Bcl-X_L prevents serum deprivation-induced oxidative stress mediated by Romo1

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Abstract. B-cell lymphoma-extra large (Bcl-X_L) has been known to suppress serum deprivation-induced cell death, while reactive oxygen species modulator 1 (Romo1) is responsible for a serum deprivation-induced increase in reactive oxygen species (ROS). Therefore, we investigated whether Bcl-X_L expression could inhibit the serum deprivation-induced increase in ROS and cell death, which are mediated by Romo1. We found that Bcl-X_L expression effectively blocked serum deprivation- and Romo1-triggered ROS generation. Bcl-X_L also inhibited apoptotic cell death induced by both serum deprivation and oxidative stress. From these results, we suggest that increased Bcl-X_L expression, which is observed in many cancer cells, confers resistance to oxidative stress in the cancer cells by suppressing Romo1-mediated oxidative stress.

Introduction

A major function of Bcl-X_L is to suppress apoptosis by inhibiting the release of cytochrome c from mitochondria (1,2). Bcl-X_L is also known to prevent apoptosis from a variety of external stimuli (3). One such stress that induces cell death is serum deprivation. Serum contains growth factors, hormones, attachment and spreading factors, minerals, trace elements, lipids, and various other factors that are necessary for cell growth, differentiation, transport, attachment, spreading, pH maintenance, and protease inhibition (4). Therefore, serum withdrawal causes the cells to stop growing. Serum deprivation also induces apoptosis, and ROS generation contributes to this cell death in serum-starved cells (5-7). Indeed, serum deprivation increases the cellular levels of ROS and activates caspase activity. Apoptosis induced by growth factor withdrawal is suppressed by antioxidant treatment (8), and Bcl-X_L expression inhibits serum deprivation-triggered cell death (9-11).

Romo1 is reported to induce mitochondrial ROS generation through complex III of the mitochondrial electron transport chain (12,13). Romo1 expression is up-regulated in most cancer cells and in senescent cells, and it is induced by external stimuli including serum deprivation and 5-FU (6,13,14). Tumor necrosis factor (TNF)-α-induced ROS production is also associated with Romo1. TNF-α treatment triggers the interaction between TNF complex II and the C-terminus of Romo1 exposed to the outside of the mitochondrial outer membrane (15). Simultaneously, Romo1 recruits Bcl-X_L to reduce the mitochondrial membrane potential, resulting in ROS generation and apoptosis. Romo1 is also reported to be responsible for serum deprivation-induced ROS production (6). Romo1 knockdown suppresses serum deprivation-induced ROS increase and apoptotic cell death. In the present study, therefore, we investigate whether Bcl-X_L expression inhibits serum deprivation-induced ROS increase and cell death, which are mediated by Romo1.

Materials and methods

Cell culture and reagents. Human embryonic kidney (HEK) 293 cells and HeLa cervical carcinoma cells were cultured in Dulbecco's modified Eagle's media (DMEM, Gibco-Invitrogen, Grand Island, NY). WI-38 VA13 human lung fibroblasts were cultured in Eagle's minimal essential media (EMEM, Gibco-Invitrogen). All media contained 10% heat-inactivated FBS (Gibco-Invitrogen), sodium bicarbonate (2 mg/ml; Sigma-Aldrich, St. Louis, MO), penicillin (100 units/ml), and streptomycin (100 μg/ml; Gibco-Invitrogen). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Sigma-Aldrich. 2',7'-Dichlorofluorescein diacetate (DCF-DA) and MitoSOX were obtained from Molecular Probes (Eugene, OR). For serum deprivation

