosphorylated, but not dephosphorylated, Bcl-2 simultaneously suppresses lysosomal leak, PLA2 activation and apoptosis in oxidant-challenged J774 cells.

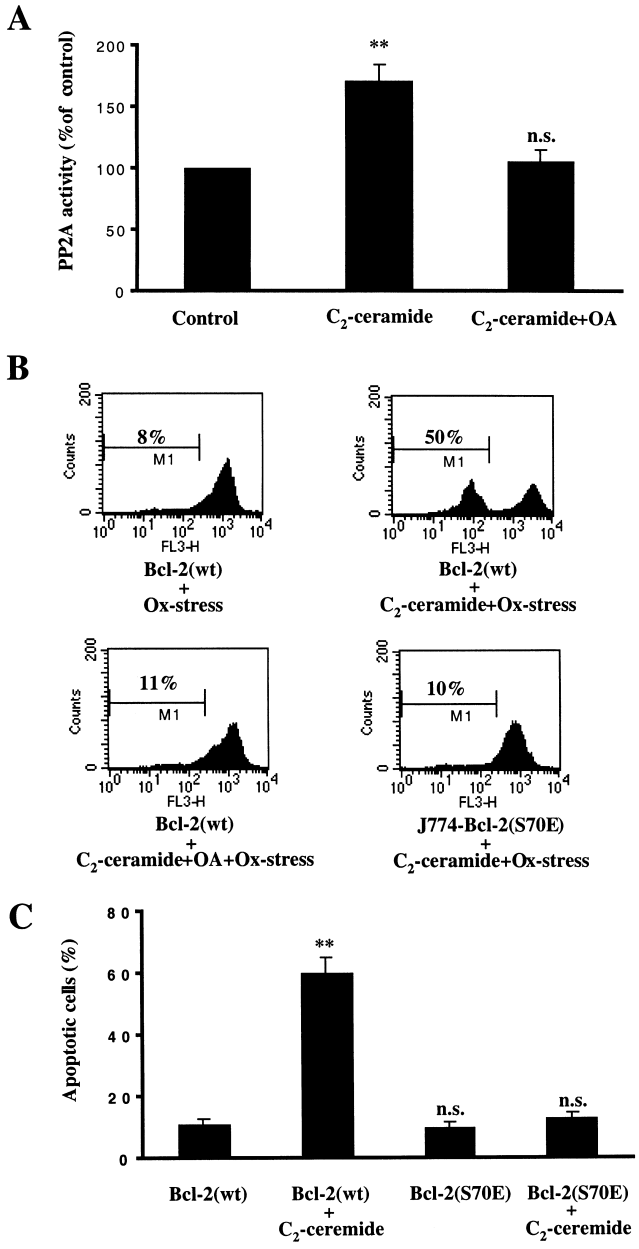

Fig. 4. Activation of PP2A abolishes the protective effect of Bcl-2 on oxidative stress-induced lysosomal rupture. J774-Bcl-2(wt) and J774-Bcl-2(S70E) cells were pretreated with 25 μM C_2-ceramide, or 25 μM C_2-ceramide together with 50 nM OA for 3 h. PP2A activity was determined following this pre-incubation period (A). PP2A activity was significantly increased (vs. control) following C_2-ceramide pretreatment ($P < 0.01$, $n = 3$) while cells pre-incubated with C_2-ceramide+OA did not differ significantly from control. The cells were then exposed to oxidative stress for 1 h and subsequently incubated under standard culture conditions for another 8 h before being exposed to AO (B) or propidium iodide (C), and analyzed by cytofluorometry as described in Section 2. In B, M1 = 'pale cells' with reduced numbers of intact lysosomes. Representative examples are given of three independent experiments. In B and C, the C_2-ceramide+Ox-stress group was significantly different from the other two groups ($P < 0.01$; $n = 3$).

