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Upregulation of Bcl2 inhibits apoptosis-driven BAX insertion but favors BAX relocalization in mitochondria

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ABSTRACT

Protein–protein interactions between the Bcl2 family proteins regulate apoptosis. An imbalance of this interaction network due to the upregulation of the proto-oncogene *Bcl2* leads to a resistance to apoptosis associated with tumor formation. Bcl2 overexpression inhibits BAX oligomerization and mitochondrial outer membrane (MOM) permeabilization. However, Bcl2 effects on earlier steps of BAX-mediated apoptosis are not fully understood. Bcl2 overexpression inhibits BAX insertion into the MOM but spontaneously increases BAX relocalization to the mitochondria. Also, a physical interaction between BAX and Bcl2 is necessary for these two effects to occur. Taken together, these results suggest upregulated Bcl2 stabilizes BAX loose binding to mitochondrial membranes, inhibiting its insertion into the MOM and consequently cytochrome *c* release.

Structured summary
MINT-7945271: *BAX* (uniprotkb:Q07813) *physically interacts* (MI:0915) with *Bcl-2* (uniprotkb:P10417) by *anti bait coimmunoprecipitation* (MI:0006)

1. Introduction

Apoptosis is a cell death program in which mitochondria play a central role. During early apoptosis, permeabilization of the mitochondrial outer membrane (MOM) allows the release of pro-apoptotic factors, like cytochrome *c*, from the intermembrane space (IMS) into the cytosol. Cytochrome *c* release promotes and amplifies the apoptotic cascade; and is considered the commitment step of programmed cell death [1–4]. Importantly, this event is highly regulated through specific interactions between proteins of the Bcl2 family [1,2,4–9]. In this family, effector proteins such as BAX are essential to the machinery allowing the permeabilization of the MOM. On the other hand, anti-apoptotic members such as Bcl2, inhibit this process by directly binding to the pro-apoptotic effector proteins [5]. Finally, BH3-only proteins relay apoptotic stimuli to the mitochondria by interacting with both effectors and anti-apoptotic Bcl2 family members [1,2,5]. Therefore, when challenged by an apoptotic stimulus, the combined signaling within the Bcl2 family dictates the immediate fate of the cell, i.e., to induce permeabilization of the MOM or not [1].

An imbalance inside the Bcl2 family proteins interaction network, e.g., through the variation of the expression level of its components, may cause pathophysiological situations. In this respect, genetic events leading to the overexpression of the *Bcl2* proto-oncogene are associated with tumor formation and more particularly B-Cell non-Hodgkin's lymphoma [10–12]. Also, prolymphocytic cell lines overexpressing Bcl2 exhibit both resistance to mitochondrial apoptosis and the ability to induce lymphoma after injection in mice [10,13–15]. Interestingly, it was recently shown that an over-abundance of the Bcl2 protein caused the sequestration of some pro-apoptotic BH3-only proteins in mitochondria. This accumulation of activator BH3-only proteins has been described as a 'priming for death', and this concept is currently used to develop new strategies to selectively kill cancer cells [16,17]. Finally, Bcl2 overexpression induces inhibition of mitochondrial apoptosis that requires a physical interaction between Bcl2 and the pro-apoptotic protein BAX [18,19].

Early apoptosis consists in a specific chain of events exquisitely regulated by Bcl2 family proteins which are leading to MOM permeabilization. The translocation of cytosolic BAX to the mitochondria occurs in response to an apoptotic stimulus. BAX then undergoes a conformational change that leads to the exposure of its N-terminus, and is inserted into the MOM [1,2,4–9]. BAX was also shown to form oligomers in mitochondrial membranes during apoptosis [20]. These BAX oligomers are essential constituents of MAC, the apoptotic channel responsible for cytochrome *c* release [15,21,22]. On the other hand, Bcl2 is an anti-apoptotic protein normally inserted into the MOM. It is now well established that

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an excess of Bcl2 causes lymphomagenesis and results in the inhibition of BAX oligomerization and MAC activity [10–15,23]. However, the effects of Bcl2 on the earlier steps of the BAX-mediated apoptotic pathway remain poorly understood.

