Communication

Regulation of Bcl-xl Channel Activity by Calcium*

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Recent studies have demonstrated that the anti-apoptotic proteins, Bcl-2 and Bcl-xl, with the carboxy-terminal hydrophobic domain removed, form cation-selective channels in the lipid bilayer reconstitution system. However, the regulatory properties of these channels are unknown. In this study, we investigated the ion-conducting properties of full-length Bcl-xl in the lipid bilayer reconstitution system. Our findings indicate that Bcl-xl forms a cation-selective channel that conducts sodium but not calcium and that Bcl-xl channel activity is reversibly inhibited by luminal calcium with a half-dissociation constant of 60 μM. This calcium-dependent regulation of the Bcl-xl channel provides new insights into the roles of calcium and Bcl-2-related proteins in the programmed cell death pathway.

Bcl-2 and its related proteins are critical regulators of programmed cell death or apoptosis (1, 2). Composed of both anti- and pro-apoptotic members, this family of proteins functions in a programmed cell death pathway common to most multicellular organisms. It is generally believed that the ratio of death antagonists, such as Bcl-2 and Bcl-xl, and death agonists, such as Bax and Bcl-xs, plays a major role in the fate of the cell following an apoptotic stimulus (3). The anti-apoptotic proteins have also been suggested to function by interacting with caspases/CED-4 homologs (4, 5). Most of the Bcl-2-related proteins contain a hydrophobic carboxyl-terminal sequence that anchors the protein to membranes of organelles, including the endoplasmic reticulum (ER), mitochondrial and nuclear, which play important roles in apoptosis (6–9). Despite their importance in regulating development and homeostasis in multicellular organisms, the exact physiological function(s) of these proteins remain elusive.

One of the current theories is that Bcl-2 family members regulate ion fluxes. This idea is supported by evidence that Bcl-2 overexpression prevents calcium redistribution from the ER to mitochondria following growth factor withdrawal (10) and inhibits apoptosis-associated calcium waves (11) and nuclear calcium uptake (12). Bcl-2 overexpression has also been shown to enhance the uptake of calcium by mitochondria (13) and preserve mitochondrial transmembrane potential (14). Our previous data have demonstrated that Bcl-2 overexpression is associated with a reduction in the transient elevation of cytosolic calcium induced by thapsigargin-mediated ER calcium ATPase inhibition (15). Furthermore, Bcl-2 overexpression maintains calcium homeostasis and calcium-dependent protein processing in the ER of thapsigargin-treated cells (16).

Recently, the x-ray and NMR structure of Bcl-xl was shown to resemble the physical structure of ion channel-forming bacterial toxins, such as diphtheria toxin and colicin Ia (17). Subsequently, Bcl-xl, Bcl-2 and Bax, have been shown to form ion channels using the lipid bilayer reconstitution system (18–21). However, these studies all utilized proteins from which the carboxy-terminal hydrophobic domain had been deleted. Previous studies of the channel forming bacterial toxin, colicin Ia, showed that the channel formed by protein which lacks the hydrophobic transmembrane domain differs in conductance from the one formed by the intact colicin Ia (22). Thus, it is presently unclear whether the ion channel forming properties of the truncated Bcl-2 family members necessarily correspond to those of the intact proteins. Furthermore, it seems paradoxical that Bcl-2 and Bcl-xl, which function to inhibit apoptosis, should structurally and functionally resemble bacterial toxins whose main purpose is to form pores in cell membranes and ultimately destroy cells by disrupting ion homeostasis. This suggests that the ability of Bcl-2 and Bcl-xl to promote cell survival may not derive solely from their ability to form a channel. An explanation to this paradox may lie in understanding how Bcl-2 and Bcl-xl channel activity is regulated.

In light of our recent evidence that depletion of cellular calcium abrogates the anti-apoptotic effect of Bcl-2 (16), we set out to examine whether calcium is able to regulate the channel activity of anti-apoptotic Bcl-2 family members in a lipid bilayer reconstitution system. In the present report, we show that full-length Bcl-xl, produced in a bacterial expression system, forms a monovalent cation-selective channel that conducts sodium but not calcium. Significantly, we observed that Bcl-xl channel activity is inhibited by calcium, indicating for the first time that Bcl-xl forms a calcium-regulated cation channel.

