Pro-apoptotic Cleavage Products of Bcl-xL Form Cytochrome c-conducting Pores in Pure Lipid Membranes*

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During apoptotic cell death, cells usually release apoptotic proteins such as cytochrome c from the mitochondrial intermembrane space. If Bcl-2 family proteins induce such release by increasing outer mitochondrial membrane permeability, then the pro-apoptotic, but not anti-apoptotic activity of these proteins should correlate with their permeabilization of membranes to cytochrome c. Here, we tested this hypothesis using pro-survival full-length Bcl-xL and pro-death Bcl-xL cleavage products (ΔN61Bcl-xL and ΔN76Bcl-xL). Unlike Bcl-xL, ΔN61Bcl-xL and ΔN76Bcl-xL caused the release of cytochrome c from mitochondria in vivo and in vitro. Recombinant ΔN61Bcl-xL and ΔN76Bcl-xL, as well as Bcl-xL, cleaved in situ by caspase 3-possessed intrinsic pore-forming activity as demonstrated by their ability to efficiently permeabilize pure lipid vesicles. Furthermore, only ΔN61Bcl-xL and ΔN76Bcl-xL, but not Bcl-xL, formed pores large enough to release cytochrome c and to destabilize planar lipid bilayer membranes through reduction of pore line tension. Because Bcl-xL and its C-terminal cleavage products bound similarly to lipid membranes and formed oligomers of the same size, neither lipid affinity nor protein-protein interactions appear to be solely responsible for the increased membrane-perturbing activity elicited by Bcl-xL cleavage. Taken together, these data are consistent with the hypothesis that Bax-like proteins oligomerize to form lipid-containing pores in the outer mitochondrial membrane, thereby releasing intermembrane apoptotic factors into the cytosol.

Proteins of the Bcl-2 family are key regulators of programmed cell death in multicellular organisms. Some members of this family, including Bax, Bak, Bok/Mtd, Bad, Bik/Nbk, Bid, Bim/Bod, and Hrk promote apoptosis, whereas others, including Bcl-2, Bcl-xL, Bcl-w, Bcl-1/A1, Mcl-1, and Boo/Diva inhibit apoptosis (1). All these proteins share one to four conserved Bcl-2 homology domains (BH) designated BH1, BH2, BH3, and BH4 (1, 2). In addition, Bcl-2 family members can possess a C-terminal hydrophobic amino acid sequence that helps localize them to intracellular membranes, primarily the outer mitochondrial membrane (1, 2). The activity of Bcl-2 family proteins can be modulated not only at the transcriptional level but also by post-translational modifications (1, 3). For example, various cellular proteases have been shown to cleave Bcl-2, Bcl-xL, Bid, Bax, and Bad producing C-terminal fragments with potent pro-apoptotic activity (4–23). Bcl-xL can be cleaved by caspase 3 after aspartate 61 and 76 and by calpain after alanine 60, converting Bcl-xL from an anti-apoptotic factor to a pro-apoptotic factor (5, 6, 11).

Cumulative evidence indicates that Bcl-2 relatives function, at least in part, by regulating the release of proteins enclosed in the mitochondrial intermembrane space. Current models propose that Bcl-2 family proteins exert this function either by forming pores in mitochondrial membranes themselves, or by modulating endogenous mitochondrial channels through protein-protein interactions (1, 24–26). Of note, these two mechanisms of action are not necessarily mutually exclusive. The pore-forming function was first proposed based on the structural similarity of Bcl-xL and the pore-forming domain of bacterial toxins such as colicins and the diphtheria toxin (27). More recently, Bid, Bax, and Bcl-2 have shown similar structural patterns (28–31). Consistent with the structural similarity to bacterial toxins, Bcl-2 family proteins can permeabilize artificial lipid bilayer membranes, with pro-apoptotic members generally showing higher capacity for pore formation than anti-apoptotic members (32–40). Here, we report that pro-apoptotic C-terminal cleavage fragments of Bcl-xL, but not their full-length anti-apoptotic counterpart, permeabilize both mitochondria and pure lipid bilayer membranes to cytochrome c.