In this study, we report that resistance to mitochondrial apoptosis conferred by Bcl2 overexpression is primarily associated with inhibition of BAX insertion into the MOM. Interestingly, however, mitochondrial BAX levels were significantly increased in non-apoptotic cells overexpressing Bcl2; up to levels equivalent to those observed in apoptotic parental cells. Also, this spontaneous increase in Bcl2-mediated BAX relocalization at the mitochondria was dependent upon the ability of Bcl2 to physically interact with BAX. Taken together, these results suggest upregulated Bcl2 stabilizes BAX loose binding to mitochondrial membranes, inhibiting its insertion into the MOM and consequently cytochrome *c* release.

2. Materials and methods

2.1. Cells, growth conditions, and induction of apoptosis

The hematopoietic FL5.12 parental, Bcl2 overexpressing, and Bcl2-G145E overexpressing cell lines were grown as previously described [15]. Apoptosis was induced by 12 h of IL-3 withdrawal [14,15].

2.2. Cytochrome c release assay

Cytosolic cytochrome *c* from digitonin-permeabilized normal or apoptotic cells was quantified by ELISA (Quantikine; R&D Systems, Mineapolis, MN), as described by [21,22].

2.3. Isolation of mitochondria

Mitochondria were isolated and resuspended in mitochondrial buffer (230 mM mannitol, 70 mM sucrose, 1 mM EDTA, 5 mM HEPES-KOH, pH 7.4), as previously described [15].

2.4. Isolation of MOM-inserted BAX

Membrane-inserted mitochondrial proteins were isolated by centrifugation after removing the proteins loosely bound to the mitochondrial outer membrane by an alkaline treatment with 0.1 M Na_2CO_3, pH 11, as described in [20].

2.5. Whole cell protein extraction and sub-cellular fractionation

Whole cell protein extraction: Total cell proteins were solubilized by an incubation of 30 min in lysis buffer (PBS supplemented with 1% (v/v) Triton X-100). *Sub-cellular fractionation*: Cytosolic and mitochondrial fractions were obtained by differential centrifugation, as previously described by [13].

2.6. Antibodies

Primary antibodies: anti Bax (N20, Santa Cruz Biotechnology, Inc., 1:2500), anti VDAC1 (Ab-1, Calbiochem, 1:2500), anti Bcl2 (C2, Santa Cruz Biotechnology, Inc., 1:2000). Secondary antibodies: goat anti-rabbit (1:5000) and anti-mouse (1:2500) HRP-coupled (Santa Cruz Biotechnology, Inc.).

2.7. Calculation of the amount of mitochondrial and MOM-inserted BAX

Non-saturated Western blots for BAX and VDAC1, a protein natively inserted in the mitochondrial outer membrane, were scanned and quantified with Image J software. The average ratio of BAX relative to VDAC1 was calculated for each condition. These BAX/VDAC1 ratios were normalized to that of the parental condition which was arbitrarily fixed at 1.

2.8. Immunoprecipitation assays

Mitochondrial proteins were solubilized in mitochondrial buffer containing 2% CHAPS as previously described [20]. Total soluble BAX was detected by Western blot, and quantified by densitometry using increasing amount of recombinant BAX (a gift from Bruno Antonsson) as standards [21]. After quantification, the same amounts of total BAX were immunoprecipitated with an antibody recognizing a BAX epitope known to be exposed during early apoptosis (N20-AC, Santa Cruz Biotechnology, Inc.), as previously described by [21]. Alternatively, these lysates were also incubated with an antibody against all forms of BAX (5B7, Sigma–Aldrich, St. Louis, MO).

3. Results

3.1. Bcl2 overexpression inhibits BAX insertion but does not modify mitochondrial BAX levels during apoptosis

It is now well established that Bcl2 overexpression inhibits apoptosis through the inhibition of MAC formation and activity [13,15,23]. However, the effects of an excess of Bcl2 on earlier steps of BAX-mediated apoptotic pathway begged further investigation. In this study, early apoptosis was induced in pro-lymphocytic FL5.12 cells by 12 h of IL-3 withdrawal [24,25] in order to analyze the effects of Bcl2 overexpression on mitochondrial cytochrome *c* release; and BAX translocation, conformational change (resulting in the exposure of BAX N-terminal region), and insertion into the MOM.

