

Phosphorylation of BCL-2 regulates ER Ca^{2+} homeostasis and apoptosis

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Phosphorylation of BCL-2 within an unstructured loop inhibits its antiapoptotic effect. We found that phosphorylated BCL-2 predominantly localized to the endoplasmic reticulum (ER) and tested whether phosphorylation would control its activity at this organelle, where Ca^{2+} dynamics serve as a critical control point for apoptosis. Phosphorylation greatly inhibits the ability of BCL-2 to lower $[\text{Ca}^{2+}]_{\text{er}}$ and protect against Ca^{2+} -dependent death stimuli. Cells expressing nonphosphorylatable BCL-2^{AAA} exhibited increased leak of Ca^{2+} from the ER and further diminished steady-state $[\text{Ca}^{2+}]_{\text{er}}$ stores when compared to cells expressing BCL-2^{wt}. Consequently, when BCL-2 is phosphorylated, Ca^{2+} discharge from the ER is increased, with a secondary increase in mitochondrial Ca^{2+} uptake. We also demonstrate that phosphorylation of BCL-2 inhibits its binding to proapoptotic family members. This inhibitory mechanism manifested at the ER, where phosphorylated BCL-2 was unable to bind proapoptotic members. $[\text{Ca}^{2+}]_{\text{er}}$ proved coordinate with the capacity of BCL-2 to bind proapoptotic BH3-only members, further integrating the apoptotic pathway and Ca^{2+} modulation. Unexpectedly, the regulation of ER Ca^{2+} dynamics is a principal avenue whereby BCL-2 phosphorylation alters susceptibility to apoptosis.

The EMBO Journal (2004) 23, 1207–1216. doi:10.1038/sj.emboj.7600104; Published online 4 March 2004

Subject Categories: differentiation & death

Keywords: apoptosis; BCL-2; calcium; endoplasmic reticulum; phosphorylation

Introduction

Apoptosis is a highly regulated pathway in mammalian cells in which the pro- and antiapoptotic proteins of the BCL-2 family constitute an important control point upstream of irreversible damage to cellular constituents including organelles (reviewed in Adams and Cory, 2001; Martinou and Green, 2001). The antiapoptotic members such as BCL-2

possess sequence conservation within four domains (BH1–4), and bind and sequester proapoptotic proteins. Proapoptotic BCL-2 family proteins can be subdivided into 'multidomain' proteins such as BAX and BAK, which display homology within BH1–3 domains, and the 'BH3-only' proteins, which only possess sequence homology within this amphipathic α -helix, which serves as the critical death domain. The ratio between the antiapoptotic and proapoptotic BCL-2 family members helps determine the susceptibility of cells to a death signal (Oltvai *et al.*, 1993). Genetic and biochemical studies indicate that multidomain BAX and BAK function as a gateway to the intrinsic death pathway operating at both the mitochondria and endoplasmic reticulum (ER). The upstream BH3-only members respond to select death signals and subsequently trigger the conformational activation of BAX and BAK, inducing their intramembranous homo-oligomerization and permeabilization of the mitochondrial outer membrane (Wei *et al.*, 2001). Released intermembranous proteins include cytochrome *c*, which complexes with Apaf 1 and caspase-9 to form a postmitochondrial apoptosome that activates effector caspases (Li *et al.*, 1997). Conversely, BCL-2 can sequester activated BH3-only proteins, and thus inhibit the activation of BAX and BAK (Cheng *et al.*, 2001).

In the absence of BAX and BAK, cells are resistant to a wide variety of death signals including agents that release Ca^{2+} from intracellular stores, such as the lipid second messengers arachidonic acid and C_2 -ceramide as well as oxidative stress. BAX and BAK also localize to the ER, and cells deficient in BAX, BAK have reduced resting $[\text{Ca}^{2+}]_{\text{er}}$, which accounts for their resistance to Ca^{2+} -dependent death stimuli (Scorrano *et al.*, 2003). Reciprocally, expression of the antiapoptotic protein BCL-2 protects cells from death by thapsigargin, an irreversible inhibitor of the sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase (SERCA) responsible for uptake of Ca^{2+} from the cytosol into the ER lumen (Lam *et al.*, 1994). BCL-2 is also found at the ER (in addition to mitochondria and nuclear membrane), and its overexpression resulted in reduced $[\text{Ca}^{2+}]_{\text{er}}$, accompanied by an increased leak of Ca^{2+} from the ER stores (Foyouzi-Youssefi *et al.*, 2000; Pinton *et al.*, 2000). Several studies found that ER-targeted BCL-2 demonstrated selective protection against specific stimuli, suggesting that subcellular location may influence the antiapoptotic role of BCL-2 (Zhu *et al.*, 1996; Wang *et al.*, 2001). Organelle-specific correction of the ER or mitochondrial defect in BAX, BAK doubly deficient cells indicates that many intrinsic death signals require ER-released Ca^{2+} as well as mitochondrial-based BAX or BAK to restore fully apoptosis (Scorrano *et al.*, 2003). Thus, regulation of ER calcium levels may prove to be a common general mechanism whereby cells adjust sensitivity to diverse death stimuli.

BCL-2 family proteins are frequently regulated by post-translational modifications that control their activity and conformation. Antiapoptotic BCL-2 is phosphorylated on specific residues within an unstructured loop in response to diverse stimuli including treatment with chemotherapeutic

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Received: 19 August 2003; accepted: 12 January 2004; published online: 4 March 2004

taxanes (Haldar *et al*, 1998; Ling *et al*, 1998; Scatena *et al*, 1998), survival factor addition (May *et al*, 1994; Poommipanit *et al*, 1999), or PKC activation (Ruvolo *et al*, 1998). In most instances, phosphorylation of BCL-2 has been associated with its inactivation, in that deletion of the loop region or mutation of select phosphorylation sites is reported to enhance the antiapoptotic activity of BCL-2 (Chang *et al*, 1997; Haldar *et al*, 1998; Srivastava *et al*, 1999; Yamamoto *et al*, 1999). However, in another cell system, phosphorylation is reported to augment the activity of BCL-2 to bryostatin-1 (May *et al*, 1994). As multiple kinases have been reported to phosphorylate BCL-2, the functional consequence of BCL-2 phosphorylation should be examined in the context of a given cell type and specific signal.