EXPERIMENTAL PROCEDURES

All lipids were obtained from Avanti Polar Lipids (Alabaster, AL). KCl, HEPES, EDTA, Triton X-100, Nonidet P-40, horse heart cytochrome c, and dextrans of different molecular weights with or without fluorescein isothiocyanate (FITC) labeling were supplied by Sigma Chemical Co. (St. Louis, MO). 8-Aminonaphtalene-1,3,6-trisulfonate (ANTS) and p-xylene-bis-piridinium bromide (DPX) were from Molecular Probes (Eugene, OR). DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; LUV, large unilamellar vesicle(s); ANTS, 8-amino-1,3,6-trisulfoflavine; TLPS, trilissamine rhodamine B-labeled 1,2-dioleoylphosphatidylethanolamine; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; zVAD-tmk, benzofurancarboxy-val-Ala-Asp-fluoromethyl ketone.

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‡ The abbreviations used are: BH, Bcl-2 homology domain; GST, glutathione S-transferase; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; LUV, large unilamellar vesicles; ANTS, 8-amino-1,3,6-trisulfoflavine; TLPS, trilissamine rhodamine B-labeled 1,2-dioleoylphosphatidylethanolamine; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; zVAD-tmk, benzofurancarboxy-val-Ala-Asp-fluoromethyl ketone.
lar Probes (Eugene, OR). Sephadex G-10, Sephacryl S-300, and Super-
dex 200 were purchased from Amersham Pharmacia Biotech (Piscat-
away, NJ). Caspase 3 (catalog no. 201-038-C005, without CHAPS
detergent) and zVAD-fmk were obtained from Alexis (San Diego, CA).
The polyclonal anti-Bcl-xL H62 antibody and the monoclonal anti-FLAG
antibody were obtained from Santa Cruz, CA, and the monoclonal anti-cytochrome c 65791A and 7H8.2C-12 antibodies were from PharMingen (San Diego, CA). The monoclonal anti-Bcl-xL 2A1 antibody was a generous gift from Dr. Larry Boise
(University of Miami). LipofectAMINE was obtained from Invitrogen
(Carlsbad, CA).

Proteins—Plasmids encoding glutathione S-transferase (GST)-Bcl-xL fusion proteins were constructed by sub-
cloning human Bcl-xL cDNA into pGEX2T (Amersham Pharmacia Bi-
tech). A stop codon was inserted after Bcl-xL amino acid 212 to generate GST-ΔC20Bcl-xL. The coding sequence for Bcl-xL amino acids 62-233 and 77-233 plus an initiation codon were amplified by polymerase chain reaction to construct GST-ΔN61Bcl-xL and GST-ΔN76Bcl-xL, respectively. The presence of the deletions was confirmed by DNA se-
quencing. All constructs were produced as GST fusion proteins from pGEX vectors using Escherichia coli DH1α as the host strain. A 50-m1
overnight culture was used to inoculate 1 liter of Luria-Bertani me-
dium, which was further incubated at 37 °C until an A600 nm value of 0.4
was achieved. The cells were induced with 0.1 mM isopropyl-β-D-thio-
galactopyranoside, and incubated at 30 °C for 3 h with 1 mM NADH
and NADPH, harveting by centrifugation. Then, cells were resuspended in a 10-m1
phosphate-buffered saline (PBS) solution with 1% Triton X-100 (w/v) and protease inhibitors, and disrupted by sonication. The resulting
lysate was centrifuged at 12,000 × g for 15 min at 4 °C to pellet cellular
dehesion. Glutathione-agarose beads were added to the supernatant and incubated at 4 °C with gentle rotation for 3 h. The beads were washed
twice with 50 ml of ice-cold PBS without Triton X-100, packed in a 1-m1
column, and washed again with 10 ml of PBS. After incubation with
thrombin for 3 h at room temperature, cleaved proteins were eluted with 5 ml of PBS containing 0.5% Nonidet P-40. To 1-m1 elution frac-
tions 200 µl of prewashed detergent-binding beads (Calbio-
chem, San Diego, CA) were added, followed by incubation for 30 min at
4 °C, and removal of the beads by centrifugation. After repeating this process five times, the supernatants were dialyzed against PBS buffer
(10-kDa cutoff dialysis membrane), and finally, protein samples were
concentrated using a Centricron BIOMAX-10 filter. Purified proteins
were characterized by SDS-PAGE in 4–20% Tris-glycine gels (Invitro-
gen) followed by Colloidal Blue staining (Pierce, Rockford, IL).

