Bcl-X$_L$ affects Ca^{2+} homeostasis by altering expression of inositol 1,4,5-trisphosphate receptors

Chi Li*, Casey J. Fox*, Stephen R. Master*†‡, Vytautas P. Bindokas§, Lewis A. Chodosh*†‡, and Craig B. Thompson*†‡¶

*Abramson Family Cancer Research Institute, †Department of Cancer Biology, and ‡Department of Medicine, University of Pennsylvania, Philadelphia, PA 19104; and §Department of Neurobiology, Pharmacology and Physiology, University of Chicago, Chicago, IL 60637

An oligonucleotide-based microarray analysis of 9,500 genes and expressed sequence tags (ESTs) demonstrated that the type 1 inositol 1,4,5-trisphosphate receptor (IP$_3$R) was significantly down-regulated in Bcl-X$_L$-expressing as compared with control cells. This result was confirmed at the mRNA and protein levels by Northern and Western blot analyses of two independent hematopoietic cell lines and murine primary T cells. Bcl-X$_L$ expression resulted in a dose-dependent decrease in IP$_3$R protein. IP$_3$R expression is regulated as part of a mitochondrion-to-nucleus stress-responsive pathway. The uncoupling of mitochondrial oxidative phosphorylation resulted in induction of binding of the transcription factor NFATc2 to the IP$_3$R promoter and transcriptional activation of IP$_3$R. Expression of Bcl-X$_L$ led to a decreased induction of both NFATc2 DNA binding to the IP$_3$R promoter and IP$_3$R expression in response to the inhibition of mitochondrial oxidative phosphorylation. The Bcl-X$_L$-dependent decrease in IP$_3$R expression also correlated with a reduced T cell antigen receptor ligation-induced Ca^{2+} flux in Bcl-X$_L$ transgenic murine T cells, and microsomal vesicles prepared from Bcl-X$_L$-overexpressing cells exhibited lower IP$_3$-mediated Ca^{2+} release capacity. Furthermore, reintroducing IP$_3$R into Bcl-X$_L$-transfected cells partially reversed Bcl-X$_L$-dependent anti-apoptotic activity. These results suggest that even under non-apoptotic conditions, expression of Bcl-2-family proteins influences a signaling network that links changes in mitochondrial metabolism to alterations in nuclear gene expression.

Proteins of the Bcl-2 family play critical roles in the regulation of programmed cell death (1–3). In response to stimuli such as growth factor withdrawal, loss of cellular attachment, or DNA damage, cells from multicellular organisms can initiate their own death through apoptosis. Under such conditions, the expression of anti-apoptotic Bcl-2 family members, including Bcl-2 and Bcl-X$_L$, can prevent the initiation of an apoptotic response and maintain the survival and/or promote the recovery of the cell. In contrast, overexpression of pro-apoptotic family members such as Bax and Bak promotes the initiation of apoptosis in response to cytotoxic insults. These results have demonstrated that Bcl-2 proteins function to regulate the apoptotic response. However, it is less clear whether Bcl-2 proteins play roles in regulating cellular physiology under non-apoptotic conditions.

Bcl-2 and Bcl-X$_L$ are constitutively expressed and are localized to the endoplasmic reticulum (ER), perinuclear, and outer mitochondrial membranes. On the basis of their localization, Bcl-2 and Bcl-X$_L$ have been hypothesized to regulate mitochondrial physiology and/or Ca^{2+} metabolism (4, 5). Alternatively, these anti-apoptotic proteins may function primarily to oppose the pro-apoptotic functions of two other family members required for the initiation of programmed cell death, Bax and Bak (1–3). Both Bax and Bak exist in an inactive conformation in healthy cells, and they undergo conformational changes in response to apoptotic stimuli (6–8). These conformational changes then enable their participation in events that result in apoptosis.

To address whether the anti-apoptotic protein Bcl-X$_L$ influences cellular physiology in the absence of apoptotic stimuli, we have carried out an oligonucleotide-based microarray analysis of 9,500 murine genes and expressed sequence tags (ESTs) to identify global changes in gene expression in response to Bcl-X$_L$ expression. Highly reproducible changes in the expression of several genes involved in the metabolism of calcium and other divalent cations were identified. The most dramatic change was observed for the type 1 inositol 1,4,5-trisphosphate (IP$_3$) receptor (IP$_3$R), an intracellular Ca^{2+} channel gated by the secondary messenger IP$_3$. IP$_3$R is responsible for the mobilization of Ca^{2+} from intracellular stores in response to signals from receptors on the plasma membrane (9, 10). One response to the release of Ca^{2+} through IP$_3$R is enhanced coupling of mitochondrial oxidative phosphorylation rate to increased cellular demand as a consequence of activation of a signal transduction cascade (5, 11). We observed a dose-dependent reduction of IP$_3$R protein in cells that overexpress Bcl-X$_L$, which further correlated with a reduction in receptor-induced Ca^{2+} flux and with a reduced ability to release Ca^{2+} upon IP$_3$ treatment of ER vesicles. Also, reduction of mitochondrial membrane potential increased the binding of the transcription factor NFATc2 to the IP$_3$R promoter and IP$_3$R expression. This effect was compromised by Bcl-X$_L$ overexpression. These data provide new insights into how intracellular Ca^{2+} homeostasis is affected by Bcl-X$_L$ expression. This study suggests that overexpression of Bcl-X$_L$ leads to a reduction in IP$_3$R levels, through down-regulation of a mitochondrion-to-nucleus stress signaling pathway. Transient transfection of IP$_3$R was able to partially reverse Bcl-X$_L$-mediated resistance to apoptosis.

Materials and Methods

Cell Lines, Mice, and Reagents. The FL5.12 murine pro-B cell line, the 2B4.11 murine T cell hybridoma line, and pLck-Bcl-X$_L$ transgenic mice were described (12). Pharmacological agents were *myo*-inositol 1,4,5-trisphosphate, phorbol 12-myristate 13-acetate (PMA), and ionomycin (Sigma), and Indo-1 acetoxymethyl ester and propidium iodide (Molecular Probes). Antibodies were anti-CD3 (145-2C11) (PharMingen), type 1 IP$_3$R-specific polyclonal antibody (pAb; the kind gift of Darren Boehning and Suresh K. Joseph), type 3 IP$_3$R-specific mAb (Transduction Laboratories, Lexington, KY), pAbs against sarcoplasmic reticulum/ER Ca^{2+} ATPase 2 (SERCA2) and actin and mAb against NFATc2 (Santa Cruz Biotechnology), pAb against calreticulin (StressGen Biotechnologies, Victoria, Canada), goat anti-hamster pAb (Pierce), and pAb against Bcl-X$_L$ (12).