In cycling Jurkat cells, BCL-2 is normally phosphorylated at G₂/M. Moreover, diverse agents that induce a G₂/M cell cycle block including paclitaxel, vincristine, vinblastine, and nocodazole result in the phosphorylation of BCL-2. JNK kinase appears to be responsible for phosphorylation of Thr69, Ser70, and Ser87 within the unstructured loop. Mutation of these residues in the BCL-2^{AAA} variant enhanced antiapoptotic activity in response to taxol and anti-Fas treatment (Yamamoto *et al*, 1999). Thus, phosphorylation can inactivate BCL-2 at G₂/M, acting as a physiological means of regulating apoptotic susceptibility during the cell cycle. However, little is known concerning how the phosphorylation of BCL-2 actually affects susceptibility to cell death.

As the regulation of ER Ca²⁺ dynamics is a control point for apoptosis (Demaurex and Distelhorst, 2003; Orrenius *et al*, 2003), we asked whether the phosphorylation of BCL-2 might exert its influence on apoptosis by affecting the regulation of ER Ca²⁺. Unexpectedly, we found that phosphorylated BCL-2 predominantly localized to the ER, warranting further testing of this thesis. Data presented here identify a mechanism whereby phosphorylation of BCL-2 inhibits its antiapoptotic activity by altering Ca²⁺ dynamics at the ER.

Results

Nonphosphorylatable BCL-2^{AAA} demonstrates enhanced protection against Ca²⁺-dependent death stimuli

Phosphorylation of BCL-2 has been noted to inhibit its antiapoptotic function (Chang *et al*, 1997; Haldar *et al*, 1998; Srivastava *et al*, 1999; Yamamoto *et al*, 1999). The microtubule-damaging agent paclitaxel arrests cells at G₂/M resulting in phosphorylation of BCL-2, and Jurkat T cells expressing a nonphosphorylatable BCL-2^{AAA} (T69A, S70A, S87A) were found to be more resistant to taxol-induced cell death (Yamamoto *et al*, 1999) (Figure 1A). To assess the spectrum of death stimuli to which nonphosphorylatable BCL-2 displays enhanced protection, we compared Jurkat T cells expressing comparable levels of BCL-2^{wt} or BCL-2^{AAA} with a control line bearing an empty neomycin selection vector (Vector) (Yamamoto *et al*, 1999). The BCL-2^{AAA}-expressing cells proved more resistant than BCL-2^{wt}-expressing cells to several intrinsic pathway death stimuli including thapsigargin, staurosporine, and etoposide, as well as extrinsic pathway anti-Fas antibody treatment (Figure 1A).

To extend these studies, we reconstituted BCL-2^{null} murine embryonic fibroblast cell lines (MEFs) with comparable levels of stably expressed BCL-2^{wt} or BCL-2^{AAA} (Figure 1B).

We then tested the response of the cell lines to the lipid second messenger arachidonic acid, in which apoptosis is heavily dependent on the release of Ca²⁺ from the ER followed by activation of mitochondrial permeability transition (Scorrano *et al*, 2001). BCL-2^{AAA}, as compared to BCL-2^{wt}, further improved survival of MEFs (Figure 1B) and Jurkat cells (Figure 1C) following exposure to 30 μM arachidonic acid. The oxidant H₂O₂ also induces a Ca²⁺-dependent death (Scorrano *et al*, 2003), and Jurkat cells expressing BCL-2^{AAA} were significantly more protected than cells expressing BCL-2^{wt} when treated with H₂O₂ (Figure 1D). This body of data suggests that phosphorylation of BCL-2 can substantially inhibit its ability to protect against Ca²⁺-dependent death stimuli.

Jurkat or reconstituted MEF cells expressing BCL-2^{wt} or BCL-2^{AAA} were subjected to subcellular fractionation, revealing that slower-migrating phosphorylated BCL-2 species localized principally to the light membrane fraction (enriched for ER) rather than the mitochondria-enriched heavy membrane fraction (Figure 1E). The predominant localization of phosphorylated BCL-2 to the ER suggests a testable thesis that phosphorylation of BCL-2 might control its activity at this organelle.

Nonphosphorylatable BCL-2^{AAA} reduces the level of released Ca²⁺

An increasing body of evidence suggests that modulation of ER Ca²⁺ levels is an important mechanism by which BCL-2 family members can regulate the susceptibility of a cell to apoptosis (Nakamura *et al*, 2000; Orrenius *et al*, 2003; Scorrano *et al*, 2003). As BCL-2^{AAA} demonstrated enhanced protection from Ca²⁺-dependent death stimuli, we investigated the Ca²⁺ dynamics of BCL-2^{null} MEFs compared to those corrected with BCL-2^{AAA} or BCL-2^{wt}. Cells were treated with thapsigargin, which irreversibly inhibits the SERCA pump responsible for uptake of Ca²⁺ from the cytosol into the ER lumen. This results in the passive release of Ca²⁺ from ER stores and an increase in cytosolic Ca²⁺, $[Ca^{2+}]_i$, which we measured with the fluorescent dye Fura-2. The restoration of BCL-2^{wt} protein lowered the $[Ca^{2+}]_i$ of BCL-2^{null} MEFs elicited by thapsigargin (Figures 2A and B). Of note, cells corrected with BCL-2^{AAA} as compared to BCL-2^{wt} demonstrated significantly further reduced $[Ca^{2+}]_i$ in response to thapsigargin ($P \leq 0.05$) (Figures 2A and B).

The extent of capacitative Ca²⁺ entry was assessed by the addition of extracellular Ca²⁺. Prior studies reported that cells overexpressing BCL-2 displayed decreased capacitative Ca²⁺ entry (Foyouzi-Youssefi *et al*, 2000; Pinton *et al*, 2000). BCL-2^{null} cells demonstrated greater capacitative Ca²⁺ entry than cells reconstituted with BCL-2^{wt} (Figure 2A), indicating a role for BCL-2 at physiological levels. The capacitative entry was comparable for BCL-2^{AAA} and BCL-2^{wt} cells (Figure 2A).