Release of Cytochrome c from Mitochondria—For the immunofluo-
rescence studies, 2 × 106 NIH 3T3-BHK cells were transiently transfected with 1 µg of the plasmid pDNA using LipofectAMINE. 10 h post-transfection, cells were immunostained with se-807 anti-FLAG and 65791A anti-cytochrome c antibodies, followed by rhodamine-conjugated and fluorescein isothiocyanate (FITC)-conju-
gated secondary antibodies, respectively (Chemicon, Temecula, CA). Cells were analyzed with a Noran OZ CLSM confocal microscope system with a ×60 objective (Nikon, Tokyo, Japan). Fluorescence studies were performed as described previously (41). Mitochon-
dria were isolated from livers of male Harlan Sprague-Dawley rats as
described by Rickwood et al. (42), followed by two washes in 210 mM
manit, 70 mM sucrose, 0.5 mM EDTA, and 10 mM HEPES (pH 7.2).
Isolated mitochondria (50 µg of protein) were incubated with recombi-
nant Bcl-xL protein (1 µM) in 50 µl of 125 mM KCl, 0.02 mM EDTA, 5 mM
sodium succinate, 10 mM HEPES-KOH, pH 7.2, 5 mM NaHPO4, 5 µM
rotenone, for 30 min at 30 °C. Reaction mixtures were centrifuged at
14,000 × g for 10 min, mitochondrial membrane pellets corresponding
to 25 µg of protein and the corresponding volume of supernatants
were separated by SDS-PAGE on 4–20% Tris-glycine gels, and their
respective cytochrome c contents were estimated by immunoblotting
using 7H8.2C-12 using the ECL method (Amersham Pharmacia Biotech).

Vesicle Preparation—Large unilamellar vesicles (LUV) were formed
through two polycarbonate membranes with 0.1-µm pores (Nucleopore,
San Diego, CA). Unencapsulated ANTS/DPX was separated from LUV
using prepacked Sephadex G-10 columns. Unfrapped FD-4, FD-10,
FD-40, FD-70, and cytochrome c (see below) were removed on a
Sephacryl S-400 HR column, and the cytochrome c and FD-70 contents
of eluted fractions (1 ml) were determined by absorbance and by fluo-
rescence measurements, respectively.

Size Exclusion Chromatography—Experiments were performed in a Superdex-200 (15/54) column equilibrated with 100 mM KCl, 10 mM
HEPES, 0.2 mM EDTA (pH = 7.0), with or without 2% (w/v) CHAPS
(J.T. Baker, Phillipsburg, NJ) at a 1 mM flow rate. The column was
 calibrated using protein gel-filtration standards (Bio-Rad, Hercules,
CA), having the following elution peak volumes: bovine thyroglobulin,
670,000 Da, 31.8 ml; bovine γ-globulin, 158,000 Da, 43.2 ml; avian
ovalbumin, 44,000 Da, 53.2 ml; myoglobin, 17,800 Da, 61.8 ml; and
cytochrome C, 12,400 Da, 65.1 ml. Liposomes were rehydrated with the column was
29.4 ml, as determined by blue dextran 2000 elution. Samples of 300 µl
were loaded onto the column followed by collection of 2-ml elution
fractions. Aliquots of individual fractions were subjected to SDS-PAGE
in 16% Tris-glycine gels (Invitrogen), and finally, Bcl-xL proteins were
visualized by immunoblotting using 2A1 antibody.
were performed in planar lipid bilayer membranes formed by the Mueller-Rudin technique across a 250-μm diameter hole, as described previously (45). Lipid composition was diphytanoylphosphatidylcholine/diphytanoylphosphatidyleserine (1/1, v/v) or DOPC/DOPA/DOPS (1/1/1, v/v). The solution bathing the membrane contained 100 mM KCl, 0.2 mM EDTA, 10 mM HEPES (pH 7.0). Membrane thinning was monitored visually and by capacitance measurements. After planar membrane formation, a glass micropipette filled with a solution containing the protein was brought into close contact until a gigaseal (resistance > 20–100 GΩ) was formed between the glass in the micropipette tip and the lipid bilayer within it. Then, a constant potential difference was applied to the patched membrane area, and the resulting current was filtered (5-KHz corner frequency, H900 active filter, Frequency Devices, Haverhill, MA) and stored (100-μs sampling rate). Membrane lifetime experiments were done in a Lucite chamber with a hole of ~550 μm (Warner Inst., Hamden, CO). Purified proteins were added to the aqueous sub-phase, and the solution was stirred for 5 min to ensure good mixing. A software program (BROWSE, available upon request) was modified to apply voltage pulses and facilitate membrane lifetime measurements.