IL-3 withdrawal triggered the release of cytochrome *c* by mitochondria of parental FL5.12, leading to a fourfold increase of cytosolic cytochrome *c* in this cell line (i.e., from 30 ± 1 to 120 ± 10 pmol cytochrome *c*/million cells, $n = 4$, not shown). This release was about 90% reduced in Bcl2 overexpressing FL5.12 cells (Bcl2) (Fig. 1A); a result which was in agreement with Bcl2 inhibition of MOM permeabilization during apoptosis [13,15,23]. BAX translocation was estimated by quantification of the total mitochondrial BAX content. In agreement with previous reports [15], mitochondrial BAX content increased in parental cells after IL-3 withdrawal (Fig. 2A). Interestingly, the amount of BAX in mitochondria was similar in both parental and Bcl2 overexpressing cells (Bcl2 cells) after IL-3 withdrawal (Fig. 1B). The change of conformation of BAX was detected using antibodies against a specific N-terminus epitope known to be exposed during early apoptosis [26]. Similar to that observed for total BAX, BAX conformational change levels increased in apoptotic parental cells (Fig. 2D). In addition, mitochondria of IL-3 starved parental and Bcl2 cells did not show a significant difference in the amount of BAX which underwent a change of conformation (Fig. 1C). Finally, the amount of BAX inserted into the MOM was about 70% reduced in Bcl2 cells (Fig. 1D). Taken together, all these results showed that, during apoptosis, mitochondrial cytochrome *c* release, BAX insertion, conformational change, and insertion were inhibited by Bcl2 overexpression. However, the similar BAX levels in mitochondria of both cell types strongly suggested that Bcl2-mediated strong inhibition of BAX insertion was crucial in inhibiting cytochrome *c* release.

3.2. Bcl2 overexpression increases BAX relocalization and change of conformation in mitochondria in the absence of apoptotic stimulus

The fact that both total and conformationally changed (N20 positive) BAX levels were similar in parental and Bcl2 cells after