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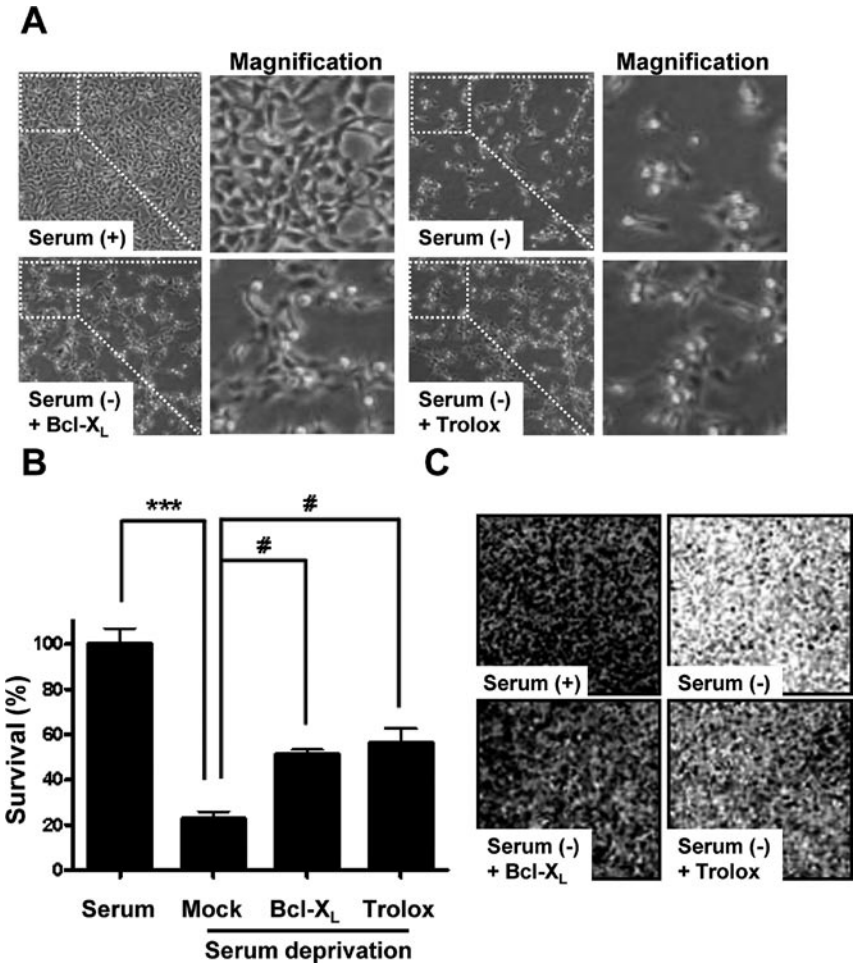

Figure 1. Serum deprivation-induced cell death in cells expressing Bcl-X_L. (A) After HEK 293 cells were transfected with vector or Bcl-X_L for 6 h, serum was removed from the cells for 48 h, and the cells were then counted under inverted light microscopy. Magnified images of the white dot boxed areas are shown on the right. Cells were treated with trolox (1 μM, 24 h) as a positive control. Magnified images of the white dot boxed areas are shown on the right. (B) The number of HEK 293 cells was counted after serum deprivation for 48 h. $^{***}p<0.001$; $^{\#}p<0.05$ by one-way ANOVA. (C) After WI-38 VA13 cells were transfected with vector or Bcl-X_L for 6 h, the serum was removed from the cells for 48 h, and the cells were stained with 1% methylene blue in methanol.

experiments, HEK 293, HeLa and WI-38 VA13 cells were washed twice with serum-free media and further incubated in DMEM or EMEM with 0.05% FBS for 48 h.

Measurement of cell growth and DAPI staining. For cell growth assays, cells were stained with 1% methylene blue in methanol for 30 min. Cells were observed under an inverted light microscope (Olympus IX50, Olympus, Tokyo, Japan). For DAPI staining, cells were fixed in phosphate-buffered saline (PBS, Gibco-Invitrogen) with 3.7% paraformaldehyde (Sigma-Aldrich) at 4˚C for 10 min. After washing three times, they were incubated with 0.5 μg/ml DAPI solution in PBS buffer for 20 min. After incubation, cells were washed with PBS twice and were examined with a fluorescence microscope under UV light. For quantification of apoptosis number, 100-200 cells were monitored in each experiment.

