

Suppression of IP₃-mediated calcium release and apoptosis by Bcl-2 involves the participation of protein phosphatase 1

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Received 2 May 2006; accepted 10 July 2006

Abstract

The involvement and potential interdependence of inositol trisphosphate (IP₃) receptors and Bcl-2 in the regulation of Ca²⁺ signaling is not clear. Here, we have explored the mechanism(s) of how Bcl-2 suppresses the IP₃-sensitive Ca²⁺ release in MCF-7 cells focusing on the possible role of protein phosphatase 1 (PP1). We found that through influences on protein–protein interaction, Bcl-2 may alter the balance between the effects of phosphatase (PP1) and kinase (PKA) on the IP₃ R1 signaling complex. Using various experimental approaches including phosphatase inhibition and RNAi, we show that Bcl-2 by competing with IP₃R1 for the binding of PP1 can reduce the IP₃-mediated calcium signal and protect cells from mitochondrial dysfunction and cell death.

Key words: endoplasmic reticulum, Ca²⁺ stores, Bcl-2, IP₃ receptors, protein phosphatase 1, apoptosis

Abbreviations: IP₃, inositol 1,4,5-trisphosphate; IP₃R, IP₃ receptor; Tg, Thapsigargin; ER, endoplasmic reticulum; PP1, protein phosphatase 1; PKA, protein kinase A.

Introduction

Endoplasmic reticulum (ER) is a major intracellular Ca²⁺ signaling site. Early studies from various laboratories have indicated that ER Ca²⁺ homeostasis is essential for cell survival and that depletion of the ER Ca²⁺ can lead to apoptosis (reviewed in ref. 1, 2). There is now convincing evidence that unrestricted release of Ca²⁺ from the ER followed by mitochondrial Ca²⁺ uptake is an important

signal for the activation of the apoptotic process [1–4]. These apoptosis-associated Ca²⁺ fluxes are regulated by members of the Bcl-2 family of proteins (reviewed in ref. 4, 5). It seems that the anti-apoptotic protein Bcl-2 can regulate ER calcium release by two different pathways. First, Bcl-2 lowers total ER Ca²⁺ pool so there is less Ca²⁺ to be released [6–9]. Second, Bcl-2 does not affect the total ER Ca²⁺ pool, but instead does not allow as much Ca²⁺ to be actively released through the inositol

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1,4,5-trisphosphate receptors (IP3R) [10]. While these effects of Bcl-2 appear to conflict with each other [11], it remains possible that they are not mutually exclusive. Abundant evidence indicates that Bcl-2 can simultaneously utilize multiple mechanisms in order to achieve maximal protection of cell survival [12]. For example, Bcl-2 can lower ER Ca^{2+} pool via both the downregulation of capacitative Ca^{2+} influx (CCE) [6, 9], and the increase of ER Ca^{2+} leakage [6, 7, 9]. In addition, Bcl-2 can interact with IP3R to restrict the release of ER Ca^{2+} [10]. However, it remains unclear whether the inhibition of the IP3R channel by Bcl-2 [10] is a direct effect due to the physical interaction between the two, or an indirect effect via interactions with intermediary proteins.

IP3Rs are intracellular calcium release channels on the ER. There are three forms of IP3R, all of which are ligand-gated channels activated by the second messenger IP3. IP3R channel function is modulated via cross-talk with other signaling pathways including those mediated by kinases and phosphatases (reviewed in ref. 13 and 14). For example, IP3R1 from cerebellum forms a macromolecular complex that includes the cAMP-dependent protein kinase (PKA) and the serine/threonine protein phosphatase PP1 [15, 16]. PKA can activate while PP1 can inhibit the activity of recombinant IP3R1 reconstituted into planar lipid bilayers [16]. Therefore, ER Ca^{2+} release via the IP3R1 channel may be regulated by a balance in the activities of PP1 and PKA. In addition to the IP3-induced calcium release during cell activation, there is also a “passive leak” mechanism on the ER that is regulated by Bcl-2 in resting cells [17]. It has been proposed that Bcl-2 may exert a protective action by lowering ER calcium pool through this basal leak mechanism [6–9]. However, the molecular nature of this passive leak has not been identified. A recent study has shown that proapoptotic Bax and Bak can also regulate the calcium leak from the ER [18]. The various published results can be rationalized if Bcl-2 affects IP3R phosphorylation indirectly by somehow modulating the effect of PP1 and/or PKA on IP3R. Thus, the regulation of IP3R phosphorylation status (or PP1/PKA balance) by Bcl-2 appears critical for our understanding of the Bcl-2 action at the ER.

While it has been suggested that Bcl-2 can function as a targeting protein for PP1 [19, 20], how does PP1 and Bcl-2 collaborate to control the IP3R1-mediated calcium signaling is not known. In the present study, we explore the mechanism by which Bcl-2 suppresses the IP3-mediated Ca^{2+} release and focus on the interactions of Bcl-2, PP1, and IP3R1 in MCF-7 cells. Using various experimental approaches including phosphatase inhibition and RNAi, we show that Bcl-2 by competing with IP3R1 for the binding of PP1 can reduce the IP3-mediated calcium signal and protect cells from mitochondrial dysfunction and apoptosis.

Material and methods

Cell culture and Bcl-2 mutants

MCF7 epithelial cell lines were grown in alpha-MEM (Sigma) supplemented with 10% FBS [21, 22]. HeLa cells were grown in DMEM with 10% FCS. Transient transfection of Bcl-2 in HeLa cells were performed using Lipofectamine (Invitrogen) according to the manufacturer's instruction. MCF7 cell lines overexpressing human wild type Bcl-2 or an empty PrCCMV vector as a control were established by stable transfection [23] and are referred to here as wtBcl-2 or CMV cell line. Plasmids encoding point mutation of Bcl-2 in which G 145 was exchanged for A (G145A) was generated using Quickchange mutagenesis (Stratagene) and verified by sequencing. Bcl-2 mutants with restricted localization to the ER (Bcl2-cb5) or to the mitochondria (Bcl2-Acta) were generated as described [21, 22].

Measurement of ER Ca^{2+} release in intact or permeabilized cells

Measurement of ATP-induced Ca^{2+} release in intact cells using the fluorescent dye Fura-2 was described previously [24]. MCF7 or HeLa cells freshly detached from culture dish ($\sim 2 \times 10^6$ cells) were loaded with Fura 2/AM (3 μM) in Buffer A containing (in mM) 5.4 KCl, 137 NaCl, 0.44 KH_2PO_4 , 4.2 NaHCO_3 , 0.34 Na_2HPO_4 , 1 MgCl_2 , 2 CaCl_2 , 5 Hepes (pH 7.4), 11.1 D-glucose, and 0.1% bovine serum albumin (BSA). After 30 min, cells were rinsed in Buffer A without Ca^{2+} . Fura-2 fluorescence was recorded continuously at 25 °C in a spectrofluorometer (Photon Technology International) at excitation wavelengths of 340 and 380 nm and an emission wavelength of 500 nm. After establishing the base line, agonist ATP (0.1 mM) was added to monitor the agonist-induced Ca^{2+} release.

Measurement of IP3-induced Ca^{2+} release was carried out in permeabilized cells using a procedure that preserves the functional integrity of the calcium coupling between ER and mitochondria [25, 26]. Briefly, MCF7 cells at $\sim 80\%$ confluence were harvested by trypsinization followed by centrifugation. The cells (1×10^7) were suspended in 2 ml of respiratory buffer (125 mM KCl, 2 mM K_2HPO_4 , 1 mM MgCl_2 , 20 mM Hepes, pH 7.0) containing the dye Fura-PE3 (0.2 μM), 5 mM glutamate, 5 mM malate, and the plasma membranes were permeabilized by the addition of digitonin (final concentration 0.01%). Prior to experimentation, the respiratory buffer was treated with Chelex 100 (BioRad) to lower the ambient $[\text{Ca}^{2+}]$ [25]. The medium free $[\text{Ca}^{2+}]$ did not exceed 300–400 nM, which represented the background

due to contamination of Ca^{2+} in the water. Fluorescence was monitored using 340 and 380 nm excitation and 500 nm emission. The experiments were carried out with the addition of CaCl_2 ($2\ \mu\text{M}$) to fill the ER store [25]. The dynamics of Ca^{2+} release was then monitored by the addition of IP3 ($10\ \mu\text{M}$). The standard equation: $K_d[(R-R_{\min})/(R_{\max}-R)] \cdot S_f/S_b$ was used to convert the fluorescence ratio to Ca^{2+} concentration [8].