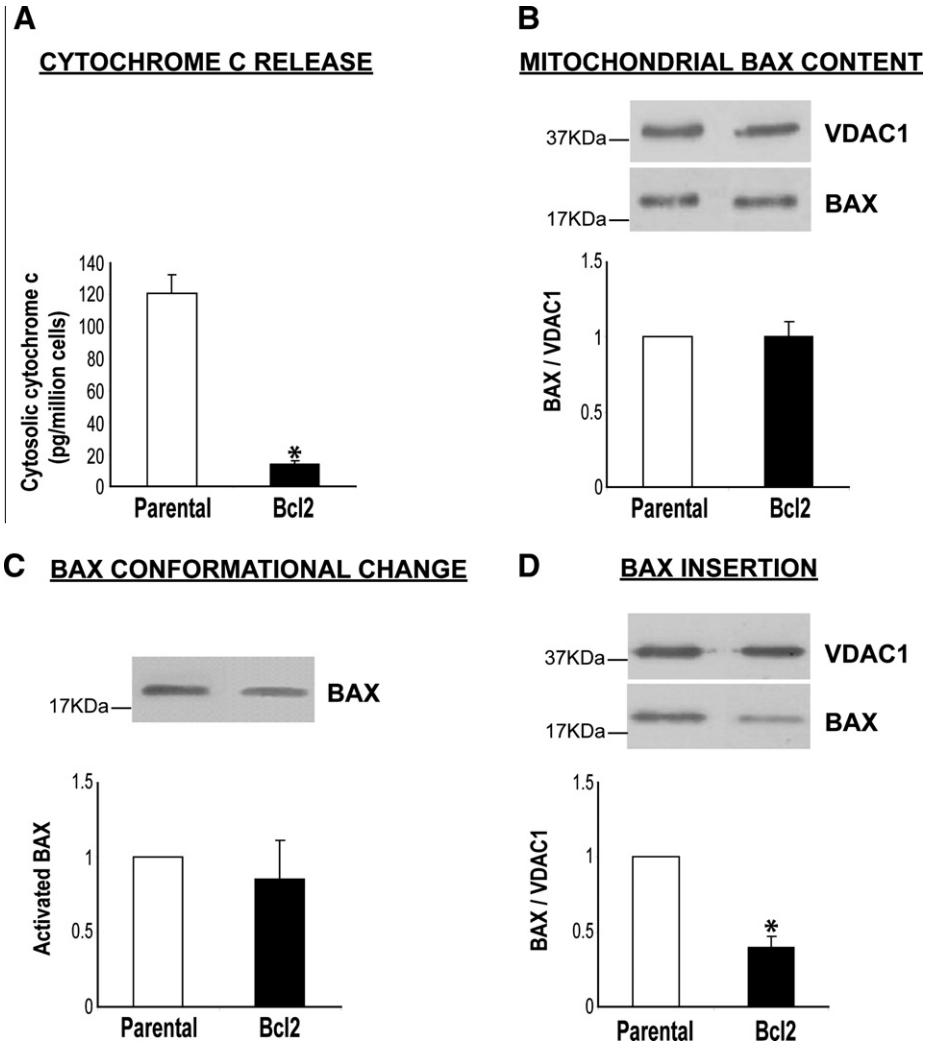

Fig. 1. Effects of Bcl2 overexpression on early mitochondrial markers of the BAX-mediated apoptotic pathway. Apoptosis was induced by 12 h of IL-3 withdrawal in parental (open bars) and Bcl2 overexpressing (Bcl2, closed bars) FL5.12 cells. Data from Bcl2 cells were quantified by densitometry, normalized relative to parental cells (see Section 2), and shown as ±S.E.M. Statistically significant differences, if any, between parental and Bcl2 overexpressing conditions, were determined by Student's *t*-test and shown as *. (A) Cytosolic cytochrome *c* from digitonin-permeabilized apoptotic parental and Bcl2 cells was quantified by ELISA as described in Section 2 (* corresponds to $p < 0.05$, $n = 4$). (B) *Mitochondrial BAX content.* The presence of mitochondrial BAX and VDAC1 in isolated mitochondria was detected by Western blot [top], and the average mitochondrial BAX/VDAC1 ratio was determined as described in Section 2 ($n = 17$) [bottom]. (C) *BAX conformational change.* Total mitochondrial proteins were solubilized in the presence of 2% CHAPS and equal amounts of total BAX were immunoprecipitated with an antibody recognizing a conformation of BAX associated with early apoptosis (N20). The immunoprecipitated BAX was detected by Western blot [top], and was quantified by densitometry ($n = 15$) [bottom]. (D) *BAX insertion.* Inserted BAX and VDAC1 were immunodetected in sodium carbonate-treated mitochondria [top] as described in Section 2. The average mitochondrial BAX/VDAC1 ratio was quantified by densitometry (* corresponds to $p < 0.05$, $n = 18$) [bottom].

apoptotic stimulation (Fig. 1B and C) suggested that Bcl2 was a positive regulator of mitochondrial BAX content. In agreement with this hypothesis, mitochondrial BAX content in Bcl2 cells grown in the presence of IL-3 (normal Bcl2 cells) was twofold higher than that in normal parental cells, reaching values similar to those observed in apoptotic parental cells (Fig. 2A). This Bcl2-mediated BAX increase in mitochondria was associated to a concomitant decrease of cytosolic BAX levels as indicated by a higher mitochondrial BAX/cytosolic BAX ratio in Bcl2 cells (Fig. 2B). Also, this effect was not due to an increase of the total cellular BAX content as Bcl2 overexpression had no influence on the total amount of BAX per cell (Fig. 2C). These data indicate that the higher levels of mitochondrial BAX content in Bcl2 cells was associated to a mitochondrial relocalization of BAX which was independent of the presence of an apoptotic stimulus. Interestingly, mitochondria of Bcl2 cells grown in the presence of IL-3 also showed similar N20 positive BAX amounts to that of apoptotic parental cells; i.e., BAX amounts which were between two and threefold higher than those of control parental cells (Fig. 2D). All these results indicated that an increase of mitochondrial Bcl2 content was able to stimulate BAX mitochondrial relocalization and change of conformation in the absence of an apoptotic stimulus.