Nonphosphorylatable BCL-2^{AAA} expression results in diminished mitochondrial Ca²⁺ uptake

Mitochondria are closely juxtaposed to the ER, and modulate the Ca²⁺ response in various signaling pathways following release of Ca²⁺ from the ER (Hajnóczky *et al*, 1995; Rizzuto *et al*, 1998). In addition, mitochondrial Ca²⁺ uptake following ER discharge has been shown to contribute significantly to the susceptibility of a cell to Ca²⁺-dependent death stimuli (Bernardi, 1999). Therefore, we tested whether reconstitution of cells with BCL-2^{AAA}, which alters $[Ca^{2+}]_i$ following ER

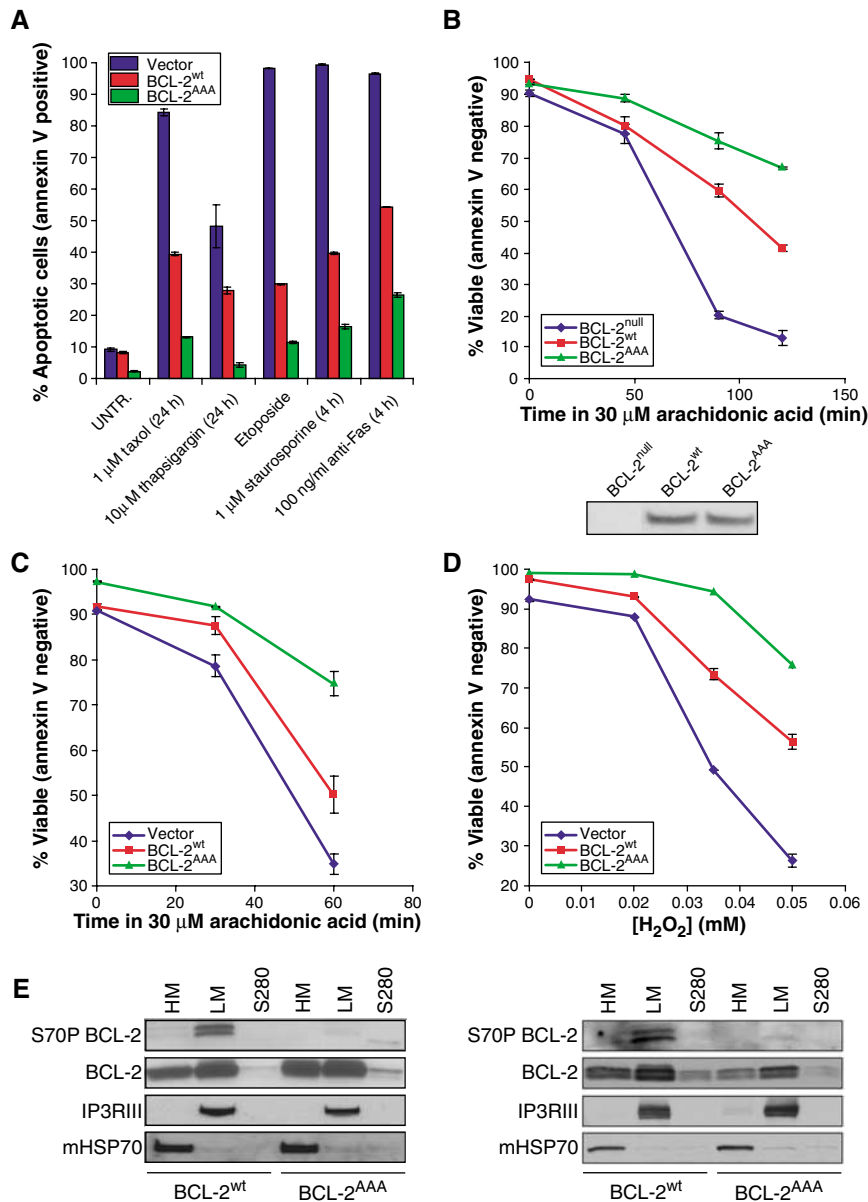


Figure 1 BCL-2^{AAA}-expressing cells are more resistant than BCL-2^{wt}-expressing cells to calcium-dependent death stimuli. (A) Jurkat cells expressing either neo vector, BCL-2^{wt}, or BCL-2^{AAA} were treated as indicated, stained with annexin V, and analyzed by FACS. (B) MEFs or (C) Jurkat lines were treated in medium without serum with 30 μM arachidonic acid for the indicated times. (D) Jurkat cells were treated in growth medium for 17 h with the indicated concentrations of H₂O₂. (E) Untreated Jurkat (left panel) or MEF (right panel) cells were fractionated as described in Materials and methods, and then run on SDS-PAGE and Western blotted with the indicated antibodies.

discharge, also influences the mitochondrial Ca²⁺ concentration, $[Ca^{2+}]_m$, as assessed in single cells by real-time fluorescence microscopy using the fluorescent Ca²⁺ indicator Rhod-2. Passive release of ER Ca²⁺ stores by thapsigargin was followed by a transient increase in $[Ca^{2+}]_m$. Real-time fluorescent images were acquired (Supplementary data, Movies 1–3), and fluorescence values of mitochondrial regions were quantitated. Ca²⁺ uptake by mitochondria was reduced in cells reconstituted with BCL-2^{wt} compared to BCL-2^{null} cells, and even further reduced with the expression of BCL-2^{AAA} (Figure 3A). Quantitation of peak $[Ca^{2+}]_m$ following thapsigargin (Figure 3B) revealed a 50% increase above the baseline for BCL-2^{null} cells. Reconstitution with BCL-2^{wt} reduced this response by approximately half (~22% above baseline), and BCL-2^{AAA} even further reduced Ca²⁺ uptake

(~13% above baseline). Thus, nonphosphorylatable BCL-2^{AAA} demonstrates greater reduction of both intracellular Ca²⁺ (Figure 2B) and peak mitochondrial uptake of Ca²⁺ (Figure 3B) following release of ER stores, when compared to BCL-2^{wt} protein.

We next asked whether the alteration in peak $[Ca^{2+}]_m$ observed in BCL-2^{AAA}-expressing cells reflected a primary aberration in the capacity of mitochondria to uptake Ca²⁺, or was secondary to their lower $[Ca^{2+}]_i$ following release of ER Ca²⁺ stores. We permeabilized the plasma membrane of Rhod-2-loaded cells with 0.001% digitonin and perfused the cells with buffers of fixed $[Ca^{2+}]_i$. The kinetics and extent of Rhod-2 fluorescence were comparable in BCL-2^{null}, BCL-2^{wt}, and BCL-2^{AAA} cells, and could be blocked by ruthenium red, an inhibitor of the mitochondrial calcium uniporter