MATERIALS AND METHODS

Plasmid Preparation—A full-length human bcl-xl cDNA (23) was polymerase chain reaction-amplified, using flanking primers (forward = 5′-GAGGTGACTGCTCTGCGAGAAGACCGG-3′ and reverse = 5′-GGGGCATGGCGCTGAGTTCATTTCCGACTGAAGAGTGAG-CC′-3′, start/stop codons are underlined) to introduce a XhoI site in the 5′ end and a XhoI site in the 3′ end. The XhoI and Xhol-digested, gel-purified polymerase chain reaction product was subcloned into the pProex-1 expression vector (Life Technologies, Inc.) containing a 6-histidine (6×His) sequence, which allows affinity purification of expressed 6×His-Bcl-xl fusion protein. The entire bcl-xl cDNA sequence was identical to the published sequence with the exception of two conservative changes, A70G and V152A.

Protein Purification—A single colony of Escherichia coli, transformed with the full-length bcl-xl cDNA, was cultured at 37 °C in LB
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**Fig. 1. Purification of full-length Bcl-xl protein.** The full-length human bcl-xl cDNA was cloned into the pProex-1 vector, with a 6xHis tag added to the 5′ end. The Bcl-xl protein was purified using nickel-agarose affinity chromatography. The proteins were analyzed by SDS-PAGE on a 12.5% gel, followed by Western blot. **A:** lane 1, solubilized extracts after high speed centrifugation; lane 2, fraction eluted with 50 mM imidazole; lane 3, fraction eluted with 80 mM imidazole; lanes 4–8, fractions eluted with 300 mM imidazole. The fraction containing the highest amount of Bcl-xl protein was silver-stained, which revealed a single band of ~31 kDa, indicating that the protein was >95% pure (**B**). The eluted fraction containing the purified Bcl-xl has an apparent molecular mass of ~31 kDa, indicating that the protein was ~31 kDa.

Medium with 100 μg/ml ampicillin. Induction was carried out at an A₆₀₀ of 0.7 with 1 mM isopropyl-β-D-thiogalactopyranoside at 37 °C for 5 h before harvesting cells by centrifugation. The cells were pelleted and resuspended in 2–5 volumes of sonication buffer (50 mM Na₃PO₄ (pH 8.0), 300 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride). Following sonication (1-min bursts/1-min cooling/200–300 watts), the cells were centrifuged at 20,000 × g for 1 h at 4 °C. The pellet was resuspended and solubilized as described previously by Loo and Clarke (24) with minor modifications. Briefly, the pellet was resuspended in 0.3 ml of buffer A (50 mM Na₃PO₄, pH 8.0, 500 mM NaCl, 50 mM imidazole, and 20% (v/v) glycerol). The proteins were then solubilized at 4 °C by adding 1 ml of solubilization buffer (buffer A + 1% (w/v) CHAPS). Insoluble material was removed by centrifuging at 16,000 × g for 15 min. The supernatant was applied to the nickel-nitrilotriacetic acid column that was pre-equilibrated with 0.5 ml of nickel-agarose (Qiagen) in buffer B (buffer A + 0.1% (w/v) CHAPS). The column was washed twice with 0.6 ml of buffer B and with 0.6 ml of buffer C (10 mM Tris-Cl (pH 7.0), 50 mM NaCl, 80 mM imidazole (pH 7.0), 0.1% (w/v) CHAPS, and 20% (v/v) glycerol). The 6x-His-tagged Bcl-xl proteins were eluted with 0.25 ml of buffer D (10 mM Tris-Cl (pH 7.0), 500 mM NaCl, 300 mM imidazole (pH 7.0), 0.1% (w/v) CHAPS, and 20% (v/v) glycerol) in multiple fractions. Typically, cells from 100 ml of culture yielded 0.5–3.0 mg of Bcl-xl protein. Purified proteins were then characterized by SDS-PAGE (12.5% gels), followed by silver staining (Bio-Rad) and Western blotting. Western Blot Analysis—Immunoblots were performed as described previously (16). Briefly, full-length 6x-His-tagged Bcl-xl proteins were mixed with the sample buffer (200 mM Tris-Cl (pH 6.7), 10% SDS, 5% β-mercaptoethanol, 15% glycerol, 0.01% bromphenol blue) and separated on a 12.5% linear SDS-PAGE. The proteins were then transferred to a nitrocellulose membrane and blotted with the Bcl-xl polyclonal antibody (Santa Cruz Biotechnology) and horseradish peroxidase-linked secondary antibody using the ECL detection system (Amersham Pharmacia Biotech).