RESULTS

Pro-apoptotic Bcl-xL Cleavage Products Release Cytochrome c from Mitochondria—Because the pro-apoptotic activity of Bcl-2 family proteins appears to rely on their ability to release cytochrome c from mitochondria, we first studied the effect of the pro-apoptotic C-terminal portion of Bcl-xL on cytochrome c distribution in living cells. Expression of ΔN61Bcl-xL in BHK cells led to a diffuse cytochrome c staining pattern indicating that cytochrome c was no longer confined to the intermembrane space of the mitochondria, but rather it was free in the cytosol (Fig. 1a, filled arrows). In the control neighboring untransfected cells, cytochrome c remained punctuate (open arrows). In agreement with the immunofluorescence studies, subcellular fractionation of BHK cells transfected with ΔN76Bcl-xL, but not Bcl-xL, showed cytochrome c in the cytosolic fraction (Fig. 1b). These results are similar to those obtained previously using the pro-apoptotic cleaved form of Bcl-2 (41). To investigate whether the release of cytochrome c was a direct or indirect effect of Bcl-xL cleavage products on mitochondria, we tested the ability of purified, recombinant ΔN61Bcl-xL and ΔN76Bcl-xL (Fig. 1c) to release cytochrome c from isolated rat liver mitochondria. Incubating isolated mitochondria with recombinant ΔN61Bcl-xL or ΔN76Bcl-xL, but not Bcl-xL, led to cytochrome c release (Fig. 1d). Taken together, these data suggest that, similar to other Bcl-2 family proteins with pro-apoptotic activity (13–16, 20–23, 46–51), C-terminal Bcl-xL cleavage fragments act directly on mitochondria to induce cytochrome c release.

Pro-apoptotic Bcl-xL Cleavage Products Possess Intrinsic Pore-forming Activity—To assess whether pro-apoptotic forms of Bcl-xL increase membrane permeability, we studied their effect on pure lipid vesicles loaded with the fluorophore ANTS and its quencher, DPX. ΔN61Bcl-xL and ΔN76Bcl-xL induced fast and extensive ANTS release from LUV, and ΔN76Bcl-xL, but not Bcl-xL, led to cytochrome c release (Fig. 2a). Anti-apoptotic Bcl-xL caused little ANTS release from LUV, and ΔC20Bcl-xL, a Bcl-xL construct lacking the C-terminal hydrophobic domain proposed to function as a membrane-anchor domain, had no effect. Of note, ΔN76Bcl-xL and ΔN61Bcl-xL did not induce ANTS release through vesicle fragmentation or aggregation, because they caused no significant changes in the static or dynamic light scattering of the LUV suspension (data not shown). To examine whether the different membrane-permuting effects of these proteins corresponded to their membrane-binding affinities, proteins were incubated with LUV, then free and membrane bound proteins were separated by
In the absence of LUV, Bcl-xL and its fragments remained in the bottom fractions of the sucrose gradient. However, after incubation with the liposomes, most of the Bcl-xL, N61Bcl-xL, and N76Bcl-xL floated to the top fraction of the gradient, demonstrating that they bound to LUV (Fig. 2a, right). ∆C20Bcl-xL did not bind to LUV at neutral pH but did bind to LUV at low pH. In the course of these experiments we noted that the capacity of Bcl-xL, N61Bcl-xL, and N76Bcl-xL to release ANTS from LUV depended on the amount of DOPG present in the liposomes (Fig. 2b, left). This effect was likely due to the higher affinity of Bcl-xL proteins for LUV containing negatively charged lipids (Fig. 2b, right).