3.3. Bcl2 overexpression induces a spontaneous increase of BAX mitochondrial content which depends on Bcl2 ability to interact with BAX

As protein–protein interactions are essential to the regulation of Bcl2 family proteins, we finally tested the impact of Bcl2/BAX interaction on the increase of mitochondrial BAX content and change of conformation observed in Bcl2 cells. For that purpose, these two parameters were assessed in mitochondria from FL5.12 cells overexpressing a Bcl2 mutant (G145E cells) which is unable to physically interact with BAX [19] (Fig. 3A). The spontaneous

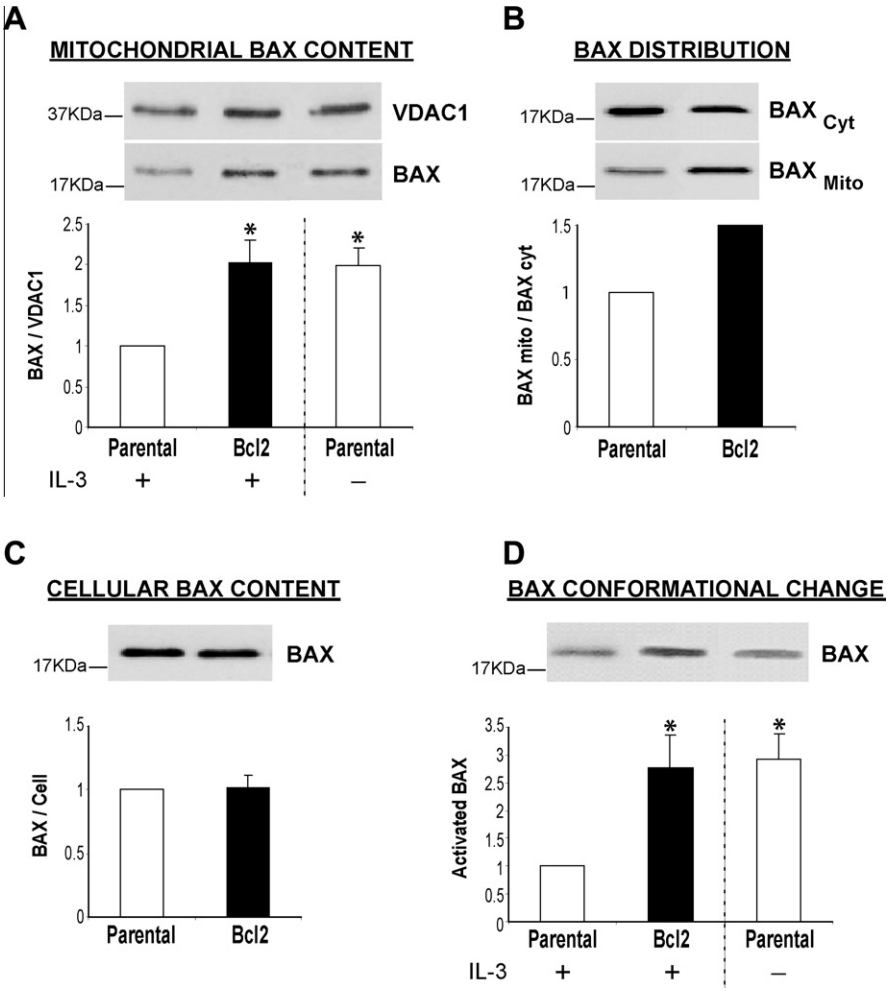

Fig. 2. Bcl2 overexpression enhances both BAX translocation and change of conformation in mitochondria of normal cells. Parental (open bars) and Bcl2 overexpressing FL5.12 cells (Bcl2, closed bars) were grown in the presence (B and C), or in the presence or absence of IL-3 (A and D). Data from Bcl2 cells were quantified by densitometry, normalized relative to parental cells (see Section 2), and shown as ±S.E.M. Statistically significant differences, if any, between parental and Bcl2 overexpressing conditions, were determined by Student's *t*-test and shown as *. (A) *Mitochondrial BAX content.* The presence of mitochondrial BAX and VDAC1 in isolated mitochondria was detected by Western blot [top], and the average mitochondrial BAX/VDAC1 ratio was quantified by densitometry as described in Section 2 (* indicates $p < 0.05$, $n = 17$) [bottom]. (B) *BAX distribution.* BAX immunodetection in cytosolic (BAX_{Cyt}) and mitochondrial (BAX_{Mito}) fractions from parental and Bcl2 cells [top]. Histogram shows the mitochondrial/cytosolic BAX signal ratio for both parental and Bcl2 cells [bottom]. These results are representative of three independent experiments. (C) *Cellular BAX content.* Total proteins from the same amounts of parental and Bcl2 cells were solubilized in the presence of 1% Triton X-100. BAX was immunodetected [top] and quantified by densitometry. This quantification was normalized per cell ($n = 14$) [bottom]. (D) *BAX conformational change.* Total mitochondrial proteins were solubilized and immunoprecipitated with an antibody recognizing a conformation of BAX associated with early apoptosis (N20) as described in Fig. 1C. Immunoprecipitated activated BAX was detected by Western blot [top] and quantified by densitometry (* corresponds to $p < 0.05$, $n = 15$) [bottom].