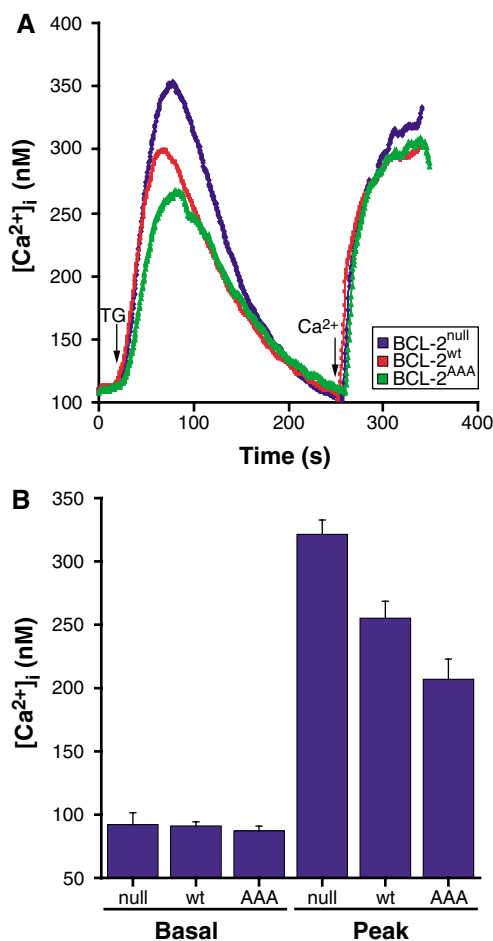


Figure 2 BCL-2^{wt} lowers levels of releasable ER Ca²⁺, and nonphosphorylatable BCL-2^{AAA} mutants further reduces levels. (A) MEF lines stably transfected with various BCL-2 constructs were loaded with the cytosolic calcium indicator Fura-2, and pulsed with 200 nM thapsigargin (TG) in the absence of extracellular calcium. After baseline was reached, calcium was added back to a final concentration of 2 mM free Ca²⁺. (B) For the curves shown in (A), basal and peak cytosolic levels of calcium were measured following TG-induced release of ER stores (average of 10 experiments).

(Figure 3C). Thus, there is no inherent difference in mitochondrial Ca²⁺ handling in these cells, and the diminished level of $[Ca^{2+}]_m$ in BCL-2^{AAA} cells appears to be secondary to the lower $[Ca^{2+}]_i$ to which the mitochondria are exposed.

Steady-state ER calcium levels are reduced in BCL-2^{AAA}-expressing cells

In order to measure directly the concentration of Ca²⁺ within the ER, $[Ca^{2+}]_{er}$, we used aequorin, a calibratable Ca²⁺ photoprotein reporter that was selectively targeted to this subcellular location (erAEQ) (Pinton *et al*, 2000). We transfected erAEQ into BCL-2^{null}, BCL-2^{wt}, or BCL-2^{AAA} MEFs, depleted $[Ca^{2+}]_{er}$ to <20 μM, and then allowed them to refill by addition of Ca²⁺ to the perfusate. Luminescence of erAEQ indicated a marked reduction in the steady-state $[Ca^{2+}]_{er}$ reached when BCL-2^{null} MEFs (618 ± 104 μM) were reconstituted with BCL-2^{wt} (317 ± 75 μM), which was even further reduced if reconstituted with BCL-2^{AAA} (186 ± 20 μM) (Figure 4A).

Steady-state $[Ca^{2+}]_{er}$ reflects an equilibrium between active Ca²⁺ uptake and diffusion from the ER. Inhibition of the

ER calcium pump SERCA with 2,5-di-(tert-butyl)-1,4-benzohydroquinone (tBuBHQ) enables the rate of leak of Ca²⁺ from the ER to be measured. After steady-state $[Ca^{2+}]_{er}$ was determined, we added tBuBHQ to the cells, and calculated the change in $[Ca^{2+}]_{er}$ over time as a measure of the rate of leak ($d[Ca^{2+}]_{er}/dt$). At each $[Ca^{2+}]_{er}$ for which the rate of leak was measured, the stepwise decrease in steady-state $[Ca^{2+}]_{er}$ (Figure 4A) correlated with an increased rate of Ca²⁺ leak from the ER (Figure 4B). These data revealed that reconstitution of BCL-2^{null} cells with BCL-2^{wt} increased the rate of Ca²⁺ leak, while the nonphosphorylatable BCL-2^{AAA} enhanced the leak even further.

Phosphorylation of BCL-2 at the ER inhibits its binding to proapoptotic BCL-2 family proteins

It has been suggested that expression of antiapoptotic BCL-2 or BCL-X_L may alter levels of various calcium handling proteins such as the ER Ca²⁺ uptake pump SERCA, calreticulin (Vanden Abeele *et al*, 2002), or IP₃ receptor (Li *et al*, 2002). Therefore, we wished to test whether levels of such ER Ca²⁺ handling proteins might account for observed differences between cells expressing BCL-2^{AAA} and BCL-2^{wt}. In general, protein levels of the tested ER Ca²⁺ handling proteins including calreticulin, calcineurin, GRP 94, IP₃R1, IP₃R3, and SERCA 2 appeared comparable in Jurkat cells or Jurkat with BCL-2^{wt} or BCL-2^{AAA} as well as MEFs that are BCL-2^{null} or reconstituted with BCL-2^{wt} or BCL-2^{AAA} (Figure 5A).

We next tested whether phosphorylation of sites within the unstructured loop would affect another activity of BCL-2, its capacity to bind BH3 domains of proapoptotic members. Phosphorylation of recombinant GST-BCL-2^{wt} by JNK substantially reduced its ability to bind recombinant multidomain BAX or the BH3-only protein BID (Figures 5B and C; ~70% reduction). Thus, phosphorylation of BCL-2 in this purified system diminished its binding to both multidomain and BH3-only proapoptotic family members.

Immunoprecipitation (IP) of total BCL-2 from solubilized lysates of Jurkat cells expressing BCL-2^{wt} resulted in co-precipitation of substantial amounts of BAX. However, when the phosphorylated fraction of BCL-2 was selectively immunoprecipitated with a BCL-2 S70-P-specific antibody, it did not co-precipitate BAX (Figure 5D). Of note, nocodazole-treated, G₂/M-arrested cells had more phosphorylated BCL-2 than aphidicolin-treated, G₁/S-arrested cells.

One principal role of BCL-2 in preventing apoptosis is to bind and sequester BH3-only proapoptotic proteins such as BIM, BID, and BAD (Cheng *et al*, 2001). As most phosphorylated BCL-2 resides at the ER (Figure 1E), we compared the ability of ER-localized BCL-2^{wt} and BCL-2^{AAA} to bind BH3-only proteins. The IP of nonphosphorylatable BCL-2^{AAA} from ER-enriched light membrane fractions of MEFs co-precipitated substantially more BIM than did BCL-2^{wt} protein (Figure 5E). This was noted when ER fractions were solubilized in either NP-40, which maximizes interactions, or in CHAPS, which does not induce conformational changes of BCL-2 members. While BIM was the predominant BH3-only protein of those tested, BID and BAD were also more highly associated with nonphosphorylated BCL-2. Together, this compilation of studies indicates that phosphorylation of ER-based BCL-2 can substantially reduce its binding to multidomain and BH3-only proapoptotic members.