Northern and Western Blots. Total RNA from the indicated cell lines was purified by using Trizol (Life Technologies). RNA (10 μg) was resolved on formaldehyde/agarose gel and Northern blotting was performed as described (12). Whole cell extracts were prepared in RIPA buffer (0.15 M NaCl/10 mM Tris·HCl, pH 7.2/1% Nonidet P-40/1% sodium deoxycholate/0.1% SDS) with protease inhibi-

Table 1. The top five increased and the top five decreased genes in Bcl-X$_L$ as compared with control FL5.12 cells

	Bcl-X$_L$ > Neo			Bcl-X$_L$ < Neo		
Rank	Gene	GenBank accession no.	Fold increase	Gene	GenBank accession no.	Fold decrease
1	Bcl-X$_L$	U10102	5.7	IP$_3$R1 (*Itpr1*)	X15373	4.6
2	Calgranulin B (S100a9)	M83219	3.6	Kidney-derived protease-like protein (*Kdap*)	AA108747	4.4
3	X-linked lymphocyte-regulated 4 (*Xlr4*)	AA190046	2.4	c-*Myb*	M13990	3.1
4	Hemoglobin α, chain 1 (*Hbaa1*)	C79755	2.0	Malic enzyme, supernatant (*Mod1*)	AA189662	2.6
5	Growth factor receptor bound protein 7 (*Grb7*)	AA216863	1.8	Selinium-binding protein 1 (*Selenbp1*)	M320325	2.5

RNA hybridization to oligonucleotide microarrays and data analysis were performed as described in *Materials and Methods.*

tors, and protein concentrations were determined by BCA protein assay (Pierce). Western blotting was performed as described (12).

Complementary RNA (cRNA) Hybridization to Oligonucleotide Microarrays and Data Analysis. Total RNA from FL5.12 cell lines was prepared by the cesium chloride method. cRNA preparation and sequential hybridization to Affymetrix Test 2, and murine 11K sub A and sub B microarrays were performed according to the manufacturer's recommendations. The intensities of individual probe cells were determined by using GENECHIP 3.3 software (Affymetrix, Santa Clara, CA). Background subtraction, probe cell scaling (to normalize between arrays), and calculation of gene expression values and pairwise fold changes were performed in a manner similar to that recommended by the manufacturer. All microarray experiments were performed in triplicate.

Measurement of Primary T Cell [Ca²⁺]$_i$. Murine splenic T cells were purified as described (12). The purified T cells were resuspended in RPMI medium 1640 with 1% fetal bovine serum and loaded with 2 μM Indo-1 in the presence of either 5 or 10 μg/ml anti-CD3 Abs at 30°C for 30 min. Loaded cells were washed and resuspended in RPMI 1640. The measurement of cytosolic free Ca²⁺ concentration ([Ca²⁺]$_i$) was performed on an LSR flow cytometer (Becton Dickinson). Briefly, after stable basal [Ca²⁺]$_i$ was achieved, cross-linking goat anti-hamster Ab (5 μg/ml) was added to cells, and 10 min later, 1 μg of ionomycin was added. Data analysis was performed by using FLOWJO software (Tree Star, San Carlos, CA), and [Ca²⁺]$_i$ was calculated from the equation $[Ca^{2+}]_i = K \cdot (R - R_{min})/(R_{max} - R)$, where the values of R_{min} and R_{max} were determined from *in situ* calibration.

Microsomal Vesicle Preparation and Ca²⁺ Uptake and Release Measurement. Microsomal vesicles and the reaction buffer were prepared as described (13), with some modification. Free Ca²⁺ in the reaction buffer was calibrated to the indicated concentrations by using the Ca²⁺ indicator calcium green–1 and a calcium calibration kit (Molecular Probes) on a FluoroMax-2 spectrofluorimeter (Instruments S.A., Edison, NJ). Experiments were carried out at 30°C with 1 μM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) and 3 μCi/ml ⁴⁵Ca²⁺ (NEN Life Science; 1 μCi = 37 kBq) in the buffer, and three aliquots containing 5 μg of microsomal vesicles were taken out at the indicated time points.

Gel Mobility-Shift Assays. Nuclear extracts were prepared as described (14), with minor modifications. Binding reactions and electrophoretic mobility-shift assays were performed as described (14). Gel mobility-shift probes included: the murine IP$_3$R1 NFAT binding site 2, 5′-GACACCCGGGGAAAGTT-TGTGGAATGAATACGT (14), and Oct-1 consensus binding sequence, 5′-TGTCGAATGCAAATCACTAGAA (Santa Cruz Biotechnology).

Transient Transfection Death Assays. The assays were performed as described (15). The reporter β-galactosidase (β-gal) activity was measured by using a Galacto-Light Plus kit (Tropix, Bedford, MA)

on a TR717 luminometer (Tropix). Percentage of cytotoxicity was calculated with the equation: cytotoxicity (%) = 100 − (100·experimental β-gal/control β-gal).