Crosslinking experiment and Western blotting

For crosslinking, MCF7 cells were incubated for 30 min with 1 mM BMH (Pierce Chemical) in media at $37\ ^\circ\text{C}$. The cells were trypsinized, removed from the dish and washed in PBS. The cells were lysed in buffer B (1% Triton, 0.1% SDS, 50 mM Tris pH 8.0, 150 mM NaCl, and 1 mM each of EDTA, EGTA, PMSF, and Aprotinin to inhibit proteases) and then incubated on ice for 30 min. Cell lysate was cleared by centrifugation at $10,000 \times g$ for 10 min. Protein concentration was measured using the Bradford assay (BioRad) and lysate containing 15–25 μg of protein was boiled in sample buffer and separated by SDS-PAGE gel (4–12%) for immunoblotting [27]. The following antibodies were used: anti-IP3R type 3 (BD Transduction Laboratories), anti-IP3R type 1, and anti-IP3R (total) recognizing all three isoforms (Calbiochem), anti-Bcl2 N-terminal (Dako), anti-PP1 α (Calbiochem), anti-SERCA2 (Affinity BioReagent), and anti-phosphoserine (Zymed). The antibody for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Research Diagonostic Inc.

Immunoprecipitation

Cells were harvested and incubated in lysis buffer C (same as buffer B except 1% NP-40 was used in place of Triton and SDS) for 30 min on ice. The lysates (1 mg protein per IP) were pre-cleared by incubation with 25 μl Protein G agarose and control IgG (2 μg , Sigma) for 1 h to remove non-specific binding. The lysates (0.25 ml) were then incubated with the primary antibody (2 μg) overnight at $4\ ^\circ\text{C}$. Protein G agarose (10 μl) was added and the samples were incubated for 1 h. The Protein G agarose beads was collected by centrifugation ($10,000 \times g$, 1 min), washed and then boiled in sample buffer (15 μl per lane), and loaded on 4–12% SDS-PAGE gel and identified by Western blotting.

RNAi studies

SiRNA sequence corresponding to human PP1 α (GAU-CAAGUACCCCGAGAAC, GenBank accession number

NM_002708, nt 333–351) and the control sequence corresponding to a region of firefly luciferase were obtained from Dharmacon Research (Lafayette, CO). Transfection of siRNA was carried out using DharmaFECT siRNA kit according to the manufacturer's instruction. Bcl-2 or CMV cells (40% confluent) in a 60 mm dish were changed to fresh culture medium (4 ml) for transfection. After 5–6 h of transfection, the cells were washed once and supplied with fresh culture medium for further incubation. Calcium measurement and western blots were carried out after 48 h transfection.

Densitometry and data analysis

Protein blots were scanned and the optical density of the bands quantified using the NIH image 1.61 software. Data (Mean $\pm$ SD) were analyzed using Student's *t* test and significance defined as a *p*-value of <0.05 .

Results

Reduction of the IP3-induced calcium release by Bcl-2

We have used the agonist (ATP) to stimulate ER Ca^{2+} release in various MCF7 cell lines stably expressing wtBcl-2, mutant Bcl-2 (G145A) or control vector (CMV). ATP binds to the purinergic receptor which activates the phospholipase C and generates IP3 which in turn activates the IP3R and causes the release of ER Ca^{2+} . To block Ca^{2+} influx due to the activity of the store-operated channel (termed capacitative Ca^{2+} entry) these experiments were performed in the absence of extracellular Ca^{2+} . Under this condition, the increase of cytosolic Ca^{2+} after the addition of ATP was largely due to release from the ER pool. Figure 1A showed the effect of Bcl-2 expression on the cytosolic Ca^{2+} signal elicited by ATP in MCF7 cells. ATP induced a transient elevation of Ca^{2+} for all three cell lines (each trace representing 2×10^6 cells). However, in the cells expressing wt Bcl-2, the increases evoked by stimulation with ATP were significantly smaller than in CMV controls (peak amplitude 175 ± 17 vs. 262 ± 16 nM, $n = 8$). The decreased stimulation by ATP requires functional Bcl-2 because mutation of Bcl-2 at G145 to A, a mutation known to inactivate Bcl-2 [27], was unable to reduce the Ca^{2+} signal. Summary of 8 independent experiments (Fig. 1B) indicated a significant reduction of the ATP-induced calcium signal by 40% ($p < 0.004$, $n = 8$) for the wtBcl-2 cells as compared to CMV or G145A. Similar studies were carried out using permeabilized cell system, which allows a direct assessment of the IP3-mediated calcium release (see method). Figure 1C showed that addition of IP3 to the permeabilized