Caspase 3 can cleave Bcl-xL to generate N61Bcl-xL and N76Bcl-xL (5, 6). Thus, we decided to test whether caspase 3-mediated cleavage of Bcl-xL affects its pore-forming activity. To this aim, Bcl-xL was first allowed to bind LUV containing ANTS/DPX, followed by treatment with caspase 3. Addition of caspase 3 to LUV-associated Bcl-xL increased its pore-forming activity to levels similar to those obtained with N76Bcl-xL, and this effect was eliminated by the caspase inhibitor zVAD-tmk (Fig. 3a). Furthermore, the time course of caspase-induced Bcl-xL cleavage correlated with that of ANTS release from liposomes, and zVAD inhibited both processes (Fig. 3b, and data not shown). Finally, similar to the results obtained with the recombinant C-terminal fragments, caspase-cleaved Bcl-xL required the presence of acidic lipids in LUV for efficient binding and pore formation (Fig. 3c). In short, these results indicate that recombinant pro-apoptotic Bcl-xL cleavage products as well as caspase-cleaved Bcl-xL possess endogenous pore-forming activity.

Pro-apoptotic Bcl-xL Cleavage Products Induce Release of High Molecular Weight Dextrans and Cytochrome c from LUV—Next, we explored the size of the pores formed by these proteins, using LUV containing self-quenching concentrations of FITC-dextran with molecular masses of 4 kDa (FD-4), 10 kDa (FD-10), 20 kDa (FD-20), and 30 kDa (FD-30). We found that recombinant pro-apoptotic Bcl-xL cleavage products as well as caspase-cleaved Bcl-xL possess endogenous pore-forming activity.
kDa (FD-10), 40 kDa (FD-40), and 70 kDa (FD-70). ΔN61Bcl-xL and ΔN76Bcl-xL induced release of dextran of all sizes tested but with different efficiencies (Fig. 4 and Table I). At a constant ΔN76Bcl-xL concentration, the larger the entrapped dextran, the slower the kinetics and the lower the final extents of dextran release (Fig. 4a). To investigate the dependence of marker release on pro-apoptotic protein concentration, the protein/lipid ratio was varied over a wide range, from 1/10,000 to 1/250 mol/mol (Fig. 4b). ΔN61Bcl-xL induced half-maximal releases of ANTS, FD-10, and FD-70 at about 10, 30, 50, 175, and 225 nm, respectively (Fig. 4b). Thus, the larger the encapsulated molecule, the higher the protein concentration required for release. Caspase cleaved Bcl-xL also induced release of high molecular weight dextrans, albeit with lower efficiency compared with ΔN61Bcl-xL and ΔN76Bcl-xL (Table I). However, full-length Bcl-xL did not release significant amounts of FITC-dextran from LUV. Prompted by these observations, we decided to test whether the pro-apoptotic Bcl-xL forms could directly release cytochrome c from LUV. To this aim, LUV containing cytochrome c were incubated with Bcl-xL proteins, the treated samples were filtered through a 100-kDa microcentrator, and the cytochrome c contents of the retentate and the filtrate were detected by immunoblotting. ΔN61Bcl-xL and ΔN76Bcl-xL released cytochrome c from LUV in a time- and dose-dependent manner similar to that observed with FD-10 (Fig. 5, and data not shown). On the contrary, Bcl-xL and ΔC20Bcl-xL did not cause significant release of cytochrome c. To confirm these findings and to further explore the effect of protein concentration on lipidosome permeabilization, gel filtration on a Sephacryl 400 HR column was used (Fig. 6). Pure LUV eluted in the void volume of the column, at 12–16 ml (Fig. 6a), whereas free FD-70 and cytochrome c eluted later, at 19–27 ml and 26–35 ml, respectively (Fig. 6b). When LUV containing FD-70 and cytochrome c were treated with ΔN76Bcl-xL at 1/1000 protein/lipid molar ratios, most of the FD-70 eluted together with the liposomes, while most of the cytochrome c did not co-elute with the liposomes (Fig. 6c). Thus, under these conditions ΔN76Bcl-xL efficiently released cytochrome c but not FD-70. However, at higher ΔN76Bcl-xL concentrations most of the FD-70 escaped from LUV, similar to cytochrome c (Fig. 6d). Finally, Bcl-xL released neither cytochrome c nor FD-70 (Fig. 6f). In summary, these results demonstrated that, unlike anti-apoptotic full-length Bcl-xL pro-apoptotic cleavage products of Bcl-xL formed pores in pure lipid vesicles that allowed passage of large macromolecules, such as cytochrome c and high molecular weight dextrans.