Results

Type 1 IP$_3$R mRNA Is Decreased in Bcl-X$_L$-Expressing Cells. Previous experiments have shown that the expression of Bcl-X$_L$ in FL5.12, an interleukin-3 (IL-3)-dependent lymphoid cell line, can confer resistance to a variety of death stimuli (12). Bcl-X$_L$-transfected FL5.12 cells maintain equivalent viability, cell size, mitochondrial mass, growth rate, and cell cycle time as parental or vector-transfected cells when continuously grown in IL-3-containing medium (refs. 12 and 16; data not shown). Total RNA was purified from FL5.12 cells stably transfected with either a Bcl-X$_L$-expressing plasmid or the vector control (12) 24 hr after passage into fresh IL-3-containing medium. The Bcl-X$_L$-expressing cell line used in these analyses was chosen because Bcl-X$_L$ protein levels in this cell line were comparable to those found in mitogen-activated T cells (data not shown). Isolated RNA was then hybridized to Affymetrix murine 11K oligonucleotide microarrays representing 9,500 genes and expressed sequence tags (ESTs). Data from three independent experiments were averaged, and pairwise analysis was used to identify differentially expressed genes. The expression of 17 genes was increased significantly in Bcl-X$_L$-overexpressing cells (Xl-4.1) versus control cells (Neo-5.3), whereas we identified 59 genes whose expression was lower in Xl-4.1 versus Neo-5.3 cells. The 10 genes whose expression differed most markedly in Bcl-X$_L$-overexpressing versus control cells are presented in Table 1. Bcl-X$_L$ displayed the greatest increase in Xl-4.1 cells, confirming that transfection had resulted in a significant increase in Bcl-X$_L$ expression. The type 1 IP$_3$R gene, *Itpr1*, was the top-ranked gene whose mRNA was decreased in Bcl-X$_L$-overexpressing as compared with control cells. In addition to type 1 IP$_3$R, two other subtypes of IP$_3$R (types 2 and 3) encoded by distinct genes have been identified in mammalian cells (9, 10). The type 2 IP$_3$R is expressed at a low level in FL5.12 cells (data not shown), whereas type 3 IP$_3$R expression was not detectable by microarray analyses.

We next determined whether overexpressing Bcl-X$_L$ could also similarly affect IP$_3$R expression in another cell line. Two stably transfected 2B4.11 T cell hybridoma lines were examined, the empty vector control (Neo) and the Bcl-X$_L$-expressing line (Xl-C10) (12). As reported in other systems (17, 18), Bcl-X$_L$ expression protected 2B4.11 cells from apoptosis induced by treatment with the SERCA inhibitor thapsigargin. As shown in Fig. 1*A*, the majority of Bcl-X$_L$-expressing cells remained viable after 48 hr in the presence of either 50 or 100 nM thapsigargin, whereas most control cells were dead at this time. Northern blot analysis revealed that the Bcl-X$_L$-transfected 2B4.11 cells expressed significantly less type 1 IP$_3$R than did the vector-transfected 2B4.11 cells (Fig. 1*B*).

IP$_3$R Proteins Are Reduced in Bcl-X$_L$-Expressing Cells. To confirm the Northern data, the link between Bcl-X$_L$ and IP$_3$R expression was investigated by Western blot analyses. As shown in Fig. 2*A*, FL5.12, 2B4.11, and primary T cells that overexpressed Bcl-X$_L$ had signif-
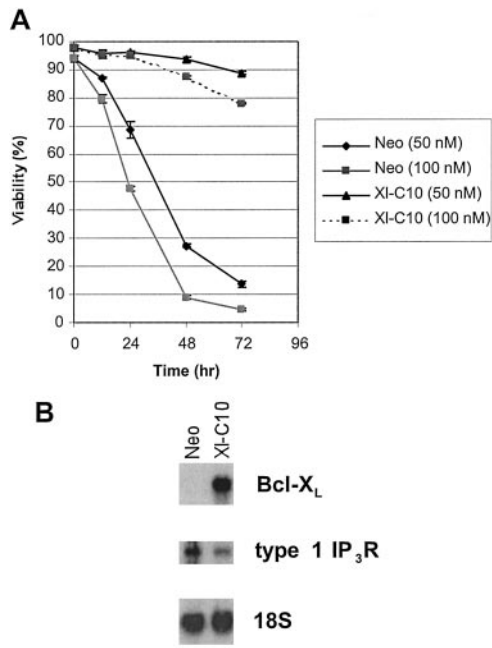
Fig. 1. Bcl-X$_L$-expressing 2B4.11 cells survive in the presence of thapsigargin and have reduced type 1 IP$_3$R mRNA. (*A*) Survival was measured by propidium iodide exclusion with either 50 nM or 100 nM thapsigargin in the cell cultures at the indicated time points. Means and standard deviations of triplicate measurements are shown. (*B*) Northern blots of total RNA purified from 2B4.11 Neo and Xl-C10 cells. The filter was hybridized with probes for Bcl-X$_L$, type 1 IP$_3$R, and 18S rRNA, respectively.

icantly lower levels of type 1 IP$_3$R protein as compared with controls. Anti-Bcl-X$_L$ and actin Western blots confirmed the levels of Bcl-X$_L$ and total protein in these samples (Fig. 2*A*). Type 2 IP$_3$R protein could not be detected in either FL5.12 or 2B4.11 cells by Western blotting. However, as shown in Fig. 2*B*, the expression of type 3 IP$_3$R was also reduced in Bcl-X$_L$-overexpressing 2B4.11 and FL5.12 cells. In contrast, the expression of SERCA2, the major pump responsible for the transfer of Ca^{2+} from the cytosol to the ER, was unaffected by Bcl-X$_L$ expression (Fig. 2*B*). Bcl-X$_L$ over-expression also did not affect the expression of calreticulin, a Ca^{2+}-binding protein in the ER lumen, or total intracellular Ca^{2+} (Fig. 2*B* and data not shown).

Thus, expression of Bcl-X$_L$ reduces the expression of at least two subtypes of IP$_3$R. We next assessed whether Bcl-X$_L$ could affect IP$_3$R expression in a dose-dependent manner. To this purpose, additional 2B4.11 cell lines expressing various amounts of Bcl-X$_L$ were made. As shown in Fig. 3, type 1 IP$_3$R was expressed at similar levels in the parental and three Neo cell lines tested. All three Bcl-X$_L$-expressing cell lines had considerably less type 1 IP$_3$R compared with the parental and Neo cell lines. Among three Bcl-X$_L$-expressing cell lines examined, the amounts of type 1 IP$_3$R were inversely correlated with the levels of Bcl-X$_L$. Parallel experiments using a Bcl-2 expression plasmid demonstrated that stable Bcl-2 expression also led to a comparable reduction in IP$_3$R expression (data not shown).