Western blot analysis. PARP cleavage was examined by Western blot analysis of cells transfected with either Bcl-X_L or Romo1. Cells were harvested and then washed with cold PBS (pH 7.4). The cells were lysed with 70 μl of cold RIPA buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 1% NP-40, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 2 μg/ml leupeptin). Equal amounts of the protein samples (20 μg) were separated by 10 or 12% SDS-PAGE and transferred onto nitrocellulose membranes. Immunodetection was performed using anti-PARP (BD Transduction Lab, San Jose, CA), anti-Bcl-X_L (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Flag (Sigma-Aldrich) and anti-β-actin antibodies (Sigma-Aldrich).

Fluorescence microscopy for measurement of ROS production. The cells were stained with DCF-DA (50 μM, Sigma-Aldrich) and MitoSOX (5 μM, Molecular Probes) for 30 min. DAPI staining was used to measure apoptotic cell death or nuclear localization. Intracellular ROS production was measured by fluorescence microscopy (Universal Imaging, Westchester, PA).

Flow cytometric analysis for measurement of mitochondrial ROS. ROS levels were measured by flow cytometry using MitoSOX. Cells were harvested, fixed with ethanol for 20 min and washed with PBS. The ROS levels were analyzed using a FACScan flow cytometer (Becton-Dickson, San Jose, CA).