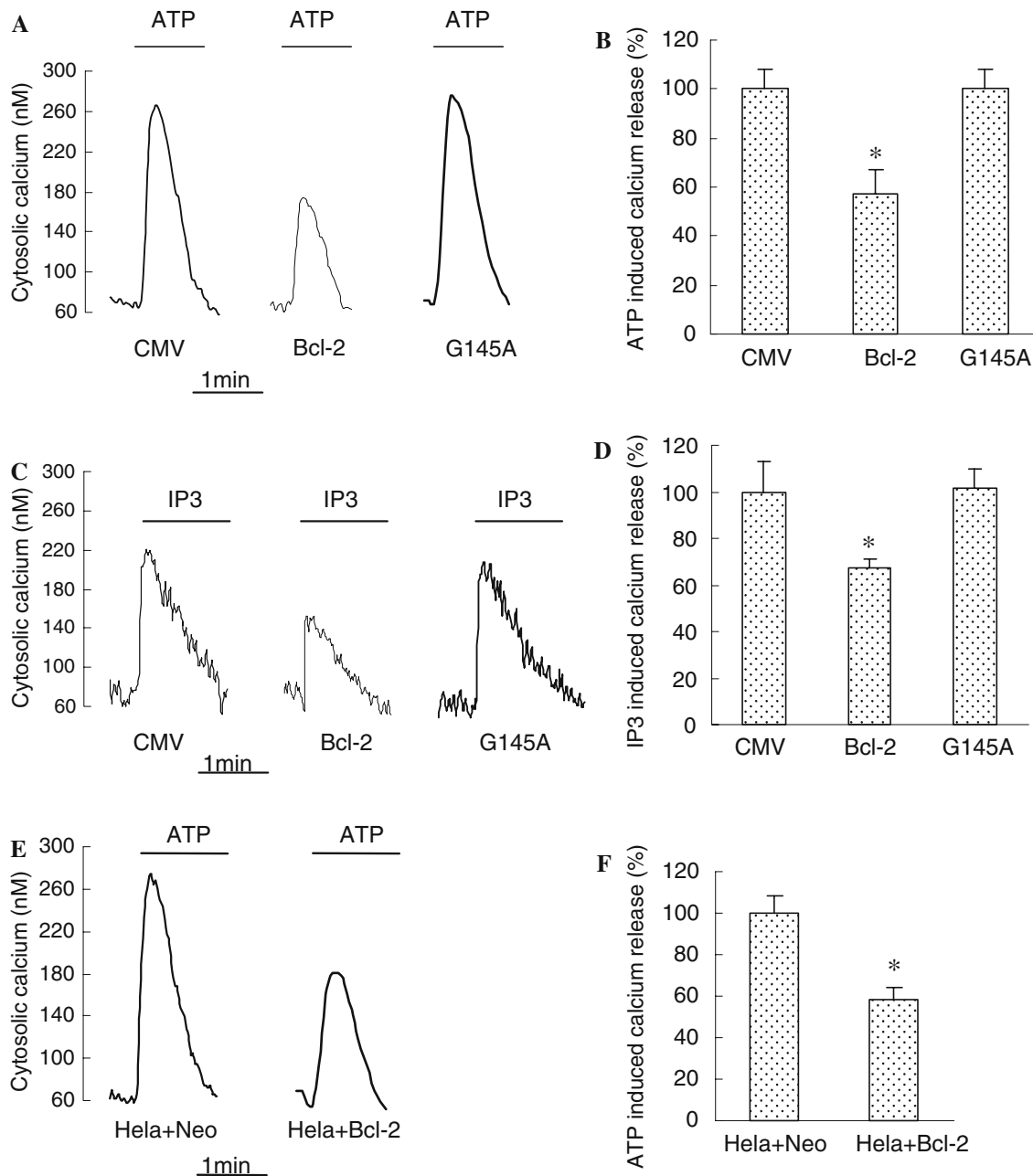


Fig. 1. Reduction of IP3-induced calcium release by Bcl-2. MCF-7 cells were stably transfected with CMV control vector, or plasmids encoding wild type Bcl-2, or inactive Bcl-2 mutant G145A. (A) Cytosolic calcium signal in CMV control, wtBcl-2 and G145A cells in response to 0.1 mM ATP. (B) Peak calcium increase in response to 0.1 mM ATP in CMV, wtBcl-2, and G145A cells represented as the percentage comparing with CMV. (C) Cytosolic calcium signal in CMV, wtBcl-2 and G145A cells in response to 10 μ M IP3. (D) Peak calcium increase in response to 10 μ M IP3 in CMV, wtBcl-2 and G145A cells represented as the percentage comparing with CMV. (E) Cytosolic calcium signal in control HeLa cells and HeLa cells overexpressing wtBcl-2, in response to 0.1 mM ATP. (F) Peak calcium increase in response to 0.1 mM ATP in control HeLa cells and HeLa cells overexpressing wtBcl-2 represented as the percentage comparing to control. Data from B, D, and F are mean $\pm$ SD from at least six to eight independent experiments (see text).

cell also led to smaller Ca^{2+} elevation from the wtBcl-2 cells as compared to control CMV or G145A cells, respectively (peak amplitude 146 ± 7 vs. 218 ± 20 for CMV or 220 ± 10 nM for G145A). Summary of 7 experiments

(Fig. 1D) indicated a significant reduction of the IP3-induced calcium release by 33% ($p < 0.007$) from the Bcl-2 cells as compared to CMV or G145A. Thus, both intact- and permeabilized-cell studies indicated a reduction of

IP3-mediated Ca^{2+} release by Bcl-2. To assure that this phenomenon was not restricted to MCF7 cells, we have also tested the Bcl-2 effect with another cell type. Figure 1E, F showed that transient transfection of Bcl-2 into HeLa cells also led to 42% decrease of ATP-sensitive calcium release than control cells transfected with vector (Neo) only (peak amplitude 187 ± 11 vs. 280 ± 23 nM, $n = 6$, $p < 0.007$). Our study of HeLa cells with whole cell suspension (Fig. 1E) agrees with the previous report of HeLa cells using cover-slips [6] indicating that different techniques of calcium measurement yielded the same results. In addition, a recent study in WEHI7.2 T cells also showed that Bcl-2 decreases IP3-mediated calcium release [10]. Therefore, the suppression of IP3-mediated Ca^{2+} release by Bcl-2 appears a general phenomenon. Similar to wtBcl-2 cells, studies of MCF7 cells expressing Bcl-2 localized to the ER (Bcl2-cb5) also indicated decreased IP3-sensitive release as compared to CMV cells (not shown). The results indicated that Bcl-2 located at the “ER membrane” is responsible for the decreased IP3-sensitive Ca^{2+} signal.

Enhancement of the ATP-induced calcium release correlates with susceptibility to apoptosis

The physiological relevance of the reduced ATP-sensitive calcium release by Bcl-2 is still not clear. Figure 2A showed the effect of Bcl-2 on the cytosolic Ca^{2+} signal elicited by ATP in MCF7 cells after treatment with staurosporine (STS, $0.5 \mu\text{M}$), a common apoptotic agent. The results (from five independent experiments) indicated that STS treatment for 2 h clearly led to a significant increase of the ATP-sensitive Ca^{2+} release in mutant G145A (Fig. 2A, left) as well as control CMV cells (not shown). In contrast, STS treatment had no effect on Bcl-2 cells (Fig. 2A, right). Comparison of the peak heights with and without STS (Fig. 2B) indicated that the increment of the ATP sensitive release was $\sim 35\%$ ($p < 0.01$, $n = 4$) for the mutant G145A or CMV (not shown), while that for the wtBcl-2 was unchanged. Similar study using permeabilized cells also indicated that STS caused increased Ca^{2+} release from the IP3-sensitive pool (not shown). A parallel study (Fig. 2C) further indicated that STS treatment led to an increase of mitochondrial cytochrome c release in the G145A but not in the Bcl-2 cells. Another study using adriamycin as apoptotic agent (Fig. 2D) also confirmed the cleavage of caspase-3/7 substrate PARP in CMV or mutant G145A cells but not in wtBcl-2 cells after 24 h treatment. The results suggest that the increased Ca^{2+} release from the IP3-sensitive pool by STS in the CMV or mutant Bcl-2 cells is associated with mitochondrial damage and susceptibility to apoptosis. In contrast, resistance to STS-induced changes in the

IP3-sensitive pool in wtBcl-2 cells is associated with protection of mitochondrial integrity and cell survival.

Regulation of the ATP-sensitive Ca^{2+} release: the effect of kinase- and phosphatase- inhibitors

IP3R1 forms a macromolecular complex that contains kinase PKA and phosphatase PP1 [15, 16], raising the possibility that the ATP-sensitive release in the MCF7 cells is regulated by a balance in the activity of these two enzymes. In order to test this hypothesis, we have investigated the effect of H89, a PKA inhibitor, and of calyculin A, a PP1/PP2A inhibitor on ATP-sensitive calcium release in intact cells. The results indicated that pretreatment of cells with H89 ($5 \mu\text{M}$) for 5 min was sufficient to increase the ATP-sensitive release for CMV (Fig. 3A) as well as for wtBcl-2 cells (Fig. 3B). The increase by H89 treatment over untreated controls was $88 \pm 12\%$ and $65 \pm 10\%$ for CMV and wtBcl-2 cells respectively ($n = 4$, $p < 0.01$). In contrast, pretreatment of cells with calyculin A (CA, 200 nM) for 5 min led to significant reduction of ATP-sensitive release for CMV cells by $35 \pm 7\%$ ($p < 0.01$, $n = 5$) but no reduction in Bcl-2 cells as compared to their respective untreated controls. Further study in permeabilized cells using a specific inhibitor of PP1 (Inhibitor-2, 10 nM , 5 min) also reduced the IP3-mediated release by 40% for CMV cells but having no effect on Bcl-2 cells (two experiments). The results are consistent with the hypothesis that protein kinase A and PP1 are involved in the control of IP3-sensitive release. Since inhibition of PP1 in CMV cells led to 35–40% reduction of the IP3-sensitive release which is similar to the 40% reduction by Bcl-2 expression seen in Fig. 1A, the results suggest that Bcl-2 may act like a PP1 inhibitor in the control of IP3R activity. This can also explain the lack of additional effect by PP1 inhibitor (CA) in the wtBcl-2 cell study (Fig. 3B). It is possible that Bcl-2 may sequester PP1 and alter the normal balance of PP1/PKA of the IP3R complex.