![Fig. 4](http://www.jbc.org/)

**Table I**

| Protein       | Protein/lipid | % Release |
|---------------|---------------|-----------|
| Bcl-xL        | 1/10,000      | 1.1 ± 0.4 (6) | ND^a |
| Bcl-xL        | 1/2,000       | 6.9 ± 1.0 (6) | 0 (4) |
| Bcl-xL        | 1/1,000       | 18.7 ± 1.4 (9) | 0 (4) |
| Bcl-xL        | 1/500         | 35.9 ± 2.5 (9) | 0.4 ± 0.2 (6) |
| Bcl-xL        | 1/250         | 53.2 ± 4.8 (7) | 2.7 ± 0.8 (5) |
| ΔN61Bcl-xL    | 1/10,000      | 8.9 ± 2.2 (6) | 4.8 ± 0.7 (5) |
| ΔN61Bcl-xL    | 1/2,000       | 63.5 ± 6.2 (6) | 27.2 ± 2.8 (5) |
| ΔN61Bcl-xL    | 1/1,000       | 91.5 ± 7.2 (6) | 50.2 ± 6.2 (5) |
| ΔN61Bcl-xL    | 1/500         | 92.7 ± 6.6 (6) | 70.4 ± 7.3 (5) |
| ΔN61Bcl-xL    | 1/250         | 93.5 ± 7.9 (4) | 82.2 ± 5.3 (5) |
| ΔN76Bcl-xL    | 1/10,000      | 14.2 ± 3.1 (8) | 9.8 ± 1.1 (7) |
| ΔN76Bcl-xL    | 1/2,000       | 73.7 ± 3.8 (8) | 54.5 ± 5.2 (7) |
| ΔN76Bcl-xL    | 1/1,000       | 91.9 ± 6.2 (9) | 85.2 ± 4.5 (9) |
| ΔN76Bcl-xL    | 1/500         | 94.9 ± 5.2 (9) | 90.2 ± 6.1 (9) |
| ΔN76Bcl-xL    | 1/250         | 92.7 ± 4.9 (7) | 93.5 ± 5.5 (8) |
| Cleaved Bcl-xL| 1/10,000      | 4.2 ± 0.8 (6) | 0.5 ± 0.2 (6) |
| Cleaved Bcl-xL| 1/2,000       | 24.2 ± 4.1 (5) | 3.1 ± 1.0 (5) |
| Cleaved Bcl-xL| 1/1,000       | 51.3 ± 5.1 (5) | 7.9 ± 2.2 (5) |
| Cleaved Bcl-xL| 1/500         | 85.2 ± 6.6 (4) | 19.4 ± 4.0 (4) |

^a ND, not determined.