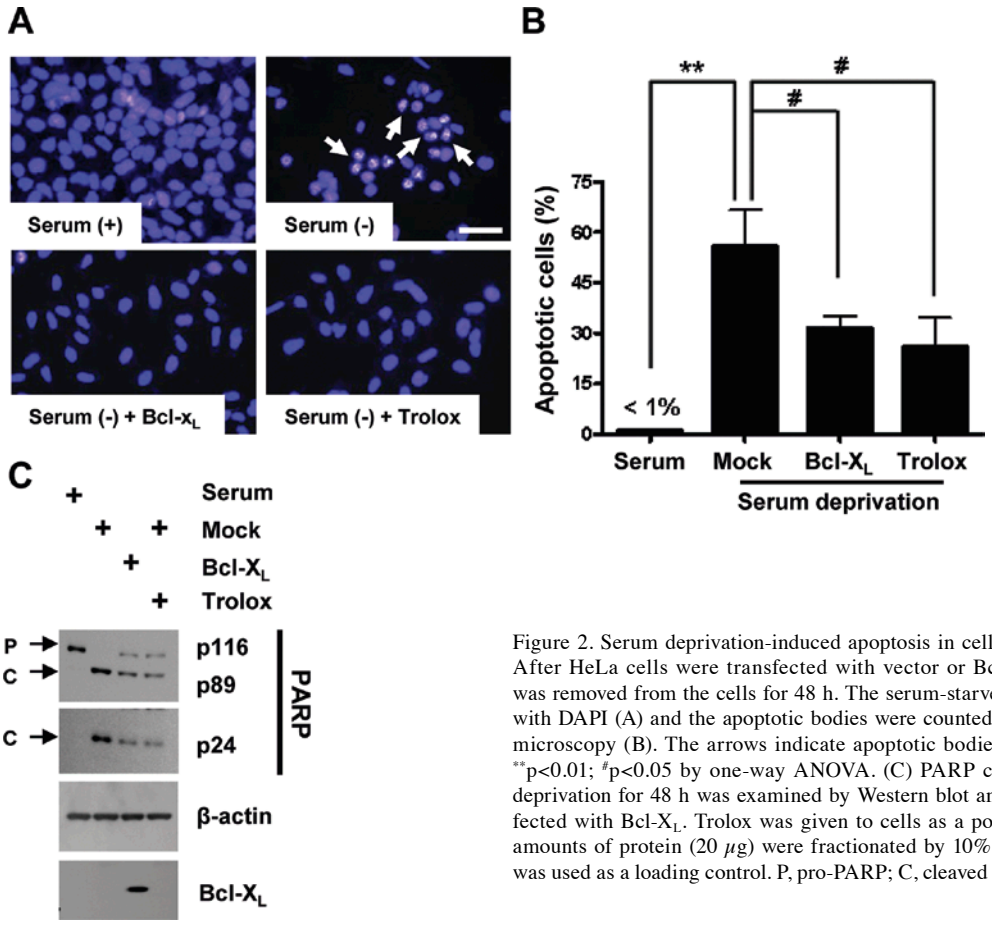

Figure 2. Serum deprivation-induced apoptosis in cells expressing Bcl-X_L. After HeLa cells were transfected with vector or Bcl-X_L for 6 h, serum was removed from the cells for 48 h. The serum-starved cells were stained with DAPI (A) and the apoptotic bodies were counted under inverted light microscopy (B). The arrows indicate apoptotic bodies. Scale bar, 20 μm. $^{**}p<0.01$; $^{\#}p<0.05$ by one-way ANOVA. (C) PARP cleavage after serum deprivation for 48 h was examined by Western blot analysis in cells transfected with Bcl-X_L. Trolox was given to cells as a positive control. Equal amounts of protein (20 μg) were fractionated by 10% SDS-PAGE. β-actin was used as a loading control. P, pro-PARP; C, cleaved PARP.

Statistical analysis. Each assay was performed in triplicate and independently repeated at least three times. Statistical significance was defined as $p<0.05$. Means, SEs, and p-values were calculated using GraphPad PRISM version 4.02 for Windows (GraphPad Software, San Diego, CA).

Results

Bcl-X_L blocks serum deprivation-triggered apoptotic cell death. To investigate whether Bcl-X_L suppressed serum deprivation-induced cell death, cells were transfected with Bcl-X_L. After transfection for 48 h, the number of serum-starved HEK 293 cells was counted under microscopy. As shown in Fig. 1A and B, Bcl-X_L inhibits serum deprivation-induced cell death. However, the cell death induced by serum deprivation was partially blocked by Bcl-X_L expression and trolox treatment. This result is consistent with previous results (5-7). This finding was confirmed in WI-38 VA13 cells (Fig. 1C). Trolox was used as a positive control antioxidant after serum deprivation.

Numerous reports demonstrate that serum deprivation induces programmed cell death characterized by DNA fragmentation and apoptotic bodies (6,16-18). To investigate the correlation between Bcl-X_L expression and inhibition of apoptosis caused by serum deprivation, DAPI staining was used to measure apoptotic cell death. We stained the cells with DAPI and visualized them with fluorescence microscopy after Bcl-X_L transfection in serum-starved HeLa cells. Bcl-X_L expression significantly blocked serum deprivation-triggered apoptotic cell death (Fig. 2A). Trolox was used as a positive control. Apoptotic bodies were quantified from three independent experiments and shown in Fig. 2B. During apoptosis, PARP, a 116-kDa nuclear poly (ADP-ribose) polymerase, is cleaved into 89-kDa and 24-kDa fragments by caspase-3. This PARP cleavage is considered to be one of the classical characteristics of apoptosis as a marker of cells undergoing apoptosis (19). As shown in Fig. 2C, cleaved PARP proteins were observed in serum-deprived cells, but Bcl-X_L expression and trolox treatment partially inhibited serum deprivation-induced apoptosis. These results demonstrate that serum deprivation-induced apoptotic cell death was blocked by Bcl-X_L expression.