increases observed for both mitochondrial BAX content and change of conformation in Bcl2 cells, were abolished in G145E cells (Fig. 3B and C). This result indicated that Bcl2 is required to physically interact with BAX, at least through its BH1 domain [19], in order to increase both total and N20 positive BAX amounts in mitochondria of non-apoptotic Bcl2 cells.

4. Discussion

Bcl2 overexpression has now long been associated with lymphomagenesis and inhibition of BAX-mediated apoptosis [10–15,23]. However, the effects of Bcl2 on early steps of intrinsic apoptosis like BAX mitochondrial relocalization, insertion and change of conformation remained poorly documented and were the main focus of this work. Prolymphocytic FL5.12 cells overexpressing Bcl2 (Bcl2 cells), known for their aptitude to induce lymphoma when injected in mice [10], were the model chosen for our study. Apoptosis was induced by 12 h of IL-3 deprivation [24,25], as classic markers of mitochondrial apoptosis from BAX translocation to detection of MAC activity were detected in those cells at this time [4,13–15,24].

Bcl2 overexpression inhibited both, BAX insertion into the MOM (Fig. 1C), and cytochrome *c* release (Fig. 1A) when cells were apoptotically challenged. These results are in agreement with the inhibitory effects of an excess of Bcl2 on MAC formation and activity during mitochondrial apoptosis [15,20,21,23]. Bcl2 overexpression was originally shown to inhibit BAX mitochondrial relocalization triggered by an apoptotic stimulus [13,14]. Also, BclxL was shown recently to inhibit both of these processes in isolated mitochondria [29]. We confirmed these results as no differences in mitochondrial total and conformationally changed BAX contents were detected between normal and IL-3 deprived Bcl2 cells (compare Figs. 1B and 2A; and Figs. 1C and 2D). However and importantly, IL-3-deprived Bcl2 cells showed also similar mitochondrial total or conformationnally changed BAX contents to those of apoptotic parental cells (Fig. 1B and C). Thus, Bcl2-mediated inhibition of