Association of IP3R1 (but not IP3R3) with Bcl-2 and PP1 at the ER

To further test the possibility that Bcl-2 may act like a PP1 inhibitor in the regulation of IP3R signaling, the (direct or indirect) interactions of Bcl-2 with relevant proteins of the IP3R complex were examined. Figure 4A shows the western blots that compared the level of expression for several key proteins in the three MCF-7 cell lines. There were no major differences in the protein level of IP3Rs and PP1 of the CMV, wtBcl-2 and mutant G145A cells. Furthermore, the expression levels of ER calcium pump

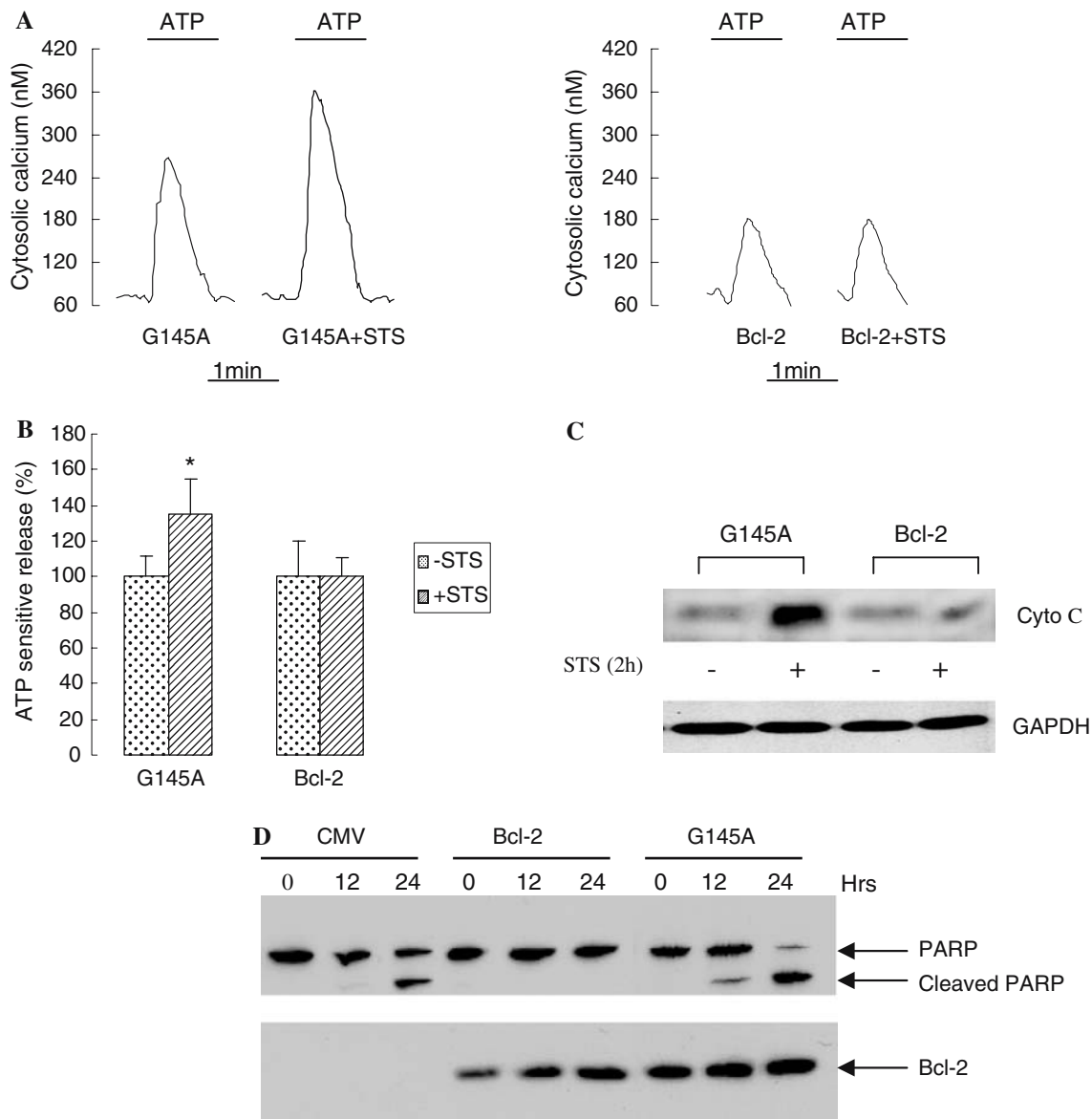


Fig. 2. Enhancement of ATP-induced calcium release correlates with susceptibility to apoptosis. (A) Cytosolic calcium signals elicited by ATP (0.1 mM) were compared in G145A cells versus wtBcl-2 cells. Cells in culture were treated with or without STS (0.5 μ M) for 2 h in the presence of caspase inhibitor ZVAD (40 μ M) and then processed for calcium measurement. The results indicate that STS treatment for 2 h led to further increase of the ATP-sensitive calcium signal in G145A cells but not in wtBcl-2 cells. (B) Summary of the experiments from (A) indicates that the increment of the ATP sensitive release by STS was $\sim$ 35% for the G145A cells while that for the wtBcl-2 cells was unchanged. (C) STS induced mitochondrial cytochrome *c* release is inhibited in wtBcl-2 cells but not in G145A cells. Cells with or without STS treatment (2 h), were gently lysed by hypotonic buffer (a kit from Biomol) and centrifuged at $12,000 \times g$ for 10 min to separate the mitochondria pellet from the cytosol. The supernatant representing the cytosolic fraction was concentrated and run on the SDS-PAGE gel for the detection of cytochrome *c* by western blotting. Equal loading of the protein sample was verified by blotting with GAPDH. The results indicate that G145A cells were sensitive to STS treatment which led to mitochondrial cytochrome *c* release, while Bcl-2 cells were resistant. (D) Adriamycin induced PARP cleavage is inhibited in Bcl-2 expressing cells but not in G145A cells. CMV, wtBcl-2, and G145A cells were incubated in charcoal-filtered medium for 6 days to reduce expression of endogenous Bcl-2. Whole cell lysates were prepared from untreated cells (0) and cells treated with 10 μ M Adriamycin for 12 and 24 h. Ten micrograms of total protein in cell lysate was separated by SDS-PAGE. Western blots were probed using anti-PARP (upper panel) and anti-Bcl-2 antibodies (lower panel). Full length PARP, cleaved PARP and Bcl-2 are indicated to the right of the panels.

(SERCA) and ER calcium binding protein (calreticulin) were unchanged, thus allowing a direct comparison of these cell lines by co-IP.

The co-IP experiments to detect the interaction of IP3R1 and Bcl-2 are shown in Fig. 4B. Using antibody to IP3R1 for co-IP (left panel), Bcl-2 was detected by western blotting

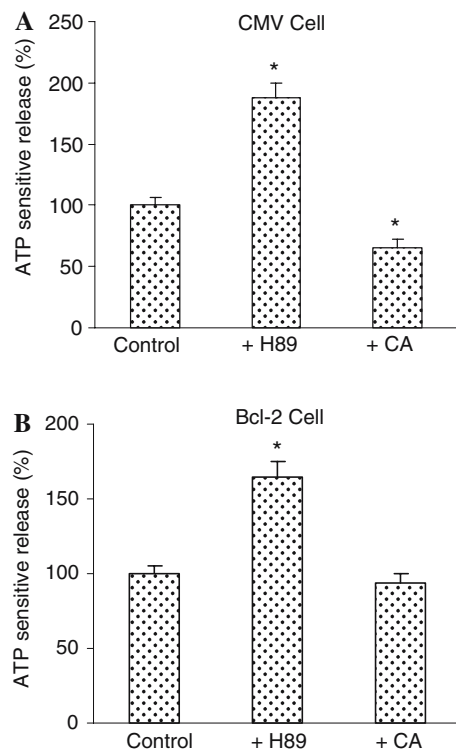


Fig. 3. The effect of kinase- and phosphatase inhibitors on the ATP-sensitive calcium release. The CMV control (A) or wtBcl-2 cells (B) were pretreated with PKA inhibitor H89 (5 μ M) or PP1/PP2 inhibitor calyculin (CA, 200 nM) for 5 min, and the ATP-induced calcium release were measured as described in Fig.1. Error bars correspond to one standard deviation, $n = 4$. The results comparing to untreated group indicate that inhibition of PKA is associated with increased ATP-sensitive calcium release for both CMV and Bcl-2 cells. However, inhibition of PP1/PP2 by CA is associated with decreased ATP-sensitive release for CMV cells but not for Bcl-2 cells.