Bcl-X_L blocks ROS increase induced by serum deprivation and Romo1 expression. To investigate whether serum deprivation-induced mitochondrial ROS generation was suppressed by Bcl-X_L expression, we first examined whether serum deprivation enhanced cellular ROS levels. HeLa cells were stained with DCF-DA and MitoSOX (mitochondrial superoxide indicator) for live cell observation in the same field. As shown in Fig. 3A, serum deprivation triggered ROS production, which was consistent with a previous report (6). Trolox was given to cells as an intracellular ROS scavenger and positive control. Next, we observed whether Bcl-X_L inhibited the ROS increase induced by serum deprivation. Mitochondrial ROS in serum-starved HEK 293 cells were

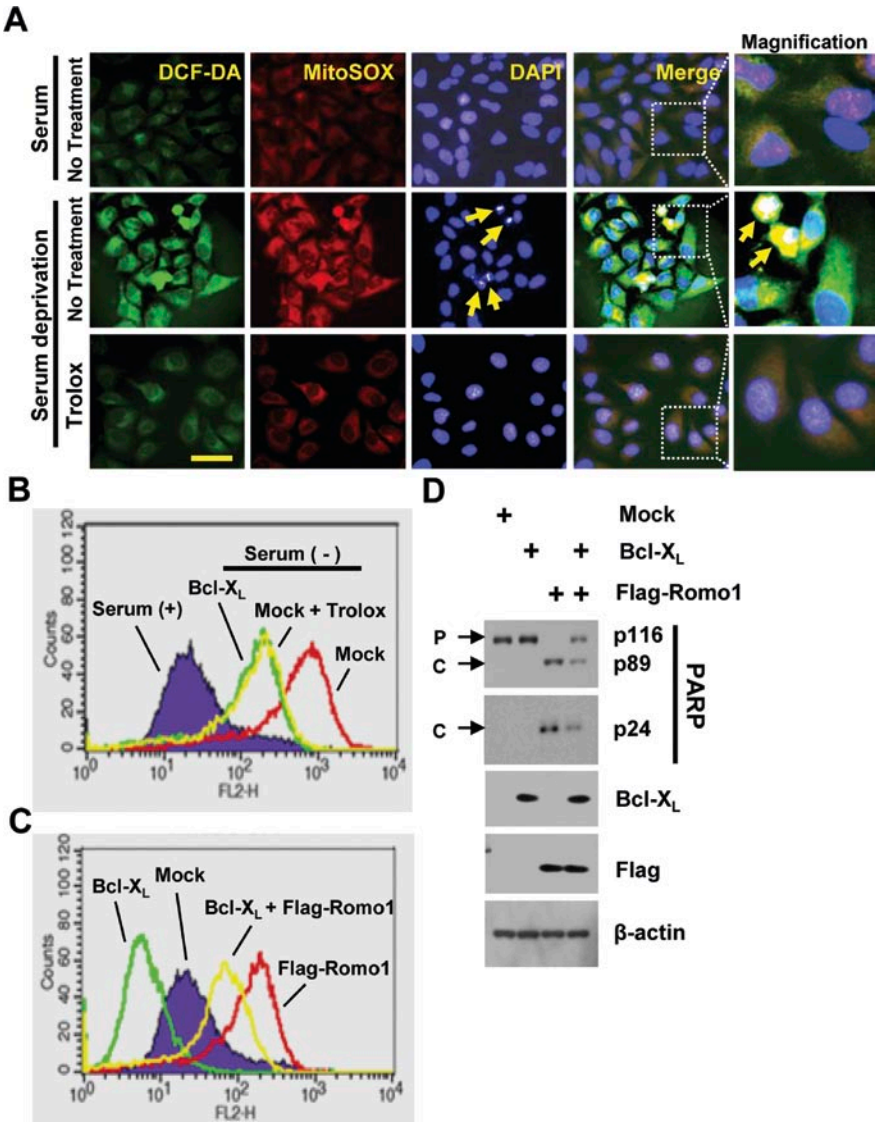

Figure 3. ROS measurements and Romo1-induced apoptosis in cells expressing Bcl-X_L. (A) The serum-starved HeLa cells were stained with DCF-DA (50 μM) or MitoSOX (5 μM) for 30 min and the increase in ROS was observed by fluorescence microscopy. Magnified images of the yellow dot boxed areas are shown on the right. Arrows indicate apoptotic bodies. The scale bars are 20 μm. (B) After HEK 293 cells were transfected with vector or Bcl-X_L for 6 h, the cells were serum starved for 48 h and then stained with MitoSOX. Increased ROS levels were analyzed by flow cytometry. Cells were treated with trolox as a positive control. (C) HEK 293 cells were transfected with Bcl-X_L or Flag-Romo1 and stained with MitoSOX. Increased ROS levels were analyzed by flow cytometry. (D) Western blot analysis of protein extracts from HeLa cells transfected with Bcl-X_L or Flag-Romo1. P, pro-PARP; C, cleaved PARP.