Expression of Bcl-X$_L$ Leads to Reduced Induction of NFAT DNA-Binding Activity and Type 1 IP$_3$R Expression in Response to the Impairment of Mitochondrial Oxidative Phosphorylation. Previous studies in muscle cells have demonstrated that the inhibition of mitochondrial oxidative phosphorylation results in the increased expression of the myoblast counterpart of IP$_3$R, the ryanodine receptor-1, because of activation of a mitochondrial-to-nuclear stress signaling pathway (19, 20). Bcl-X$_L$ has been reported to maintain outer mitochondrial membrane integrity by promoting the efficient coupling of electron
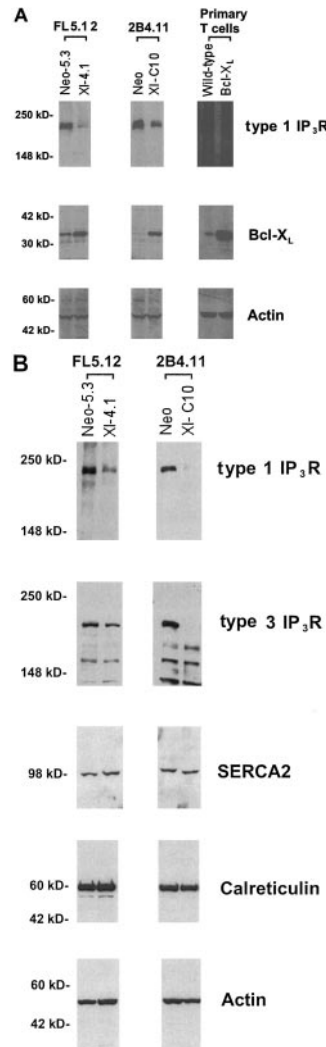

Fig. 2. IP$_3$R proteins are down-regulated in cells expressing Bcl-X$_L$. (*A*) Western blots of cell extracts made from the indicated cells. Filters were prepared as described in *Materials and Methods* and probed with antibodies against type 1 IP$_3$R, Bcl-X$_L$, and actin, respectively. The numbers at left indicate the molecular markers. (*B*) Immunoblot analysis of whole cell extracts from the cells indicated on the top. The filters were probed with the antibodies indicated on the right. The positions of molecular mass markers are indicated on the left.

transport to cytosolic substrates and ADP levels (16, 21). To determine whether Bcl-X$_L$ expression affects the mitochondrial-to-nuclear stress signaling pathway, we next tested IP$_3$R expression after treatment with carbonyl cyanide *p*-(trifluoromethoxy)phenyl-hydrazone (FCCP), a potent uncoupler of mitochondrial oxidative phosphorylation. As shown in Fig. 4*A*, 2B4.11 Neo cells gradually died in the presence of 20 μM FCCP, whereas Xl-C10 cells remained viable, although FCCP depolarized the mitochondria and reduced the mitochondrial membrane potential of both Neo and Xl-C10 cells to a similar degree (data not shown). Thirty minutes after FCCP treatment, type 1 IP$_3$R expression was significantly enhanced in both Neo and Xl-C10 cells (Fig. 4*B*). As in untreated cells, the level of type 1 IP$_3$R protein in Neo cells was nearly 3 times that in Xl-C10 cells in the presence of FCCP.

Transcriptional activation of IP$_3$R has been reported to be dependent on activation of NFAT family of transcription factors (14, 22). To determine whether changes in mitochondrial potential correlated with changes in NFAT DNA-binding activity, we examined NFAT protein levels in 2B4.11 Neo and Xl-C10 cells. The two cell lines had comparable total NFAT protein (data not shown). However, when nuclear extracts were made, less constitutive NFAT-dependent DNA binding to the type 1 IP$_3$R promoter was detected in the nuclear extract of Bcl-X$_L$-expressing cells as compared with control cells (Fig. 4*C*). Furthermore, mitochondrial uncoupling led to a potent induction of NFATc2 DNA-binding activity, but not NFATc1 and NFATc3, two other NFAT family
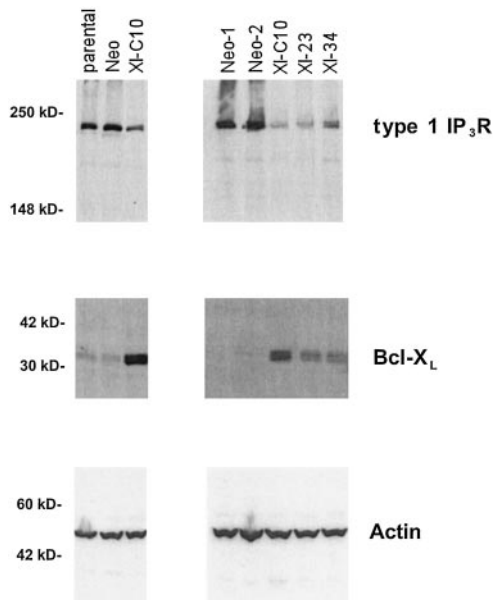
Fig. 3. Bcl-X$_L$ regulates type 1 IP$_3$R expression in a dose-dependent manner. Cell extracts from the indicated 2B4.11 cells were prepared, and the expression of type 1 IP$_3$R was analyzed by Western blotting. Positions of molecular mass markers are shown on the left.

members found in lymphocytes as demonstrated by gel-shift assays (Fig. 4C and data not shown). The level of NFAT induction was suppressed in Bcl-X$_L$-expressing cells. However, Bcl-X$_L$ expression did not suppress the level of NFAT DNA-binding activity induced by treatment with phorbol 12-myristate 13-acetate and the calcium ionophore ionomycin.

T Cell Receptor (TCR)-Mediated Ca^{2+} Flux Is Reduced in Bcl-X$_L$ Transgenic Primary T Cells. The mobilization of IP$_3$-sensitive Ca^{2+} stores through IP$_3$R plays a central role in TCR-mediated T cell activation

(23, 24). Therefore, we next determined whether reduced IP$_3$R expression in Bcl-X$_L$-expressing transgenic T cells (Fig. 2A) had a functional impact on the mobilization of ER Ca^{2+} after TCR cross-linking. Ca^{2+} flux assays were performed on live cells by using flow cytometry. The elevation of [Ca^{2+}]$_i$ induced by CD3 ligation was measured in purified splenic T cells from both wild-type and Bcl-X$_L$-transgenic mice. In both wild-type and Bcl-X$_L$-transgenic T cells, CD3 cross-linking induced a rapid elevation of [Ca^{2+}]$_i$, which subsequently declined. However, the amplitude of the [Ca^{2+}]$_i$ flux in Bcl-X$_L$-transgenic T cells was consistently lower than that in wild-type T cells (Fig. 5A). The peak [Ca^{2+}]$_i$ elevation induced by CD3 ligation in wild-type T cells was 249.6 ± 10.2 nM (n = 3), compared with 177.4 ± 15.0 nM (n = 3; P < 0.025) in Bcl-X$_L$ T cells (Fig. 5B).