in anti-IP3R1 immunoprecipitates of the wtBcl-2 and mutant G145A cells. As expected, the level of Bcl-2 in the CMV cells was very low. Control experiments using pre-immune serum (not shown) or antibody to IP3R3 (Fig. 4B, right panel) did not co-precipitate with Bcl-2, indicating that co-IP of IP3R1 and Bcl-2 was specific. Similarly, the interaction between IP3R1 and PP1 was detected by co-IP. Figure 4C showed the detection of PP1 (top blot) and IP3R1 (middle blot) in the anti-IP3R1 immunoprecipitates as expected [15, 16]. Again, the co-IP was specific for IP3R1, because PP1 was not detected in the anti-IP3R3 immunoprecipitates (bottom blot).

Importantly, Bcl-2 not only interacts with IP3R1 but also with PP1. Using antibody to PP1 for co-IP (Fig. 4D), it was possible to detect the presence of Bcl-2 (top blot) and PP1 (bottom blot) in the anti-PP1 immunoprecipitates. Control experiments using pre-immune serum (Fig. 4D, lower panel) did not lead to co-IP with Bcl-2. The results confirmed the previous report of interaction between Bcl-2 and PP1 in a

murine T cell line [19]. It appears that the amount of Bcl-2 in the anti-PP1 immunoprecipitates was increased for the wtBcl-2 cells as compared to the CMV or G145A cells (Fig. 4D, top blot). The results suggest a possible reduction of Bcl-2/PP1 interaction in the G145A cells as compared to wtBcl-2 cells. It has been shown that Bcl-2 binding to PP1 required the intact motif sequence 143-NWGRIVAFEEF-153 in the BH1 region of Bcl-2 [19]. Since the mutation G145A occurs in this binding motif, this might explain the decreased level of Bcl-2 found in the anti-PP1 precipitates of the G145A cells. In summary, the results of the co-IP experiments suggest that IP3R1 at the ER can interact with PP1 (the α isoform) as well as Bcl-2 while IP3R3 cannot. This difference in the type 1 and 3 of IP3R is consistent with the report that they serve distinct roles in Ca^{2+} signaling [28].

Knockdown of PP1 decreases the ATP-sensitive release in CMV but not in Bcl-2 cells

To further test the functional consequence of the association of PP1 and Bcl-2 with IP3R1, we have used the RNAi approach to knock down selectively the protein expression of PP1 in CMV and wtBcl-2 cells (see Methods). Figure 5A shows that transfection of siRNA for PP1 in CMV (left panel) and wtBcl-2 cells (right panel) led to ~80% reduction of PP1 expression as compared to the transfection of control siRNA. The transfection of PP1 siRNA did not result in an alteration of the Bcl-2 level as indicated by western blotting (see middle blots in Fig. 5A). The parallel assay was then carried out to measure the cytosolic calcium increase by ATP stimulation. Figure 5B shows a reduction of the ATP-sensitive release by PP1 siRNA transfection as compared to the control siRNA transfection in the CMV cells (left panel) but not in the wtBcl-2 cells (right panel). Data from five experiments (Fig. 5C) indicated a significant decrease (~40%) for CMV cells from control transfection representing $100 \pm 10\%$ to $57 \pm 8\%$ with the PP1 siRNA transfection ($p < 0.006$, $n = 5$). In contrast, there was already a reduced ATP sensitive pool in the wtBcl-2 cells, and knocking down PP1 did not result in additional reduction (Fig. 5C, right panel). Therefore, knocking down PP1 in CMV cells has an equivalent effect as Bcl-2 expression. Since the RNAi approach is more specific than the phosphatase inhibitor study (Fig. 3), the results (Fig. 5) further support the hypothesis that Bcl-2 acts like a PP1 sequester in the control of IP3R1 channel function.

Knockdown of PP1 protects CMV cells from apoptosis

Since knocking down PP1 in CMV cells has an equivalent effect as Bcl-2 expression on the ATP sensitive release