stained with MitoSOX and analyzed by flow cytometry. As shown in Fig. 3B, Bcl-X_L expression partially blocked the ROS increase induced by serum deprivation. A recent study demonstrated that serum deprivation-induced mitochondrial ROS increase and apoptotic cell death are mediated by Romo1 (6). Therefore, we explored whether mitochondrial ROS generation derived from Romo1 expression was suppressed by Bcl-X_L expression. As shown in Fig. 3C, Romo1 triggered ROS production and this result was consistent with a previous report (12). Bcl-X_L expression partially reduced the ROS increase induced by Romo1 expression. Interestingly, Bcl-X_L expression itself reduced the cellular ROS levels. We recently reported that Romo1 is responsible for apoptosis induced by serum deprivation (6). To examine whether Bcl-X_L inhibits apoptosis induced by Romo1, the cleavage of the PARP protein by Romo1 was measured by Western blot analysis in cells expressing Bcl-X_L. As shown in Fig. 3D, Romo1 expression induced PARP cleavage, which was consistent with a previous report (6). The PARP cleavage was significantly blocked by Bcl-X_L. These data indicated that serum deprivation-triggered ROS generation and Romo1-induced apoptosis are regulated by Bcl-X_L.

Bcl-X_L blocks oxidative stress-induced apoptotic cell death. Excessive oxidative stress can lead to apoptosis (20). To determine whether Bcl-X_L inhibits H_2O_2-induced apoptosis, HeLa cells were treated with H_2O_2 and the number of cells was counted by microscopy. As shown in Fig. 4A, H_2O_2 treatment caused cell death. However, Bcl-X_L partially blocked H_2O_2-induced cell death. Next, the apoptotic bodies in H_2O_2-treated cells were stained with DAPI. As shown in Fig. 4B, Bcl-X_L overexpression significantly