Microsomal Vesicles with Reduced IP$_3$R Release Less Ca^{2+} upon IP$_3$ Treatment. To investigate whether decreased expression of IP$_3$R in Bcl-X$_L$-expressing cells directly affected intracellular Ca^{2+} homeostasis, we performed ^{45}Ca^{2+} uptake and release experiments using purified microsomal vesicles. Microsomal vesicles from 2B4.11 Neo and Xl-C10 cell lines were prepared. Western blot analyses demonstrated very little mitochondrial contamination in the prepared microsomal vesicles (data not shown). As in 2B4.11 whole cell extracts, there was an inverse correlation between the amount of type 1 IP$_3$R in purified microsomal vesicles and the level of Bcl-X$_L$ expression (Fig. 6A). With 500 nM free Ca^{2+} in the reaction buffer, microsomal vesicles were allowed to accumulate ^{45}Ca^{2+} in the presence of MgATP for 30 min, at which point equilibrium was achieved (data not shown). As shown in Fig. 6B, the ^{45}Ca^{2+}-loaded microsomes were then treated with 10 μM IP$_3$. When microsomal vesicles purified from control cells were used, IP$_3$ addition resulted in the release of about 40% of total ^{45}Ca^{2+}, and this level was sustained over the remainder of the experiment. In contrast, vesicles purified from Bcl-X$_L$-expressing Xl-C10 cells release less than 25% of total ^{45}Ca^{2+} immediately after IP$_3$ addition. Unlike those prepared from control cells, vesicles purified from Xl-C10 cells gradually reaccumulated the released ^{45}Ca^{2+} so that 30 min after IP$_3$ addition, more than 90% of originally accumulated
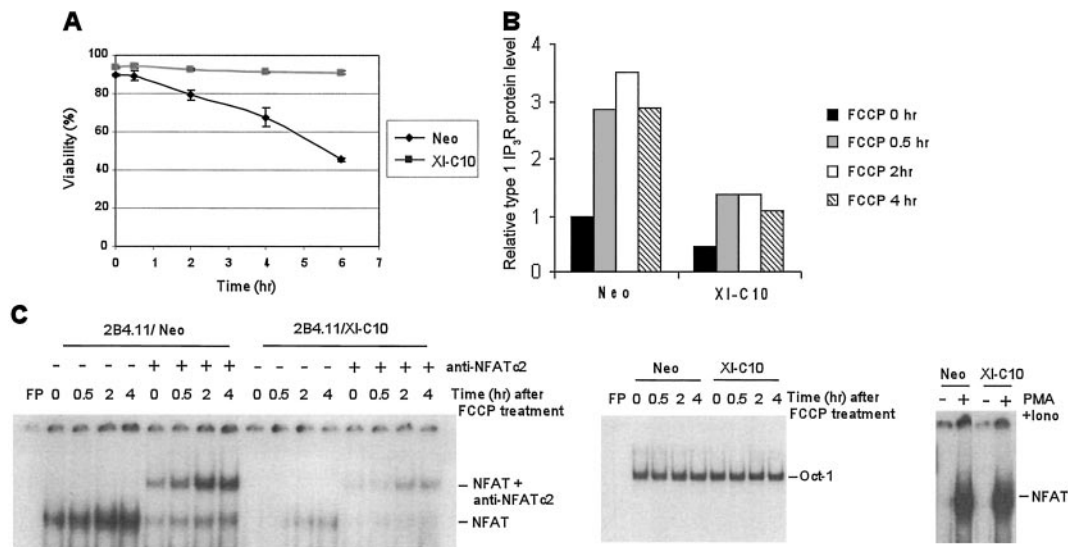

Fig. 4. Increased NFATc2 binding to type 1 IP$_3$R promoter and type 1 IP$_3$R expression in response to inhibition of mitochondrial oxidative phosphorylation is compromised by Bcl-X$_L$ expression. (A) The viability of 2B4.11 Neo and Xl-C10 cells in the presence of 20 μM FCCP was measured over time by the propidium iodide exclusion method. Means and standard deviations of triplicates are shown. (B) Whole cell extracts were prepared from the indicated 2B4.11 cells treated with FCCP. The amounts of type 1 IP$_3$R and actin on Western blot films were determined as described in *Materials and Methods*. The relative type 1 IP$_3$R protein level is the amount of IP$_3$R protein normalized to the amount of actin protein. (C) The indicated 2B4.11 cells were treated with FCCP and nuclear extracts were prepared. Gel-shift assays were performed by using end-labeled murine type 1 IP$_3$R NFAT binding site 2 or Oct-1 binding site. Gel-shift experiments with Oct-1 were used to normalize the quantity and quality of nuclear extracts. FP, free probe; Iono, ionomycin.
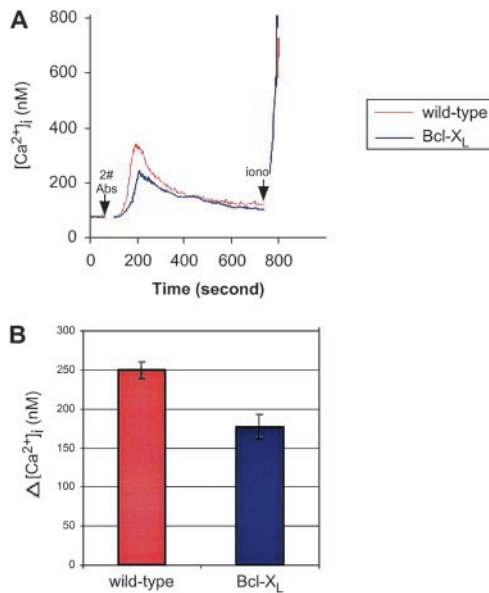
Fig. 5. TCR-mediated $[Ca^{2+}]_i$ elevation is reduced in Bcl-X$_L$ transgenic T cells. (*A*) Analysis of Ca^{2+} flux upon CD3 ligation by flow cytometry. Purified splenic T cells were incubated with Indo-1 and anti-CD3. $[Ca^{2+}]_i$ was measured by using flow cytometry, and Ca^{2+} release was induced by addition of cross-linking secondary antibodies. The $[Ca^{2+}]_i$ was calibrated as described in *Materials and Methods*. Arrows represent time when indicated reagents were added. 2# Abs, secondary antibodies; Iono, ionomycin. (*B*) Peak values of $[Ca^{2+}]_i$ elevation upon CD3 ligation shown in *A* were plotted as mean ± SEM from three independent experiments.