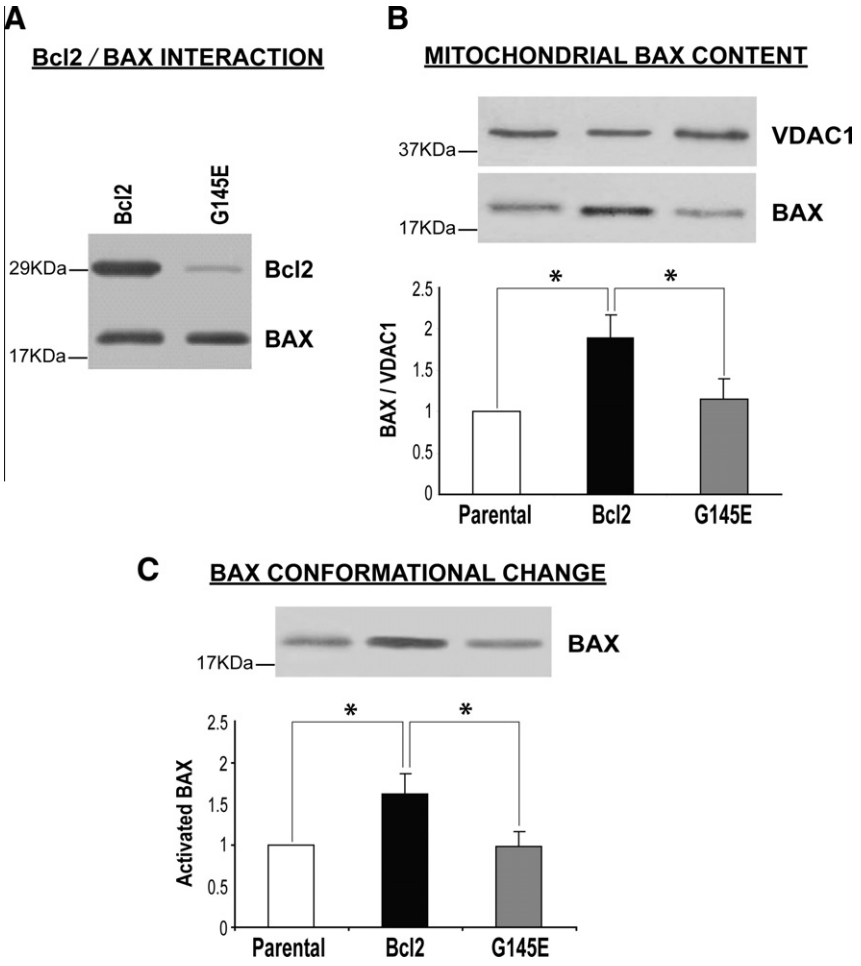

Fig. 3. The lack of Bcl2/BAX interaction abolishes Bcl2-mediated increase of BAX mitochondrial content and change of conformation. Three different FL5.12 cell types were grown in the presence of IL-3: parental cells (parental), cells overexpressing Bcl2 (Bcl2) and cells overexpressing a Bcl2 mutant which is unable to interact with BAX (G145E). Data from Bcl2 and G145E cells were quantified by densitometry, normalized relative to parental cells (see Section 2), and shown as ±S.E.M. Statistically significant differences, if any, between parental and Bcl2 overexpressing conditions, were determined by Student's *t*-test and shown as *. (A) *Bcl2/BAX interaction.* Mitochondrial CHAPS lysates from Bcl2 and G145E cells containing equal amount of BAX were immunoprecipitated with an antibody recognizing all BAX conformations (5B7). The presence of BAX and Bcl2 in the pellets containing the immunoprecipitated proteins was assessed by Western blot. The minor band in the G145E lane for Bcl2 is likely to correspond to the endogenous Bcl2 in this cell line. (B) *Mitochondrial BAX content.* Western blots show levels of BAX and VDAC1 in isolated mitochondria from parental, Bcl2 and G145E cells [top]. The average mitochondrial BAX/VDAC1 ratio was quantified by densitometry as described in Section 2 (* indicates $p < 0.05$, $n = 7$) [bottom]. (C) *BAX conformational change.* Total mitochondrial proteins from parental, Bcl2 and G145E cells were solubilized with 2% CHAPS and equal amounts of total BAX were immunoprecipitated with an anti-BAX antibody recognizing a conformation of BAX associated with early apoptosis (N20). The presence of activated BAX in the pellets containing the immunoprecipitated proteins was assessed by Western blot [top]. The average activated BAX was quantified by densitometry ($n = 11$) [bottom].