Planar Lipid Bilayer Preparation and Single Channel Recordings—Planar lipid bilayer membranes were formed across an aperture of 200 μm diameter with a mixture of phosphatidylethanolamine/phosphatidyserine/cholesterol in a ratio of 5:5:1 as described previously (25). Single channel currents were recorded with an Axopatch 200A patch clamp unit, and data analyses were performed with pClamp Software. The eluted fraction containing the purified Bcl-xl protein was either used directly or concentrated by Centriplus-30 (Amicon) and reconstituted in liposomes (phosphatidylethanolamine/phosphatidyserine/cholesterol in a ratio of 1:1:1) for the single channel measurements. The two preparations gave similar results. Purified Bcl-xl (60–100 ng) or Bcl-xl containing liposome was added to the cis-solution in the presence of asymmetric NaCl conditions at pH 7.4. The experiments were performed at room temperatures (23–25 °C). Single channel currents were recorded at 1 ms/pulse with the cut-off filter frequency set at 1 KHz.

RESULTS

The full-length Bcl-xl protein was expressed in *E. coli* following isopropyl-β-D-thiogalactopyranoside induction and purified to homogeneity using nickel-nitrilotriacetic acid affinity chromatography. The Western blot shown in Fig. 1A represents the elution profile from the nickel column. The purified Bcl-xl has an apparent molecular mass of ~31 kDa, which corresponds to

the endogenous Bcl-xl (data not shown). It has been shown previously that Bcl-xl runs slightly higher than the predicted molecular mass of 26 kDa for the full-length Bcl-xl protein (18). Silver staining of an eluted fraction revealed a single band, indicating that the preparation was >95% pure (Fig. 1B).

To characterize the single channel function of full-length Bcl-xl, purified protein was reconstituted in the lipid bilayer, and the selected current traces shown in Fig. 2 were recorded with 200 mM NaCl present in the cis-solution (to which the Bcl-xl protein was added) and 50 mM NaCl present in the trans-solution. Bcl-xl displays channel activity, producing a larger current at +40 mV than at −40 mV (Fig. 2A). In addition, the current-voltage relationship has a reversal potential of ~30 mV (Fig. 2B), which corresponds to a selectivity ratio of *P*<sub>Na*/P*<sub>K</sub> = 13.9. These findings indicate the cation-selective nature of the Bcl-xl channel. In separate experiments with a recording solution consisting of 200 mM NaCl (cis)/200 mM KCl (trans), the full-length Bcl-xl channel had a reversal potential of ~ −5 to 0 mV, which corresponds to a relative selectivity ratio of P<sub>Na*/P*<sub>K</sub> = −1.2. This value is similar to the truncated Bcl-xl channel reported by Minn et al. (18). A characteristic feature of the Bcl-xl channel is the appearance of multiple conductance states, as shown in Fig. 2C. Typically, six distinctive conductance levels could be identified, with each conductance level of ~50 picoamperes. The association of multiple conductance states with the full-length Bcl-xl channel is similar to that of the Bcl-xl channel that had the carboxyl-terminal hydrophobic domain deleted (18). This characteristic may be because of the propensity of Bcl-xl proteins to interact with one another, as this prevents us from distinguishing the current measurements from either single channels or multiple channels. At the present, we do not know the stoichiometry of the Bcl-xl channel, i.e. the number of subunits that participates in the formation of the cation-selective channel.

To confirm that the conductance shown in Fig. 2 was a property of the Bcl-xl protein, we measured its conductance in the presence of the Bcl-xl polyclonal antibody, which recognizes first 19 amino acids of the amino terminus. As shown in Fig. 3, addition of ~31 nM antibody to the cis-solution produced a significant reduction in Bcl-xl channel activity (*n* >3), whereas addition of an equivalent amount of bovine serum albumin to the cis-solution was without effect (data not shown). Furthermore, when the antibody was added to the trans-solution, no change in channel activity was observed. These findings suggest that the Bcl-xl channel activity may be regulated by protein-protein interactions at the amino terminus and confirm that the channel activity observed in the planar lipid bilayer system was indeed produced by Bcl-xl rather than any minor contaminants in the protein preparation. In addition, these data confirm that the protein is oriented in the cis-cytoplasmic trans-luminal manner.