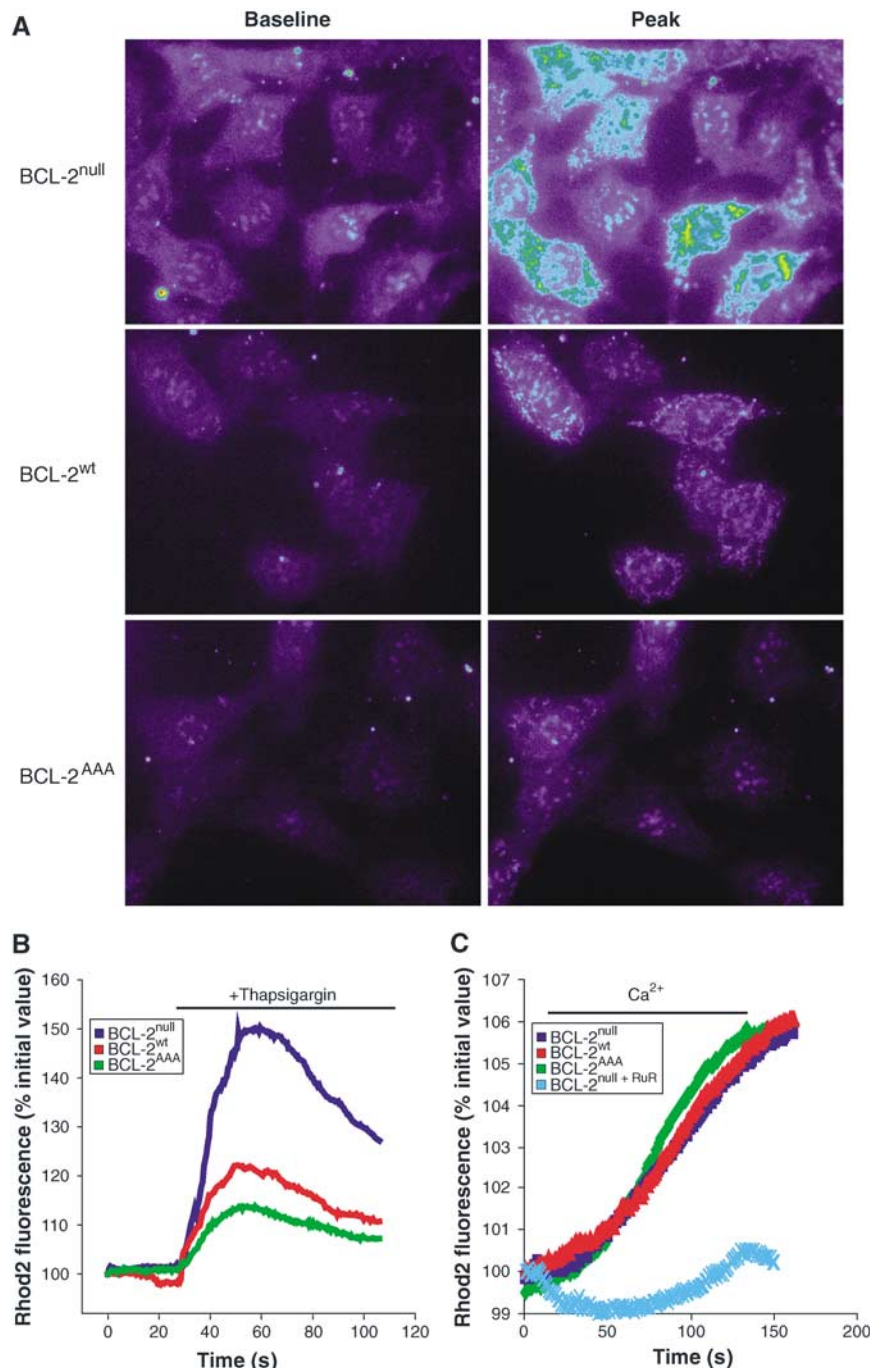


Figure 3 Mitochondria in BCL-2^{AAA}-expressing cells take up lower levels of calcium following release from the ER. **(A)** MEF lines loaded with Rhod-2 were treated with 200 nM thapsigargin, and sequential images were taken. Baseline and peak fluorescence images were selected for comparison. Complete image sequences are given in Supplementary data, Movies 1–3. **(B)** For the images in (A), mitochondrial areas were selected, and normalized fluorescence intensity values were measured over time (see Materials and methods). **(C)** MEFs loaded with Rhod-2 were permeabilized with 0.001% digitonin and exposed to 14.5 μ M Ca^{2+} . Ruthenium red (100 μ M), an inhibitor of the mitochondrial calcium uniporter, was added where indicated.

Capacity of BCL-2 to bind proapoptotic BH3-only proteins correlates with ER Ca^{2+} levels

We next asked if the capacity of BCL-2 to lower steady-state $[Ca^{2+}]_{er}$ was related to its ability to bind proapoptotic members. Fura-2 was used to measure Ca^{2+} release from the ER of Fl 5.12 cells expressing BCL-2^{wt} or BCL-2^{G145E}, a BCL-2 mutant that fails to bind either multidomain or BH3-only proapoptotic members (Yin *et al*, 1994). Strikingly, BCL-

2^{G145E} does not lower $[Ca^{2+}]_i$ (Figure 5F), correlating with its inability to bind proapoptotic BCL-2 family proteins. Like BCL-2^{wt}, BCL-X_L^{wt} also lowers $[Ca^{2+}]_i$ (Figure 5F), and a portion of BCL-X_L localizes to the ER-enriched light membrane fraction (Supplementary Figure 1). Of note, the BCL-X_L^{F131VD133A} mutant, which cannot bind BAX or BAK, but retains the ability to bind BH3-only proteins (Cheng *et al*, 2001), did substantially reduce $[Ca^{2+}]_i$ (Figure 5F). Thus,