BAX mitochondrial relocalization and change of conformation were unlikely to constitute the primary events leading to apoptosis blockade as previously suggested by [13,14]. In fact, Bcl2 overexpression was rather determining in blocking mitochondrial apoptosis downstream of BAX change of conformation and upstream of BAX insertion. This observation was in agreement with the notion that, during apoptosis, BAX conformational change occurs previous to BAX insertion, as previously suggested by [5,27] and recently indicated by [37]. Finally, and as several studies indicate BAX oligomerization occurs inside the MOM [22,28], our results also strongly suggest that Bcl2-mediated inhibition of BAX insertion is the most determining step of the process leading to the blocking of cytochrome *c* release.

Another interesting observation of our study was that the total BAX content in mitochondria from normal Bcl2 cells was higher than that of normal parental cells (Fig. 2A). This elevation was the result of a spontaneous BAX mitochondrial relocalization (Fig. 2B) that required an interaction between Bcl2 and BAX; this effect was not observed when Bcl2/BAX interaction was disrupted in the Bcl2 mutant G145E (Fig. 3A and B). Of note, transient high expression levels of Bcl2 were reported to lead to MOM permeabilization; an effect which was partially reversed by the mutation G145E [30,31]. We show however that a stable overexpression of Bcl2 is not pro-apoptotic per se, and that it even makes the cells resistant to IL-3-induced apoptosis (Fig. 1A). The latent pro-apoptotic function of Bcl2 reported in [30,31] may nevertheless contribute to the increase of mitochondrial BAX in non-apoptotic cells observed during our study (Fig. 3B and C). Our findings also suggest that Bcl2 can stabilize BAX loose association at the MOM level through direct physical interaction. Other proteins integral to the MOM, like the subunit of the protein import complex TOM, Tom22, were previously shown to have a similar function [32–34]. Importantly, these proteins are natural pro-apoptotic receptors for BAX in the mitochondria. On the other hand, Bcl2 was shown to sequester the nuclear orphan receptor Nur77 at the mitochondria; an interaction leading to the conversion of Bcl2 into a pro-apoptotic protein which eventually trigger BAX conformational change and MOM permeabilization [35,36]. The results of our experiments also suggest that Bcl2 can behave as a BAX decoy receptor, stabilizing BAX at the MOM in order to inhibit further

apoptotic steps. Moreover, BAX conformational change was also increased in mitochondria of normal Bcl2 cells in a Bcl2/BAX interaction-dependant way (Figs. 2D and 3C). Strikingly, the levels of conformationally changed BAX in mitochondria of Bcl2 cells were actually similar to those observed in mitochondria of parental cells dying by apoptosis (Fig. 1B). It has been reported that this specific form of BAX does not interact with Bcl2 [26]. It was also recently shown that BAX N-terminal exposure was not essential to BAX insertion and cytochrome *c* release, but rather correlates with mitochondrial targeting [37]. These observations indicate that BAX conformational change through the exposure of its N-terminus is not a controlling step of MOM permeabilization (as also previously suggested by [5]), but rather a marker of BAX stabilization at the MOM level. Taken together, our results then strongly suggest that Bcl2 overexpression indirectly increases BAX conformational change levels at the MOM level through BAX stabilization.

In cancer cells that overexpress Bcl2, the protein itself is often largely bound to pro-apoptotic BH3-only proteins like BIM. In such circumstances, Bcl2 and the cells are described as being 'primed for death', and this characteristic is currently exploited to design new drugs (e.g., ABT-737) which selectively kill cancer cells [17]. This phenomenon has also been described in FL5.12 cells overexpressing Bcl2 in which BIM was shown to be abnormally accumulated at the mitochondrial level [16]. Interestingly, BIM was also recently shown to directly interact with BAX; this interaction leads to membrane permeabilization and cytochrome *c* release [38]. In our study, we show that Bcl2 overexpression leads also to an abnormal accumulation of BAX in mitochondria (Figs. 2 and 3). This observation suggests that the "primed for death" status induced by Bcl2 overexpression might also involve multidomain effector members of the Bcl2 family proteins, such as BAX. The analysis of this peculiar new type of "addiction" to the anti-apoptotic proteins should then prove to be critical to understand the mechanisms by which cancer cells maintain survival.

Acknowledgments

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