To study whether the full-length Bcl-xl channel is regulated by calcium, 100 mM CaCl<sub>2</sub> was added to the trans-solution. The current traces shown in Fig. 4 were obtained with a NaCl gradient of 200 (cis)/50 mM (trans). Following the addition of 100 mM CaCl<sub>2</sub> to the trans-solution, the activities of the channel at both +60 mV and −60 mV were reduced to essentially zero, indicating that calcium inhibited the function of the Bcl-xl channel. Furthermore, because an inward current was not observed at −60 mV, the Bcl-xl channel does not appear to be permeable to calcium.

The dose-response relationship between luminal calcium concentration and Bcl-xl channel activity was examined, to further understand the effect of luminal calcium. The results indicate that calcium attenuates both the current amplitude and opening probability of the Bcl-xl channel activity, with a *K*<sub>s</sub>
In addition, the inhibitory effect was because of the action of luminal calcium, as suggested by the following observations. First, adding calcium to the cis-solution had a lesser inhibitory effect on the Bcl-xl channel current amplitude and opening probability (Fig. 5A). Second, adding 10 mM MgCl₂ to the trans-solution did not affect the channel activity (Fig. 5A). In addition, the inhibitory effect of luminal calcium was readily and consistently reversed by adding EGTA to the trans-solution (n = 5), as illustrated in Fig. 5B.

DISCUSSION

Our findings indicate that full-length Bcl-xl forms a monovalent cation selective channel, with conductance properties similar to those described previously using recombinant proteins from which the carboxyl-terminal hydrophobic domain is deleted. The purified Bcl-xl protein (60–100 ng) was added to the cis-solution of the lipid bilayer in the presence of a NaCl gradient of 200 (cis)/50 mM (trans) at pH 7.4. Consecutive current traces were recorded with test pulses of −40 to +40 mV from a holding potential of 0 mV (A). The channel was selective for cation over anion with a PNa/PCl value of 13.9, calculated from a reversal potential of −30 mV using the following formula:

$$V_{rev} = 60 \log\left(\frac{P_{Na}/P_{Cl}[Na]_o + [Cl]_i}{P_{Na}/P_{Cl}[Na]_i + [Cl]_o}\right)$$

The channel usually contained multiple conductance states, with distinctive levels of ~50 picosiemens (C).

FIG. 2. Formation of a cation-selective channel by Bcl-xl protein. The purified Bcl-xl protein (60–100 ng) was added to the cis-solution of the lipid bilayer in the presence of a NaCl gradient of 200 (cis)/50 mM (trans) at pH 7.4. Consecutive current traces were recorded with test pulses of −40 to +40 mV from a holding potential of 0 mV (A). The channel was selective for cation over anion with a PNa/PCl value of 13.9, calculated from a reversal potential of −30 mV using the following formula: $V_{rev} = 60 \log\left(\frac{P_{Na}/P_{Cl}[Na]_o + [Cl]_i}{P_{Na}/P_{Cl}[Na]_i + [Cl]_o}\right)$ (B). The channel usually contained multiple conductance states, with distinctive levels of ~50 picosiemens (C).

FIG. 3. Modulation of Bcl-xl channel by antibody against Bcl-xl. The control currents were measured at a test potential of +60 mV (left). Addition of Bcl-xl antibody to the cis-solution (~31 nM) significantly reduced the channel activity (right).

FIG. 4. Regulation of Bcl-xl channel by luminal calcium. Under condition of 200 (cis)/50 mM (trans) at pH 7.4, both outward current (at +60 mV) and inward current (at −60 mV) through the Bcl-xl channel could be measured (left). Addition of 100 mM CaCl₂ to the trans-solution resulted in reduction of channel activity at both +60 mV and −60 mV; note that the inward current is essentially absent (right).

FIG. 5. Dose-dependent inhibition of the Bcl-xl channel activity by luminal calcium. The dose-response curve was generated by adding increasing amounts of Ca²⁺ to the trans-solution (●) (A). The solid line represents the best fit according to a Hill equation, with a half-dissociation constant of 57.78 ± 26.14 µM and a Hill coefficient of 0.50 ± 0.10. Ca²⁺ (1 and 10 mM) added to the cis-solution (△) and Mg²⁺ (10 mM) added to the trans-solution (□) were less effective in the inhibition of the Bcl-xl channel activity. The inhibitory effect of trans-Ca²⁺ on the Bcl-xl channel could be reversed by the addition of 100 µM EGTA to the trans-solution (n = 5) (B).
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Based on the present findings, whether Bax and other pro-apoptotic proteins can be regulated by calcium merits immediate investigation.

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