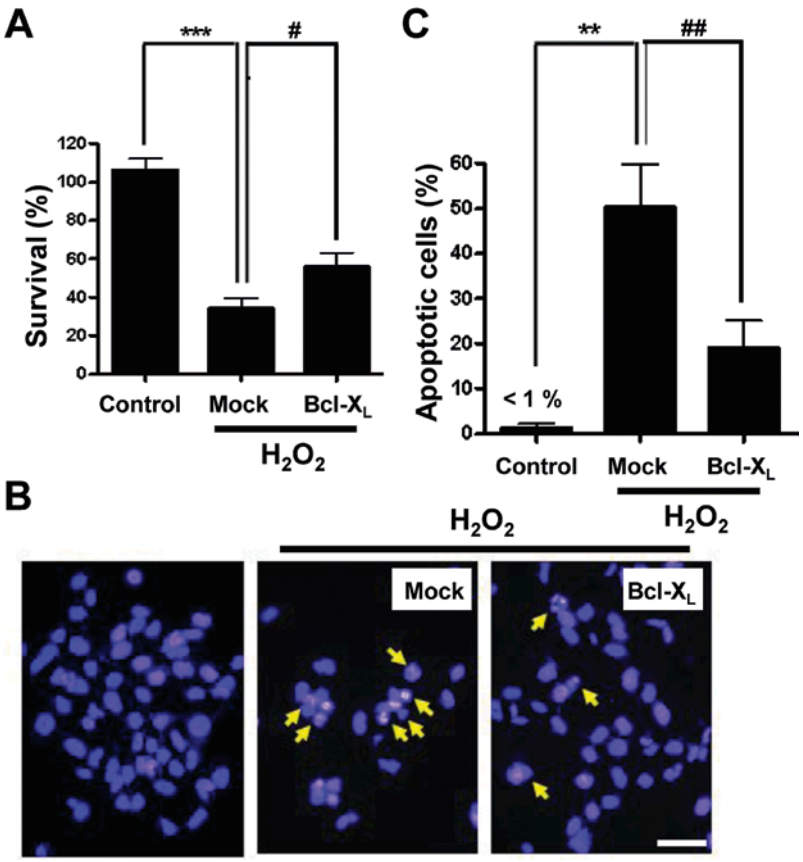

Figure 4. H_2O_2-induced apoptosis in cells expressing Bcl-X_L. (A) Cell viability in HeLa cells treated with H_2O_2. The cells were transfected with Bcl-X_L and treated with H_2O_2 (20 μM) for 24 h. Cells were counted under inverted light microscope. Results represent the means ± SE of three independent experiments performed in triplicate. ***p<0.001 vs. control; $^{\#}$p<0.05 vs. mock treated with H_2O_2 (20 μM) by one-way ANOVA. (B) The cells treated with H_2O_2 for 24 h were stained with DAPI. Arrows, apoptotic bodies. Scale bar, 20μm. (C) Quantification of the apoptotic bodies shown in (B). The data represent the average of three experiments and 200 cells were monitored in each experiment. **p<0.01 vs. control; $^{\#\#}$p<0.01 vs. mock treated with H_2O_2 (20 μM) by one-way ANOVA.

inhibited H_2O_2-induced apoptosis. Quantification of apoptotic bodies from three independent experiments revealed statistically significant differences (Fig. 4C). These results demonstrated that Bcl-X_L regulates oxidative stress-induced apoptosis.

Discussion

Many reports have demonstrated that Bcl-X_L expression inhibits cell death triggered by various stresses including serum deprivation (9-11). However, the exact mechanism by which Bcl-X_L suppresses oxidative stress-induced apoptotic cell death has been not elucidated. Recently, we identified a novel protein, Romo1, which is associated with increased cellular ROS levels in serum deprivation (6). We found that Romo1 recruits Bcl-X_L to reduce mitochondrial membrane potential, resulting in ROS generation (15). We also showed that Bcl-X_L expression effectively inhibits TNF-α-triggered ROS generation and cell death. In the present study, we identified that Bcl-X_L expression suppressed the serum deprivation/Romo1/ROS/cell death pathway. We also showed that oxidative stress-triggered cell death was blocked by Bcl-X_L expression (Fig. 4). These results demonstrate that Bcl-X_L plays an important role in homeostasis of mitochondria by preventing oxidative stress.

Romo1 is known to mediate ROS production in the mitochondria, and ROS produced by Romo1 expression induce DNA damage and cell death (12,13). ROS are generated enzymatically or by the mitochondrial electron transport chain and are removed by many intracellular antioxidant systems. However, imbalance of ROS, which is sometimes elicited by stressful conditions or infections, results in persistent oxidative stress. This chronic oxidative stress can induce single- or double-stranded DNA breaks, resulting in genomic instability that contributes to carcinogenesis (21). However, oxidative stress also triggers tumor cell death. In the present study, we demonstrate that Bcl-X_L overexpression contributes to tumor cell survival against oxidative stress. In fact, increased Bcl-X_L expression is detected in many tumor tissues and is associated with chemoresistance and poor prognosis (22,23). Therefore, we suggest that Bcl-X_L expression suppresses oxidative stress mediated by Romo1, which is triggered by external stimuli such as serum deprivation, resulting in enhanced tumor cell survival. The increased tumor cell survival contributes to chemoresistance and poor prognosis of tumor patients.

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