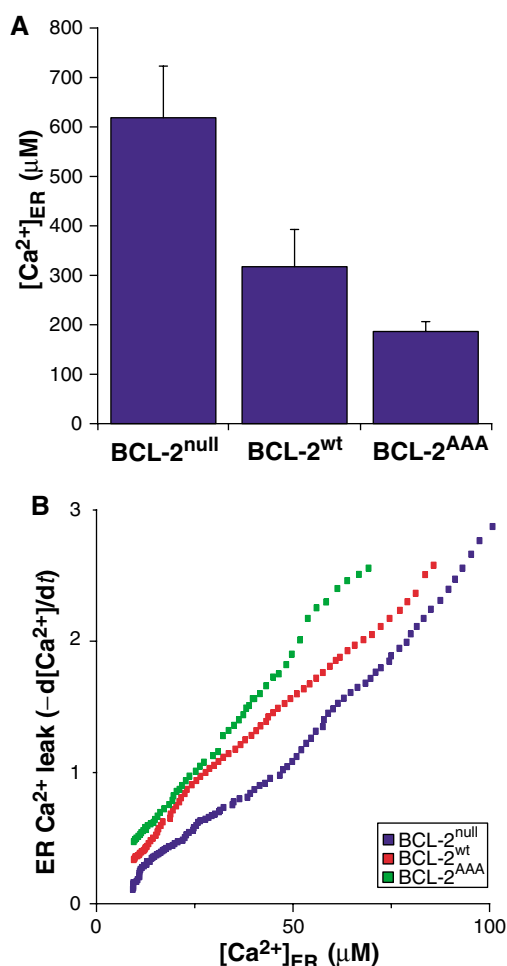


Figure 4 Cells expressing BCL-2^{AAA} have a lower peak ER calcium levels, and higher calcium leak rate from ER. **(A)** Cells transfected with ER-targeted aequorin were calcium-depleted and processed as described in Materials and methods. While monitoring aequorin luminescence, calcium was added back to obtain the peak calcium content in ER. **(B)** At peak calcium, SERCA-mediated ER calcium uptake was inhibited with tBuBHQ, and the rate of leak was determined.

both *in vivo* and *in vitro*, the capability of antiapoptotic BCL-2 or BCL-X_L to bind BH3-only proteins is coordinate with the level of releasable ER Ca²⁺ stores.

Discussion

Here we address the mechanism by which phosphorylation of BCL-2 inhibits its antiapoptotic activity. The predominant localization of phosphorylated BCL-2 to the ER (Figure 1) provided the initial insight that prompted our assessment of the role for BCL-2 phosphorylation at this gateway to apoptosis. Specifically, we asked whether phosphorylation of BCL-2 would alter its regulation of ER Ca²⁺ dynamics.

We found that nonphosphorylatable BCL-2^{AAA} displayed enhanced protection against arachidonic acid and oxidative stress, stimuli that depend on the release of ER Ca²⁺ stores (Figure 1). BCL-2^{AAA} reduced steady-state $[Ca^{2+}]_{er}$ even more profoundly than BCL-2^{wt}, which appears to reflect a further increased rate of Ca²⁺ leak from the ER when BCL-2 cannot be phosphorylated (Figure 4). Consequently, diminished release of ER Ca²⁺ results in lower intracellular Ca²⁺

($[Ca^{2+}]_i$) (Figure 2) and a secondary decrease in Ca²⁺ uptake by the mitochondria in cells expressing BCL-2^{AAA} (Figure 3). However, there was no inherent defect in mitochondrial uptake of Ca²⁺ in BCL-2^{AAA} cells when compared to BCL-2^{null} or BCL-2^{wt} expressing cells when mitochondria were exposed to Ca²⁺ (Figure 3). Thus, BCL-2 is more capable of reducing $[Ca^{2+}]_{er}$ and blocking apoptosis when it is not phosphorylated. In this regard, phosphorylation of BCL-2 reduces protection from a variety of stimuli, including taxol and etoposide (Figure 1), which can also release ER Ca²⁺ (Ueoka and Yamaguchi, 1997; Srivastava *et al*, 1999; Kidd *et al*, 2002), likely reflecting the participation of ER Ca²⁺ release in many types of apoptotic death (Scorrano *et al*, 2003).

Phosphorylation of BCL-2 has been noted to reduce binding to BAX (Poommipanit *et al*, 1999). Our data indicate that phosphorylation modulates the binding of BCL-2 to BH3-only proteins such as BID and BIM as well as multidomain proapoptotic proteins such as BAX (Figure 5B–E). Of note, deamidation within the loop region of BCL-X_L was recently shown to reduce its binding affinity for BIM (Deverman *et al*, 2002). Taken together, this suggests that post-translational modification of the loop region of antiapoptotic BCL-2 family members can regulate the binding between their pocket/groove and the BH3 domains of proapoptotic members. As *in vitro* phosphorylation of recombinant BCL-2 protein alters its binding to proapoptotic BAX or BID (Figure 5B and C), phosphorylation appears to be sufficient to induce an intramolecular conformational change that inhibits this activity.

Phosphorylation of BCL-2 has been reported in response to multiple stimuli, and apparently can be mediated by a variety of kinases, including Raf-1 (Blagosklonny *et al*, 1997), PKC α (Ruvolo *et al*, 1998), PKA (Srivastava *et al*, 1998), and JNK (Yamamoto *et al*, 1999). BCL-2 has also been noted to be phosphorylated following addition of growth factors (May *et al*, 1994; Poommipanit *et al*, 1999). In addition, v-cyclin/cdk6 can phosphorylate BCL-2 in U2OS and Cos-7 cells during G₁/S (Ojala *et al*, 2000). Of note, we detected substantial phosphorylated BCL-2 in asynchronous cultures (Figure 1E) and even some phosphorylated BCL-2 in aphidicolin-arrested (G₁/S) cells (Figure 5D). However, stress response kinases appear to be responsible for the robust phosphorylation of BCL-2 during cell cycle progression as a normal physiologic process that inactivates BCL-2 at G₂/M (Yamamoto *et al*, 1999). JNK has been reported to be activated by an ER-resident complex following ER stress (Urano *et al*, 2000), consistent with our data. We and others have noted that the JNK pathway is also responsible for phosphorylation of BCL-2 on the same residues in the loop region in response to microtubule-damaging agents, which also arrest cells in G₂/M (Haldar *et al*, 1998; Ling *et al*, 1998; Scatena *et al*, 1998). Given our results here, phosphorylation of BCL-2 during mitosis would increase $[Ca^{2+}]_{er}$, and could account for the increased G₂/M susceptibility to apoptosis noted at this checkpoint (Yamamoto *et al*, 1999).