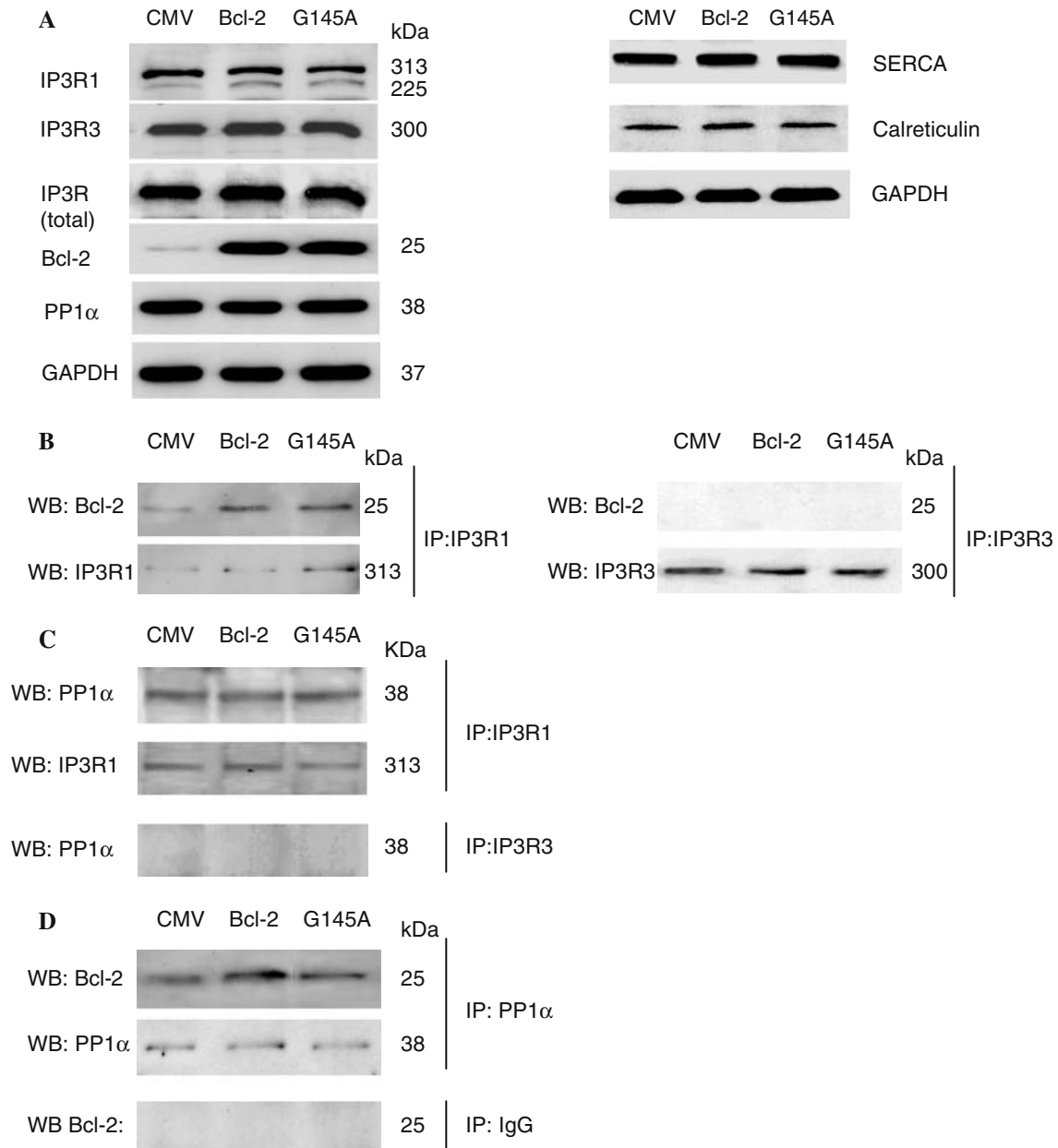


Fig. 4. Interaction of IP3R1 (but not IP3R3) with PP1 and Bcl-2 at the ER. (A) Western blots show the equivalent expression of IP3Rs, PP1(α isoform), SERCA, and calreticulin in all three MCF7 cell lines. Equal loading of samples were indicated by detection of GAPDH. Using anti-IP3R1, two bands corresponding to IP3R1 (313 kDa) and degraded IP3R1 (225 kDa) were detected. Using anti-IP3R3, a band for IP3R3 (300 kDa) was detected. Using anti-IP3R (total), both IP3R1 and IP3R3 were detected. However, the two bands were not separated. IP3R2 (MW = 300 kDa) was not identified here because the antibody for this isoform is not available commercially. (B) Interaction of IP3R1 and Bcl-2. Using anti-IP3R1 for co-IP (left panel), Bcl-2 and IP3R1 were detected in the anti-IP3R1 immunoprecipitates of CMV, wtBcl-2 and G145A cells by western blotting. The relative amount of endogenous Bcl-2 in the cell lines is shown in the CMV cells. Using anti-IP3R3 for co-IP, the right panel shows the detection of IP3R3 (but not Bcl-2) in the anti-IP3R3 immunoprecipitates by western blotting. (C) Interaction of IP3R1 and PP1 α . Using anti-IP3R1 for co-IP, PP1 α (top blot) and IP3R1 (bottom blot) were detected in the anti-IP3R1 immunoprecipitates by western blotting. (D) Interaction of PP1 and Bcl-2 detected in anti-PP1 α immunoprecipitates by western blotting. A control experiment using pre-immune serum (IgG) did not co-precipitate Bcl-2 (lower panel).

(Fig. 5C), then PP1 knockdown might also protect the cells from apoptosis. Figure 6 showed that PP1 knockdown indeed prevented CMV cells from death induced by STS

treatment (0.5 μ M). In the control group (with control siRNA transfection), STS treatment for 6 h led to reduced viability such that only $62 \pm 5\%$ of CMV cells remained

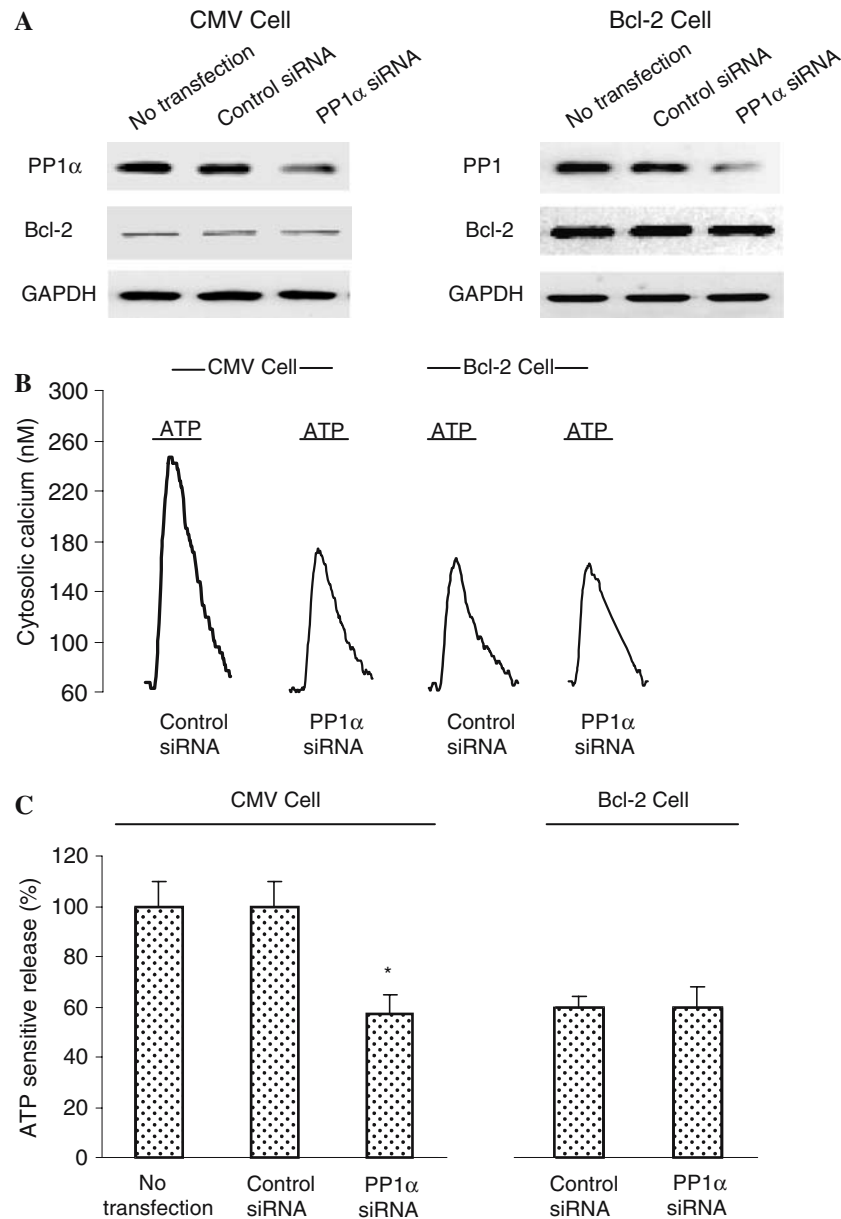


Fig. 5. Knocking down PP1 decreases the ATP-induced calcium release in CMV but not in Bcl-2 cells. CMV and wtBcl-2 cells were transfected with the PP1 α siRNA or control siRNA and used 48 h later for (A) western blotting and (B and C) assay of ATP-induced calcium release. In (A), cell lysates from cells without treatment (no transfection) were also applied to show that siRNA transfection in general had no effect on the level of PP1 (top blot) or Bcl-2 (middle blot). In (B), Calcium signals representing the ATP-induced release are compared from CMV and Bcl-2 cells. Since the Ca²⁺ trace for the non-transfected was similar to the control siRNA-transfected group, it is omitted here for clarity. (C) Quantitative analysis of the results from replicates of the experiment shown in (B). Error bars show one standard deviation, $n = 4$. ATP-induced release was shown as (PP1siRNA/control siRNA) where the control siRNA group = $100 \pm 10\%$ for the CMV cells. The results indicate that knocking down PP1 decreased the ATP-induced release to $57 \pm 8\%$ ($p < 0.03$), while siRNA transfection with a control sequence (firefly luciferase) was the same as the non-transfected group. In contrast to CMV cells, the knockdown of PP1 had no effect in the wtBcl-2 cells (Fig. 5C, right panel).

alive as indicated by the Trypan blue exclusion test ($n = 4$, $p < 0.01$) while cells without STS treatment were 100% viable. In contrast, the PP1 knockdown group was resistant

such that $90 \pm 6\%$ of CMV cells remained alive after 6 h of STS treatment. It appears that the 90% cell viability correlated well with the 80% PP1 knockdown (compare Fig. 5A

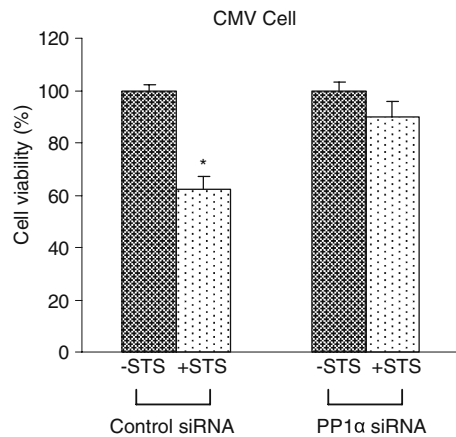


Fig. 6. Knocking down PP1 protects CMV cells from death. CMV cells were transfected with the PP1 α siRNA or control siRNA and used 48 h later for the assay of STS-induced death. After incubation with STS (0.5 μ M) for 6 h, cells were removed from the culture dish, washed once and viability assessed by counting at least 400 cells that exclude Trypan blue dye. The results indicate that knocking down PP1 protects CMV cells from STS-induced death such that 90% of cells (with PP1 knockdown) remained alive. In contrast, only 62% of cells (with control siRNA transfection) remained alive after 6 h-STS treatment.

with Fig. 6). Therefore, knocking down PP1 in CMV cells confers resistance to apoptosis such that they become anti-apoptotic like the wt Bcl-2 cells (see Fig. 2).