^{45}Ca^{2+} was reaccumulated in the microsomes. Similar results were observed with buffers containing a range of concentrations of free Ca^{2+} (data not shown).

Transfection of IP$_3$R Partially Reverses Bcl-X$_L$ Anti-apoptotic Activity.
To determine whether reduced IP$_3$R expression would account for any of the anti-apoptotic activity of Bcl-X$_L$-expressing cells, the ability of IP$_3$R expression to reverse Bcl-X$_L$ resistance to apoptosis was examined. Both 2B4.11 Neo and Xl-C10 cells were transiently transfected with either an IP$_3$R-expressing plasmid or a control vector, and the susceptibility to cell death upon thapsigargin treatment was examined. IP$_3$R expression led to a consistent but partial reduction in the ability of Bcl-X$_L$ to promote cell survival (Fig. 6*C*). IP$_3$R expression had marginal effects on cell survival of control cells.

Discussion

To study whether anti-apoptotic proteins can affect gene expression under non-apoptotic conditions, we examined gene expression by microarray analysis in stable cell lines expressing high levels of the anti-apoptotic Bcl-2-related protein Bcl-X$_L$. The type 1 IP$_3$R was found to be the most markedly decreased transcript in Bcl-X$_L$-overexpressing cells. The reduction of IP$_3$R was confirmed at both the mRNA and protein levels in several hematopoietic cell types. Depolarization of mitochondria increased both NFAT binding to the IP$_3$R promoter and the expression of IP$_3$R, both of which were compromised by Bcl-X$_L$ overexpression. Reduced expression of IP$_3$R correlated with a reduction in receptor-coupled Ca^{2+} release and a decrease in IP$_3$-responsive Ca^{2+} release from microsomal vesicles prepared from Bcl-X$_L$-expressing cells. Expression of IP$_3$R partially compromised Bcl-X$_L$'s function to promote cell survival. Together, these data provide molecular evidence that Bcl-X$_L$ expression affects intracellular Ca^{2+} homeostasis through modulation of the expression of a well-established Ca^{2+} channel, IP$_3$R.
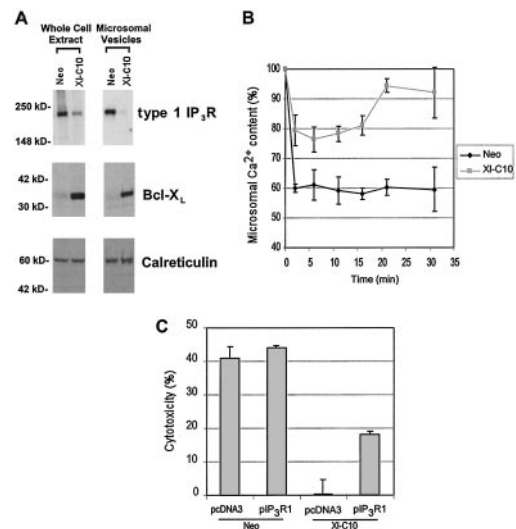

Fig. 6. Reduced amount of IP$_3$R in microsomal vesicles is associated with reduced IP$_3$-responsive Ca^{2+} release, and expressing IP$_3$R partially reverses Bcl-X$_L$ anti-apoptotic activity. (*A*) Western blots of cell extracts and microsomal vesicles prepared from the indicated 2B4.11 cell lines. The filters were probed with the indicated antibodies. The Positions of molecular mass markers are shown on the left. (*B*) Microsomal vesicles shown in *A* were used in ^{45}Ca^{2+} uptake and release experiments as described in *Materials and Methods*. After ^{45}Ca^{2+} content in microsomal vesicles reached equilibrium, 10 μM IP$_3$ was added to reactions. The microsomal ^{45}Ca^{2+} contents were measured 1, 5, 10, 15, 20, and 30 min after addition of IP$_3$. The amount of microsomal ^{45}Ca^{2+} is represented as percentage of the value at equilibrium. Means and standard deviations of triplicate measurements are shown. The data are representative of three independent experiments. (*C*) 2B4.11 Neo and Xl-C10 cells were transfected with 3 μg of pCMV-β-gal and 15 μg of the indicated expression plasmids. The cells were cultured in the absence or presence of 200 nM thapsigargin and analyzed for cytotoxicity 20 hr later as described in *Materials and Methods*. Mean and standard deviations of triplicate measurements are shown, and the data are representative of three independent experiments.

The expression of Bcl-2-related proteins has been reported to affect intracellular Ca^{2+} homeostasis (17, 25–31), although the molecular basis of these effects has remained unclear. The compensatory reduction in IP$_3$R expression in response to increase in expression of an anti-apoptotic Bcl-2 protein reported here may account for many of the effects previously noted. For example, expression of Bcl-2 has been previously reported to be associated with an altered distribution of intracellular Ca^{2+} (25), an increased mitochondrial Ca^{2+} uptake capacity (26), an enhanced ability of the ER to sequester Ca^{2+} (17, 27), a decreased total Ca^{2+} release in response to Ca^{2+}-mobilizing signals (30, 31), and a reduced ability of Bcl-2-transfected cells to initiate NFAT-dependent changes in gene expression (32). The Bcl-X$_L$-dependent reduction in IP$_3$R expression could result in a greater sequestration of Ca^{2+} in the ER, lower levels of total Ca^{2+} release in response to IP$_3$-dependent signal transduction, and a reduction in the Ca^{2+} found in the low-affinity/high-capacity mitochondrial storage pool. Finally, the ability of intracellular Ca^{2+} to induce NFAT-dependent transcription has been shown to be dependent on both the magnitude and duration of intracellular Ca^{2+} release (24). As we show here, reduction in the expression of IP$_3$R results in a decrease in both the magnitude and duration of Ca^{2+} release in response to TCR signal transduction.