Both pro- and antiapoptotic BCL-2 family members reside at the ER and influence Ca²⁺ dynamics. A principal mechanism whereby antiapoptotic BCL-2 and BCL-X_L block cell death is to bind and sequester proapoptotic BH3-only molecules (Cheng *et al*, 2001). This binding can occur at the ER (Germain *et al*, 2002; Thomenius *et al*, 2003), and as demonstrated here phosphorylated BCL-2 binds less BH3-only BIM

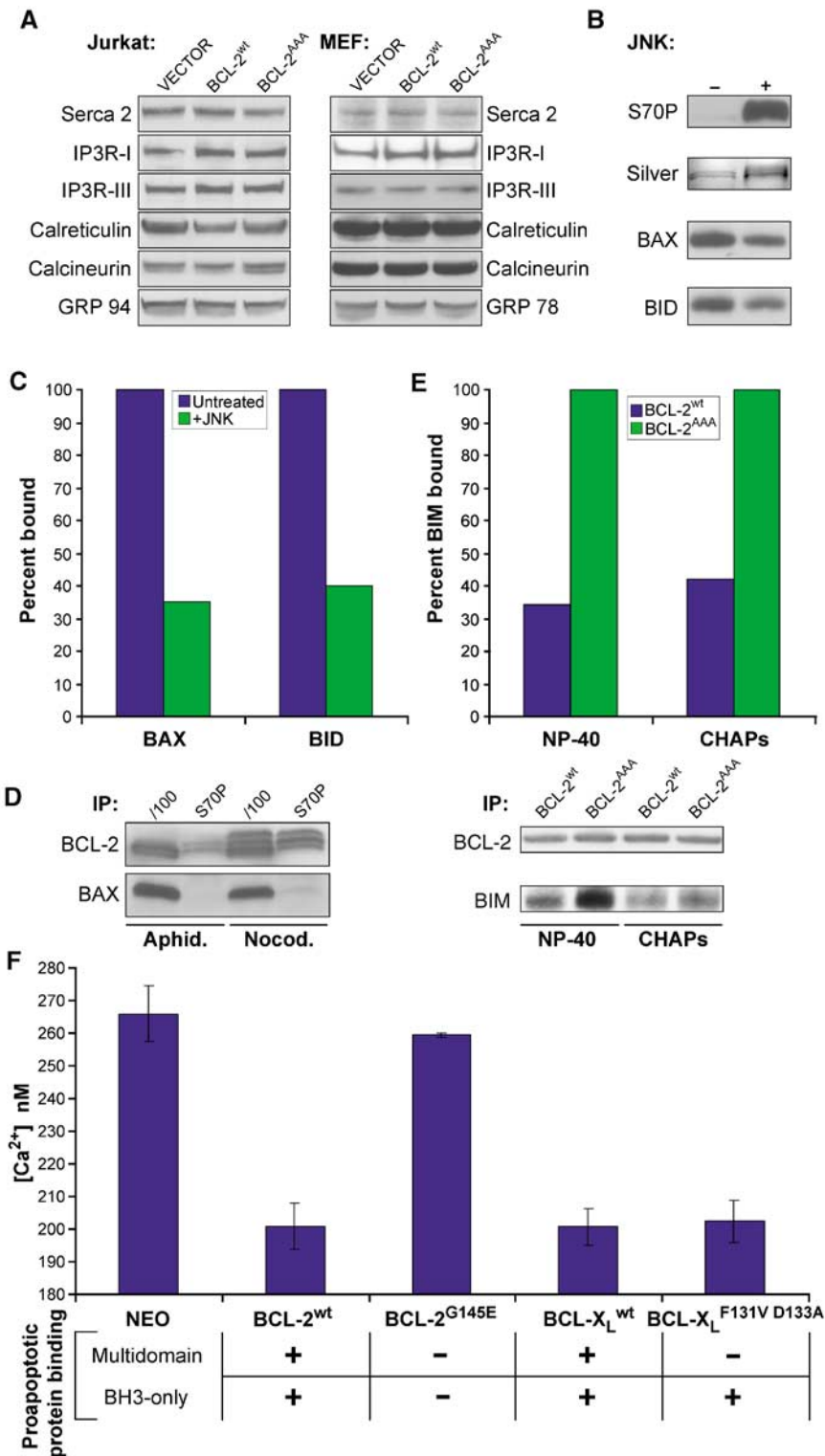


Figure 5 BCL-2 must bind to proapoptotic proteins to lower ER calcium, and this binding is prevented by phosphorylation. (A) RIPA extracts of Jurkat cell lines were analyzed for levels of calcium handling proteins. (B) GST-BCL-2 was treated with JNK kinase, run on SDS-PAGE, and either silver stained or Western blotted with anti-S70P antibody. (C) This protein was then mixed with purified BAX or BID protein in NP-40 buffer, captured with GSH beads, and association with BAX or BID was assessed by Western blot and quantitated by densitometry. (D) Jurkat cells were treated with aphidicolin or nocodazole for 16 h, and then whole-cell extracts were made in IP buffer, immunoprecipitated with the indicated antibody, and then assayed by Western blot. (E) Following subcellular fractionation, light membrane fractions from MEF lines were solubilized in NP-40 or CHAPS IP buffer, immunoprecipitated with anti-BCL-2 antibody, and assayed for associated BIM by Western blot. (F) F1 5.12 lines expressing the indicated constructs were loaded with Fura-2, and peak calcium following thapsigargin treatment was measured.

or multidomain BAX proapoptotic protein (Figure 5). The BCL-X_L^{F131VD133A} mutant that does not bind BAX or BAK but still binds BH3-only molecules still reduced releasable stores of ER Ca²⁺ (Figure 5). Conversely, the BCL-2^{G145E} mutant incapable of binding either BH3-only or multidomain proapoptotic molecules had lost its Ca²⁺ regulatory effect. This compilation of findings provides further support for a rheostat model in which the ratio of pro- to antiapoptotic BCL-2 family members also operates at the ER to coordinate Ca²⁺ dynamics. Together, our data indicate that the phosphorylation status of BCL-2 determines its capacity to bind BH3-only molecules and the releasable stores of $[Ca^{2+}]_{er}$. Overall, the control of ER Ca²⁺ dynamics appears to be a principal pathway by which the phosphorylation of BCL-2 regulates its antiapoptotic activity.

Materials and methods

Cell lines and plasmids

Fl 5.12 lines (Cheng *et al*, 2001), and Jurkat cells transfected with NEO-resistant vector, BCL-2^{wt}, or BCL-2^{AAA} (Yamamoto *et al*, 1999) have been described previously. MEFs were prepared from BCL-2^{null} mice (Veis *et al*, 1993), transformed with SV40 genomic DNA, and then transfected with either human BCL-2^{wt} or T69AS70A-S87A = BCL-2^{AAA} cloned into the MSCV puro vector (Clontech). Stable clones were selected in 1.3 µg/ml puromycin (Sigma), and grown in Iscove's modified Dulbecco's medium supplemented with minimal essential amino acids, L-glutamine, penicillin/streptomycin, and 10% fetal bovine serum. Clones with matched expression levels of BCL-2 were selected for further analysis.

Cell death assay

Jurkat or MEF cells were treated as described in the figure legends; taxol, thapsigargin, etoposide, and staurosporine were purchased from Sigma. Anti-Fas clone CH-11 is from Upstate. Arachidonic acid (Alexis Pharmaceuticals) was added to cells in RPMI without serum for the indicated times, and H₂O₂ (Sigma) was added directly to the growth medium. Annexin-V-cy-3 (Biovision) was used according to the manufacturer's protocol.