Bcl-2 binds IP3R1 indirectly at the ER

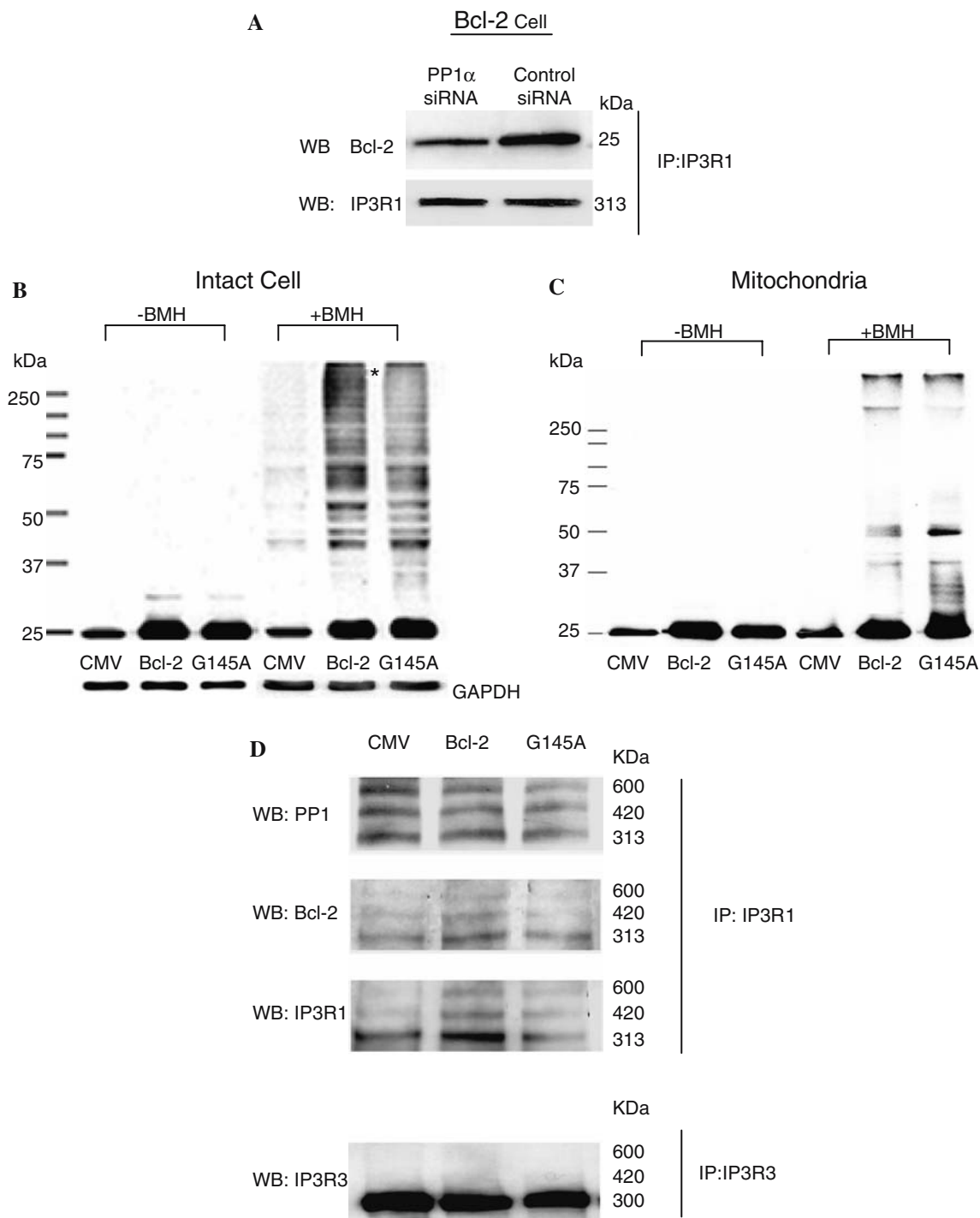
While Bcl-2 can suppress the IP3-mediated Ca^{2+} release (Fig. 1), it is unclear whether this is due to a direct or indirect action of Bcl-2. In order to distinguish these two possibilities, the effect of PP1 knock-down on the interaction between Bcl-2 and IP3R1 was examined in the wtBcl-2 cells. Using anti-IP3R1 for co-IP, both Bcl-2 (Fig. 7A, upper blot) and IP3R1 (Fig. 7A, lower blot) were detected in the anti-IP3R1 immunoprecipitates of wtBcl-2 cells. However, the amount of Bcl-2 detected was clearly reduced in samples with PP1 siRNA transfection as compared to control transfection. The combined results of Fig. 7A and Fig. 4D suggest for the first time that the interaction of Bcl-2 and IP3R1 may be an indirect effect, which depends on the presence of PP1.

Further study by cross-linking reaction was carried out to determine the association of Bcl-2 with multiple proteins *in vivo*. Fig. 7B compared the ability of Bcl-2 and G145A in the formation of high molecular weight complexes that may contain IP3R1, PP1 and Bcl-2. Addition of the cell permeable crosslinker, BMH to the wtBcl-2 cells in culture led to the detection of multiple complexes of Bcl-2 of various sizes (from 25 kDa to >600 kDa) as shown by western

Fig. 7. Bcl-2 binds to IP3R1 indirectly at the ER. (A) WtBcl-2 cells were transfected with the PP1 α siRNA or control siRNA and used 48 h later for co-IP experiments. Using anti-IP3R1 for co-IP, both Bcl-2 and IP3R1 were detected in the anti-IP3R1 immunoprecipitates by western blotting. However, the Bcl-2 level was clearly reduced in the sample with PP1 knock-down as compared to controls. Densitometry of the gel bands was used to estimate the Bcl-2/IP3R1 ratio in the two samples. The calculation indicates ~67% reduction of Bcl-2/IP3R1 ratio in the sample with PP1 knockdown. The results suggest that PP1 is required for maximal binding of Bcl-2 to IP3R1. (B) Formation of macromolecular complexes of Bcl-2 by cross-linking experiments. Intact cells in media (B), or (C) isolated mitochondria in buffer (cell lines indicated below the lanes) were incubated with the crosslinker BMH (1 mM) for 30 min, and then lysed for western blot detection of Bcl-2 and Bcl-2 complexes using an anti-Bcl-2 antibody. For SDS-PAGE, each lane was loaded with 25 μ g of protein from the cell- or mitochondrial extract. A protein band at 25 kDa indicates Bcl-2 monomer while asterisk indicates a large Bcl-2 complex (>600 kDa) that may contain IP3R1 and PP1 (see Fig. 7D). Equal loading of the proteins on the gel is indicated by GAPDH blotting. The results indicate that Bcl-2 from the wtBcl-2 cells was able to form high molecular weight (300 to 600 kDa) complexes better than the mutant Bcl-2 in the G145A cells. Fig. 7C shows that the multi-protein complexes of Bcl-2 are greatly reduced in samples from the isolated mitochondria. (D) Detection of high molecular wt complexes of IP3R1 (but not IP3R3) in MCF7 cell lysates. IP3R1 was immunoprecipitated from the lysates with anti-IP3R1 (upper panel), and the presence of IP3R1 in the precipitates confirmed by western blotting (bottom blot). Several bands including IP3R1 monomer at 313 kDa, and IP3R1 complexes at 420- and 600-kDa were detected. Although not shown here, higher MW complexes (>600 kDa) of IP3R1 were also detected. The 313-, 420-, and 600-kDa bands most likely represent the products after the dissociation of >600 kDa complexes in SDS denaturing buffer. In two other separate blots (from the same IP3R1 immunoprecipitates), the presence of Bcl-2 (middle blot) or PP1 (top blot) in the 420- and 600-kDa bands were also detected using respective antibodies. In contrast to IP3R1 (upper panel), there was no complex formation by IP3R3 (lower panel).