Bcl-X$_L$ is likely to influence IP$_3$R mRNA and protein levels indirectly. There is currently no evidence to suggest that Bcl-X$_L$ or related Bcl-2 family members have direct effects on either transcription or translation. IP$_3$R expression has been reported to depend on NFAT-dependent transcription (14, 22). Bcl-X$_L$-transfected cells have lower levels of nuclear NFAT-binding activity

for the IP$_3$R promoter. This is not due to lower total NFAT levels because Bcl-X$_L$ transfectants have levels of NFAT comparable to those of controls as assayed by Western blotting and were able to induce comparable NFAT DNA-binding activity in response to phorbol 12-myristate 13-acetate and ionomycin. Bcl-X$_L$ has been reported to maintain more efficient ADP-coupling of oxidative phosphorylation (16, 21); this would account for its ability to decrease both basal NFAT DNA-binding activity and the magnitude of the induction of NFAT DNA binding in response to mitochondrial uncoupling. Mitochondrial metabolic inhibitors have been reported to activate a mitochondrion-to-nucleus stress signaling network that leads to alterations in gene expression that then affect a wide variety of cellular processes (19, 20). In muscle cells, the gene with the greatest increase in response to activation of this pathway is the ryanodine receptor-1 (RYR-1) (20). The effect of this increased RYR-1 expression is to potentiate Ca^{2+} release and to enhance the activation of Ca^{2+}-responsive factors, including the Ca^{2+}-dependent NFAT. The results of the present study may therefore be accounted for by Bcl-X$_L$ modulation of a mitochondrial stress signaling pathway that in turn regulates the sensitivity of cytosolic Ca^{2+} signaling pathways to changes in mitochondrial metabolic function. Thus, expression of anti-apoptotic Bcl-2 family members results in the decreased expression of IP$_3$R-related ER Ca^{2+} release channels, leading to a reduction in the Ca^{2+} release in response to IP$_3$ and a reduction in the activity of Ca^{2+}-responsive transcription factors. In contrast, agents that impair mitochondrial electron transport and ATP production may cause reciprocal changes in these pathways, leading to an elevation in the expression of IP$_3$-gated channels, the potentiation of Ca^{2+}-dependent signal transduction, and enhanced activity of Ca^{2+}-dependent transcription factors such as NFAT (19, 20).

Ca^{2+} plays a regulatory role in mitochondrial ATP production by enhancing the efficiency of enzymes involved in the tricarboxylic acid cycle and electron transport (5, 11). It has been reported that close contacts between the mitochondria and ER Ca^{2+}-release channels enable high concentrations of Ca^{2+} in microdomains to be generated upon IP$_3$-mediated Ca^{2+} release from the ER, and that these high local concentrations stimulate mitochondrial respiration in response to intracellular Ca^{2+} signaling (33). This respiratory control pathway is negatively regulated by ATP. When ATP levels are low, the ability of Ca^{2+}-ATPases to sequester released Ca^{2+} into the ER is compromised, which could lead to a prolonged Ca^{2+}-induced respiratory burst, and increased Ca^{2+}-dependent signal transduction and transcription. Bcl-X$_L$ has been proposed to maintain metabolic homeostasis and ATP production by providing more efficient coupling of oxidative phosphorylation (16, 21). Enhanced cellular ATP production supported by Bcl-2 proteins would be expected to have an inhibitory effect on Ca^{2+}-dependent signal transduction by promoting more rapid ATP-dependent Ca^{2+} reuptake.

Previous studies have also suggested that IP$_3$R is directly involved in apoptosis. For example, type 1 IP$_3$R-deficient Jurkat T cells are defective in IP$_3$-induced Ca^{2+} mobilization and are resistant to apoptosis induced by a variety of death stimuli (34). However, T cells of type 1 IP$_3$R-deficient mice demonstrated Ca^{2+} mobilization in response to TCR stimulation (35). This could be the result of compensatory function of other IP$_3$R family members. Type 3 IP$_3$R is selectively increased at both the mRNA and protein levels during apoptosis, and apoptosis is blocked by expression of type 3 IP$_3$R antisense RNA (36, 37). Similarly, apoptosis induced by B cell receptor cross-linking is inhibited in cells lacking all three subtypes of IP$_3$R (38). Some evidence suggests that an IP$_3$-dependent intracellular Ca^{2+} signal may induce apoptosis by modulating the activity of the phosphatase calcineurin (39). Thus, one possible mechanism by which Bcl-X$_L$ may enhance the resistance to apoptosis is through its ability to reduce IP$_3$R expression. Consistent with the possibility, reintroduction of type 1 IP$_3$R by an expression plasmid was found to partially reverse the ability of Bcl-X$_L$ to protect cells form death. However, it is unlikely that Bcl-X$_L$'s effects on IP$_3$R expression account for all of Bcl-X$_L$ anti-apoptotic activities, particularly those involved in opposing of the pro-apoptotic activities of Bax and Bak. Current evidence suggests that Bcl-2-related proteins may protect cells from apoptosis by multiple mechanisms. Recent studies have shed light on the issue of why reduced IP$_3$R levels may promote cell survival. IP$_3$-mediated Ca^{2+} spikes induce the opening of the mitochondrial permeability transition pore, and thereby trigger cytochrome c release that initiates apoptosis (40). One intriguing possibility is that the reduced levels of IP$_3$R in cells expressing Bcl-X$_L$ release insufficient Ca^{2+} to trigger the mitochondrial permeability transition in the presence of death stimuli. The down-regulation of IP$_3$R in response to Bcl-X$_L$ expression provides another mechanism by which anti-apoptotic Bcl-2 proteins contribute to the apoptotic resistance of a cell.