Fura-2 and Rhod-2 measurements

Fura-2-AM and Rhod-2-AM were purchased from Molecular Probes. Fura-2 was loaded into cells for 30 min at 37°C in Hanks balanced salt solution (HBSS) supplemented with 10% fetal bovine serum, and then cells were washed with PBS. For assay, cells were suspended in HBSS/10% FBS, and then extracellular calcium was chelated with the addition of 2 mM EGTA. Thapsigargin (200 nM; Sigma) was added to the medium to induce the release of calcium from the ER, and cytosolic Fura-2 fluorescence was assayed on a Perkin-Elmer LS-50B spectrofluorimeter, as described previously (Scorrano *et al*, 2003).

Rhod-2 assays were performed as described previously (Scorrano *et al*, 2003). Briefly, 10⁵ cells were plated onto 25-mm round coverslips, and 24 h later cells were loaded with 4 µM Rhod-2-AM in HBSS for 3 h at 4°C. Coverslips were then mounted into the coverslip holder, washed with HBSS, and placed on the stage of a Nikon Eclipse TE200 inverted microscope equipped with a Perkin-Elmer Ultraview confocal scanner and a Hamamatsu Orca ER 12-bit CCD camera. Cells were excited using the 547 nm laser line and sequential images were taken every 500 ms with exposure times of 30 ms using a ×60 1.4 NA Plan Apo objective (Nikon) and Ultraview imaging suite (Perkin-Elmer). Images were then analyzed with Metafluor software (Universal Imaging). 20 areas of mitochondrial fluorescence for each experiment were identified in the peak fluorescent frame and the average intensity of each area was measured over the course of the experiment. These values were background subtracted, normalized for comparison, and averaged to give a curve representing the change in fluorescence over the course of the experiment. Curves representing the average of 10 experiments were compiled.

Intrinsic mitochondrial Ca²⁺ uptake ability was measured by exposing mitochondria in 0.001% digitonin-permeabilized cells to

Ca²⁺-EGTA-buffered cytosolic solutions (Molecular Probes) with a final $[Ca^{2+}]$ of 14.5 µM.

Aequorin measurements

ER aequorin reconstitution, luminescence analysis, calibration of $[Ca^{2+}]$, and leak rate measurements were performed as described previously (Pinton *et al*, 2000; Scorrano *et al*, 2003). Briefly, cells were grown on 13 mM round glass coverslips, and transfected with the erAEQ construct using Fugene 6. After 24 h, luminal calcium was depleted for 10 min at 4°C in KRB (125 mM NaCl, 5 mM KCl, 1 mM Na₃PO₄, 1 mM MgSO₄, 5.5 mM glucose, and 20 mM Hepes pH 7.4) + 1 mM EGTA, 10 µM ionomycin, and 100 µM tBuBHQ. Cells were washed twice in the same buffer without ionomycin but with 10% FCS, and then aequorin was reconstituted for 2 h at 4°C with 5 µM coelenterazine-N-AM (Molecular Probes) in KRB + 1 mM EGTA and 100 µM tBuBHQ. Coverslips were then washed with KRB/EGTA/10% FCS, and placed in a KRB/EGTA-perfused, thermostated chamber in the luminometer. Luminescence of the coverslip was measured during addition of calcium to the perfusate (KRB + 1 mM Ca²⁺). After $[Ca^{2+}]_{er}$ reached steady state, ER Ca²⁺ leak measurements were performed by perfusing cells with KRB + EGTA and 100 µM tBuBHQ.

Immunoprecipitation, subcellular fractionation, and Western blotting

IPs and binding assays were conducted in either NP-40 buffer (0.2% NP-40, 150 mM KCl, 1 mM EGTA, 50 mM Hepes pH 7.5, 5 mM MgCl₂, 50 mM NaF, 1 mM Na₃VO₄, 250 µM PMSF, and 5 µg/ml pepstatin) or CHAPS buffer (1% CHAPS, 100 mM KCl, 50 mM Hepes pH 7.5, 1 mM EGTA, 50 mM NaF, 1 mM Na₃VO₄, 250 µM PMSF, and 5 µg/ml pepstatin). IP was performed in 500 µl extract containing 750 µg protein, which was precleared by centrifugation at 16 000 g followed by exposure to 30 µl protein A/G beads (Santa Cruz). Cleared extracts were incubated with antibody for 2 hours, and captured on 30 µl beads for 1 hour, washed three times, and boiled in SDS sample buffer. Anti-human BCL-2 clone/100 antibody was purchased from Pharmingen, and N20 anti-BAX antibody was purchased from Santa Cruz. Anti-BID antibodies were described previously (Wang *et al*, 1996). Anti-S70P antibodies were generated in rabbits immunized against the following peptide (CRDPVARTpSPLQTP). For IP antibodies were used at 1 µg/100 µl, and for Western blotting antibodies were used at 1 µg/ml. Aphidicolin and nocodazole (Sigma) were added to cells for 16 h.

Subcellular fractionation was performed on 10⁹ cells. Cells were washed 1 × with PBS, suspended in HIM buffer (200 mM mannitol, 70 mM sucrose, 10 mM HEPES pH 7.5, and 1 mM EGTA), and then disrupted using a polytron cell homogenizer (Kinematica). After a crude fraction was removed at 700 g, a heavy membrane fraction was removed at 7000 g for further purification on a percoll gradient (Danial *et al*, 2003). Light membrane fraction was separated from cytosolic fraction at 280 000 g. Proteins were either solubilized in NP-40 or CHAPS buffer for IP, or in RIPA buffer (1% NP-40, 1% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM HEPES pH 7.5, 2 mM EDTA, 50 mM NaF, 200 µM Na₃VO₄, 250 µM PMSF, and 5 µg/ml pepstatin) for Western blotting.

Methods for obtaining purified GST-BCL-2 (Letai *et al*, 2002), BAX (Suzuki *et al*, 2000), and BID (Zha *et al*, 2000) have been described previously. Purified JNK (Upstate) was incubated with GST-BCL-2 for 16 h at 30°C in a buffer containing 10 mM HEPES pH 7.5, 25 mM MgCl₂, 1 mM EGTA, 1 mM ATP, and 250 µM PMSF.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank J Opferman and E Cheng for technical assistance and helpful discussions, K Yamamoto for design of the phospho-specific antibody, and E Smith for help with figure and manuscript preparation. MCB is sponsored by NIH training grant T32 CA09361. This work was supported in part by NIH grant R37CA50239. Research in LS lab is supported by Telethon (TCP012016).

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