blotting with anti-Bcl-2 antibody (Fig. 7B, lane 5). In contrast, the mutant G145A that was deficient to bind PP1 was also deficient in the formation of multiple complexes (Fig. 7B, lane 6), while the control CMV cells was least capable to form such complexes (Fig. 7B, lane 4). Additional crosslinking experiments using isolated mitochondria suggested that the formation of Bcl-2 macromolecular complexes occurs at the ER but not mitochondria (compare Fig. 7B and C).

The presence of high MW complexes that contain IP3R1, PP1 and Bcl-2 in MCF7 cell lysates can be detected in the absence of crosslinker BMH (Fig. 7D) or in the presence of BMH (not shown). When IP3R1 was immunoprecipitated from the lysates with anti-IP3R1, the presence of IP3R1 was confirmed by western blotting (see bottom blot). In addition to the IP3R1 monomer at 313 kDa, at least two additional bands (420 and 600 kDa) representing the macromolecular complexes of IP3R1 were also detected. In two other separate blots from the same IP3R1 immunoprecipitates, the presence of Bcl-2 (Fig. 7D, middle blot) or PP1 (top blot) in the 420 and 600 kDa bands were also detected using respective antibodies. In contrast to IP3R1, there was no



complex formation by IP3R3 (Fig. 7D, lower panel). The results are consistent with the notion that IP3R1 complexes at 422- and 600-kDa contain Bcl-2 and PP1. Although not shown here, the same macromolecular complexes of IP3R1 also contain PKA in agreement with the proposal that IP3R1 can function as a signaling integrator by binding to many proteins [13, 14].

Discussion

Physical interactions with other proteins appears fundamental to Bcl-2's function. Here, we have demonstrated that the anti-apoptotic action of Bcl-2 is closely related to its ability to bind PP1 and IP3R1. Direct interaction has been demonstrated between IP3R1 and PP1 [9] and also between

PP1 and Bcl-2 previously [19]. In contrast, an indirect interaction between IP3R1 and Bcl-2 is suggested here by the experiment of knocking down PP1 (Fig. 7A). We propose that Bcl-2, as a binding protein of PP1, can alter the balance of PP1/PKA in the IP3R1 complex which then results in decreased IP3-mediated Ca^{2+} release in MCF7 cells. Such mechanism of Bcl-2 action is supported by comparing the cells with wtBcl-2 expression versus that of inactive Bcl-2 (G145A) or CMV controls (Figs. 1, 3, and 5). The involvement of PP1/PKA in the regulation of IP3-mediated Ca^{2+} release is supported by the study using inhibitors (Fig. 3) or knock-down of PP1 (Fig. 5). In another study (not shown) using metabolic labeling with ^{32}P , we have also obtained evidence that IP3R1 (but not IP3R3) is phosphorylated in living cells. Taken together, the Ca^{2+} signaling studies here suggest that Bcl-2 restricts IP3R1 channel activity *in vivo*. This inhibition of the ER Ca^{2+} release by Bcl-2 can at least in part account for the observed resistance to STS-induced apoptosis (Fig. 2). While this mechanism of suppression of IP3-mediated Ca^{2+} release by Bcl-2 seems to operate in the MCF7 cells, it does not contradict with the possibility that Bcl-2 also lowers the total ER Ca^{2+} pool by the inhibition of Ca^{2+} entry via the store-operated channel [6, 9]. Indeed, we have also found a smaller ER Ca^{2+} pool by Bcl-2 in MCF7 cells and these results are in agreement with the recent report by Palmer *et al.* [8]. There was approximately 25% less Ca^{2+} released by the addition of either thapsigargin or ionomycin to the wtBcl-2 cells as compared to CMV cells or G145A cells (not shown). Consistent with the study in other cell types [6, 9], we have also detected the inhibition of the capacitative Ca^{2+} entry ($\sim 20\%$) by Bcl-2 in MCF7 cells as well as neuroblastoma cells (Kuo and Zhu, unpublished data). Thus, Bcl-2 can use multiple mechanisms to achieve maximal protection for cell survival.

In contrast to the anti-apoptotic action of Bcl-2, PP1 activity has been suggested to associate with apoptosis [20]. The involvement of PP1 in cell death is verified here in CMV cells. Knock down of PP1 in CMV cells led to decreased IP3-sensitive Ca^{2+} release and inhibition of cell death, which is typical of the wtBcl-2 cells (Figs. 5 and 6). The PP1-targeting proteins share the R/K-V/I-X-F docking motif [29]. It is noted that a similar RIGL motif is present within IP3R1 (aa 2731–2734) [9]. A similar GRIVAF motif is also present within wtBcl-2 (aa 145–160), and this sequence is required for the binding between Bcl-2 and PP1 [19]. Interestingly, this sequence is also required for interaction of Bcl-2 with Bax and BH3 only proteins. It is conceivable that wtBcl-2 may simply compete with IP3R1 for binding to PP1, while the G145A mutant is deficient to compete (Fig. 4D). This can explain why G145A cells like CMV cells are unable to suppress the IP3-sensitive Ca^{2+} release (Fig. 1).

In addition to Bcl-2, proapoptotic proteins Bax and Bak may also influence the ER Ca^{2+} store indirectly by interfering with the interaction of Bcl-2 and IP3R1 [18]. The formation of macromolecular complexes of Bcl-2 by crosslinking experiments (Fig. 7B, C) has suggested the complexity of Bcl-2 action on the ER membrane, i.e. in addition to the consideration of {IP3R1-PP1-Bcl2} as a trimolecular complex for cell survival (Figs. 4–7) the role of pro-apoptotic BH3 proteins such as Bad needs to be considered. It is possible that by binding to Bcl-2, Bad may pull Bcl-2 away from the {IP3R1-PP1-Bcl2} complex and thus increase the availability of PP1 to IP3R1. The consequence of Bad overexpression may then lead to increased IP3-mediated Ca^{2+} release which can trigger mitochondria Ca^{2+} overload and apoptosis [30]. Thus cell survival or death is regulated by a balance in ER localized kinase and phosphatase activity [31] and that Bcl-2 located on the ER membrane regulates the IP3R1 channel function by sequestering PP1. Although not shown here, this binding of Bcl-2 to PP1 and IP3R1 also appears to be related to the increased ER Ca^{2+} leakage in resting MCF7 cells. The presence of high MW complexes of IP3R1, PP1 and Bcl-2 on the ER (Fig. 7D) may also serve as a signaling integrator responsible for the inhibition of capacitative Ca^{2+} entry via the interaction of the IP3R1 complex with the store-operated channel [32, 33].

Acknowledgments

This work was supported by National Institutes of Health (HL-39481, to T.H.K.), and CIHR grant FRN 12517 (to DWA). DWA holds the Canada Research Chair in Membrane Biogenesis.

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