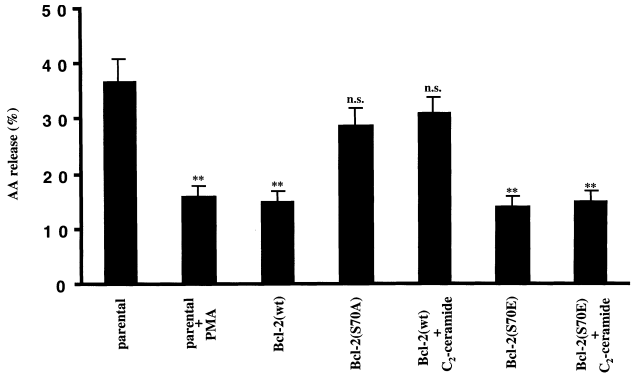

Fig. 5. Phosphorylated Bcl-2 suppresses PLA2 activation. Parental cells, and cells overexpressing Bcl-2(wt), Bcl-2(S70A), and Bcl-2(S70E) were prelabeled with ^{3}H-AA as described in Section 2, and divided into seven groups: parental cells ± 100 nM PMA for 2 h, cells overexpressing Bcl-2(wt) or Bcl-2(S70A), cells overexpressing Bcl-2(wt) and pretreated with 25 μM C_2-ceramide for 3 h, and cells overexpressing Bcl-2(S70E) and pretreated with 25 μM C_2-ceramide for 3 h. All cells were exposed to oxidative stress as described in Section 2 and then incubated for another 8 h under standard culture conditions. Finally, ^{3}H-AA acid release was assayed as described in Section 2. The parental, Bcl-2(S70A) and Bcl-2(wt)+C_2-ceramide groups did not differ significantly ($P > 0.05$) but did differ significantly from each of the other four groups ($P < 0.01$; $n = 3$ in all cases).

4. Discussion

The term programmed cell death, or apoptosis, describes the orchestrated collapse of a cell involving membrane blebbing, cell shrinkage, protein fragmentation, chromatin condensation, DNA degradation, and segmentation into apoptotic bodies which are engulfed by macrophages or neighboring cells [26]. Presently, many studies on apoptosis focus on mitochondria and the release to the cytosol of mitochondrial pro-apoptotic proteins, such as cytochrome *c*, apoptosis-inducing protein (IAP) and the second mitochondria-derived activator of caspase, direct IAP-binding protein with low p*I* (smac/DIABLO) and ensuing activation of various pro-caspases [27–31].

Lysosomal rupture is commonly regarded as a late event in cell death and as a sign of necrosis. During the last few years, however, an increasing number of reports have appeared suggesting that lysosomal rupture with release of proteases such as cathepsins B, L, H, and D may induce apoptosis. Thus, it has been reported, for example, that cathepsin B contributes to TNF-α-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome *c* [32,33], while cathepsin L was found to participate in the activation of caspase-3 [9,34], and cathepsin D may be involved in apoptosis of several types of cells [11,32,35–39]. Consequently, in the initiation of apoptosis, the stability of the lysosomal membrane may be as important as that of the mitochondrial membrane and lysosomal labilization may be a central and early upstream event in apoptosis [13]. The present results, based on two independent methods to assay alterations in lysosomal stability of living cells, lend further support to the concept that partial lysosomal rupture is a starting signal for apoptosis.

Obviously, whether or not a cell undergoes apoptosis following a particular insult depends on complex interactions between a host of anti- and pro-apoptotic proteins. In this regard, proteins belonging to the Bcl-2 gene family may have particularly potent roles [40]. The proteins in this family are characterized by a considerable amino acid sequence homology [41,42], and have been found to regulate the formation of channels that facilitate protein (e.g. cytochrome *c*) release from the mitochondria to the cytosol where pro-caspase-9 is activated [40]. Interestingly, Bcl-2 has been observed to regulate not only the permeability of the outer mitochondrial membrane but also that of the lysosomal membrane (C. Borner, personal communication and results reported herein).

Recently, the importance of post-transcriptional modification of Bcl-2 has been raised [43]. The protein is known to be phosphorylated and dephosphorylated in response to different cell death stimuli [44], and Bcl-2 has been reported to act in an anti-apoptotic fashion only in its phosphorylated form [17]. Multiple kinases have been implicated in the phosphorylation of Bcl-2, including Raf-1 kinase, PKCα, protein kinase A, and Jun N-terminal kinase/stress-activated protein kinase [20,45–47]. However, the identity of the most important kinase(s) as well as the functional significance of Bcl-2 phosphorylation in the regulation of apoptosis remain poorly defined. Bcl-2 phosphorylation has been related to both activation and inactivation of its anti-apoptotic function [43]. Of all kinases mentioned above, only PKCα has been reported to activate Bcl-2 by phosphorylation (at serine 70), while other kinases may inactivate it [17,20].

In order to extend our previous finding that Bcl-2 stabilizes lysosomal membranes in cells exposed to oxidative stress [6,48], and thus prevents apoptosis, we have now studied the activity of Bcl-2 in relation to its phosphorylation status. Our data, obtained by transfection and overexpression of Bcl-2(wt) and two mutant Bcl-2 forms, suggest that phosphorylation of Bcl-2 secondary to PKC activation makes cells resistant to oxidative stress, while PKC inactivation, or activation of PP2A, makes cells more sensitive to oxidative stress with resultant lysosomal rupture and ensuing apoptosis. These new findings lend further support to the suggestion that Bcl-2 may stabilize lysosomes, and that this activity contributes to the anti-apoptotic activity of this protein. Moreover, our results provide additional – although still correlative – evidence that lysosomal rupture is associated with the activation of PLA2. This raises the possibility that activated PLA2 may act to further destabilize the membranes of intracellular organelles (such as lysosomes and mitochondria), thereby promoting the cascade of events known as apoptosis. In this cascade, lysosomal breach may be an early upstream phenomenon.

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