1. Adams, J. M. & Cory, S. (1998) *Science* **281,** 1322–1326.
2. Reed, J. C. (1998) *Oncogene* **17,** 3225–3236.
3. Gross, A., McDonnell, J. M. & Korsmeyer, S. J. (1999) *Genes. Dev.* **13,** 1899–1911.
4. Vander Heiden, M. G. & Thompson, C. B. (1999) *Nat. Cell Biol.* **1,** E209–E216.
5. Hajnóczky, G., Csordas, G., Madesh, M. & Pacher, P. (2000) *Cell Calcium* **28,** 349–363.
6. Gross, A., Jockel, J., Wei, M. C. & Korsmeyer, S. J. (1998) *EMBO J.* **17,** 3878–3885.
7. Nechushtan, A., Smith, C. L., Hsu, Y. T. & Youle, R. J. (1999) *EMBO J.* **18,** 2330–2341.
8. Wei, M. C., Lindsten, T., Mootha, V. K., Weiler, S., Gross, A., Ashiya, M., Thompson, C. B. & Korsmeyer, S. J. (2000) *Genes. Dev.* **14,** 2060–2071.
9. Taylor, C. W., Genazzani, A. A. & Morris, S. A. (1999) *Cell Calcium* **26,** 237–251.
10. Patel, S., Joseph, S. K. & Thomas, A. P. (1999) *Cell Calcium* **25,** 247–264.
11. Duchen, M. R. (2000) *J. Physiol.* **529,** 57–68.
12. Rathmell, J. C., Vander Heiden, M. G., Harris, M. H., Frauwirth, K. A. & Thompson, C. B. (2000) *Mol. Cell* **6,** 683–692.
13. Boehning, D. & Joseph, S. K. (2000) *J. Biol. Chem.* **275,** 21492–21499.
14. Graef, I. A., Mermelstein, P. G., Stankunas, K., Neilson, J. R., Deisseroth, K., Tsien, R. W. & Crabtree, G. R. (1999) *Nature (London)* **401,** 703–708.
15. Memon, S. A., Moreno, M. B., Petrak, D. & Zacharchuk, C. M. (1995) *J. Immunol.* **155,** 4644–4652.
16. Vander Heiden, M. G., Chandel, N. S., Schumacker, P. T. & Thompson, C. B. (1999) *Mol. Cell* **3,** 159–167.
17. Lam, M., Dubyak, G., Chen, L., Nuñez, G., Miesfeld, R. L. & Distelhorst, C. W. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6569–6573.
18. Wei, H., Wei, W., Bredesen, D. E. & Perry, D. C. (1998) *J. Neurochem.* **70,** 2305–2314.
19. Biswas, G., Adebanjo, O. A., Freedman, B. D., Anandatheerthavarada, H. K., Vijayasarathy, C., Zaidi, M., Kotlikoff, M. & Avadhani, N. G. (1999) *EMBO J.* **18,** 522–533.
20. Amuthan, G., Biswas, G., Zhang, S., Klein-Szanto, A., Vijayasarathy, C. & Avadhani, N. G. (2001) *EMBO J.* **20,** 1910–1920.
21. Vander Heiden, M. G., Chandel, N. S., Li, X. X., Schumacker, P. T., Colombini, M. & Thompson, C. B. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 4666–4671.
22. Genazzani, A. A., Carafoli, E. & Guerini, D. (1999) *Proc. Natl. Acad. Sci. USA* **97,** 5797–5801.
23. Acuto, O. & Cantrell, D. (2000) *Annu. Rev. Immunol.* **18,** 165–184.
24. Lewis, R. S. (2001) *Annu. Rev. Immunol.* **19,** 497–521.
25. Baffy, G., Miyashita, T., Williamson, J. R. & Reed, J. C. (1993) *J. Biol. Chem.* **268,** 6511–6519.
26. Murphy, A. N., Bredesen, D. E., Cortopassi, G., Wang, E. & Fiskum, G. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 9893–9898.
27. He, H., Lam, M., McCormick, T. S. & Distelhorst, C. W. (1997) *J. Cell Biol.* **138,** 1219–1228.
28. Ichimiya, M., Chang, S. H., Liu, H., Berezesky, I. K., Trump, B. F. & Amstad, P. A. (1998) *Am. J. Physiol.* **275,** C832–C839.
29. Zhu, L., Ling, S., Yu, X., Venkatesh, L. K., Subramanian, T., Chinnadurai, G. & Kuo, T. H. (1999) *J. Biol. Chem.* **274,** 33267–33273.
30. Foyouzi-Youssefi, R., Arnaudeau, S., Borner, C., Kelley, W. L., Tschopp, J., Lew, D. P., Demaurex, N. & Krause, K. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 5723–5728.
31. Pinton, P., Ferrari, D., Magalhaes, P., Schulze-Osthoff, K., Di Virgilio, F., Pozzan, T. & Rizzuto, R. (2000) *J. Cell Biol.* **148,** 857–862.
32. Linette, G. P., Li, Y., Roth, K. & Korsmeyer, S. J. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 9545–9551.
33. Rizzuto, R., Pinton, P., Carrington, W., Fay, F. S., Fogarty, K. E., Lifshitz, L. M., Tuft, R. A. & Pozzan, T. (1998) *Science* **280,** 1763–1766.
34. Jayaraman, T. & Marks, A. R. (1997) *Mol. Cell. Biol.* **17,** 3005–3012.
35. Hirota, J., Baba, M., Matsumoto, M., Furuichi, T., Takatsu, K. & Mikoshiba, K. (1998) *Biochem. J.* **333,** 615–619.
36. Khan, A. A., Soloski, M. J., Sharp, A. H., Schilling, G., Sabatini, D. M., Li, S. H., Ross, C. A. & Snyder, S. H. (1996) *Science* **273,** 503–507.
37. Blackshaw, S., Sawa, A., Sharp, A. H., Ross, C. A., Snyder, S. H. & Khan, A. A. (2000) *FASEB J.* **14,** 1375–1379.
38. Sugawara, H., Kurosaki, M., Takata, M. & Kurosaki, T. (1997) *EMBO J.* **16,** 3078–3088.
39. Wang, H., Pathan, N., Ethell, I. M., Krajewski, S., Yamaguchi, Y., Shibasaki, F., McKeon, F., Bobo, T., Franke, T. F. & Reed, J. C. (1999) *Science* **284,** 339–343.
40. Szalai, G., Krishnamurthy, R. & Hajnóczky, G. (1999) *EMBO J.* **18,** 6349–6361.

CELL BIOLOGY