Table M, respectively.
**Oligomeric Status of Bcl-x<sub>L</sub> Proteins**—Bax and Bak have been proposed to form cytochrome c-conducting pores through their ability to oligomerize (39, 40, 52–54), and the pro-apoptotic forms of Bcl-x<sub>L</sub> may function in a similar manner. To test this hypothesis, we studied the oligomeric status of Bcl-x<sub>L</sub> proteins by size-exclusion chromatography on Superdex S-200. Bacterially expressed, purified full-length Bcl-x<sub>L</sub> and ΔN76Bcl-x<sub>L</sub> migrated as large complexes; no sign of Bcl-x<sub>L</sub> monomers (estimated mass, 26,063 Da) or ΔN76Bcl-x<sub>L</sub> monomers (estimated mass, 17,774 Da) was detected (Fig. 7a). In contrast, ΔC20Bcl-x<sub>L</sub> eluted in a single peak, close to its calculated monomeric molecular mass (24,020 Da), suggesting that the C-terminal hydrophobic domain of Bcl-x<sub>L</sub> is important for Bcl-x<sub>L</sub> multimerization. To study the oligomeric status of these proteins after binding to lipid membranes, they were incubated with LUV, followed by solubilization of the membranes with 2% (w/v) CHAPS. Under these conditions, Bcl-x<sub>L</sub> eluted at molecular masses ranging from 60 and 145 kDa, most consistent with dimers-pentamers of this protein, while ΔN76Bcl-x<sub>L</sub> migrated at molecular masses ranging from 35 to 100 kDa, also corresponding to multimers of two to five protein molecules (Fig. 7b).

Therefore, anti-apoptotic and pro-apoptotic forms of Bcl-x<sub>L</sub> possessed the same oligomeric status in the plane of the bilayer. Hence, oligomerization alone cannot account for the increased pore-forming activity and apoptogenicity of cleaved Bcl-x<sub>L</sub>.

ΔN61Bcl-x<sub>L</sub> and ΔN76Bcl-x<sub>L</sub> Destabilize Planar Lipid Membranes and Decrease Membrane Line Tension—To gain more insight into the structure of the pores formed by these proteins, electrophysiology of planar phospholipid membranes was used. Soon after adding ΔN76Bcl-x<sub>L</sub>, membrane conductance increased by heterogeneous fluctuations in which amplitude increased with time until the membrane became unstable (Fig. 8a). Decreased protein concentration caused similar but delayed electrical responses (data not shown). On the other hand, addition of full-length Bcl-x<sub>L</sub> induced current fluctuations of ~40–200 pS, but the membranes did not become noticeably unstable (Fig. 8b). To quantify the effect of Bcl-x<sub>L</sub> proteins on planar lipid bilayer stability, membrane lifetime experiments were performed.

ΔN61Bcl-x<sub>L</sub> and ΔN76Bcl-x<sub>L</sub>, but not Bcl-x<sub>L</sub> or ΔC20Bcl-x<sub>L</sub>, potently diminished planar membrane lifetime (Fig. 8c). By fitting the voltage dependence of membrane lifetime to a theoretical expression for lipidic pore formation (55), line tensions in the absence and presence of ΔN61Bcl-x<sub>L</sub> and ΔN76Bcl-x<sub>L</sub> were obtained (Fig. 8d). Pro-apoptotic Bcl-x<sub>L</sub> fragments effectively decreased membrane line tension in a protein-concentration-dependent fashion, reducing its original value by ~50% at 15 nM (Fig. 8e). These results are consistent with the hypothesis that pro-apoptotic cleavage fragments of Bcl-x<sub>L</sub> but not anti-apoptotic full-length Bcl-x<sub>L</sub> destabilize planar lipid bilayers through reducing membrane line tension.

**DISCUSSION**

In this work we investigated the molecular mechanism underlying the pro-apoptotic activity of cleaved Bcl-x<sub>L</sub>. Our results provide compelling evidence that cleavage of Bcl-x<sub>L</sub> markedly enhances its pore-forming activity. The C-terminal cleavage fragment of Bcl-x<sub>L</sub> found in dying cells is sufficient for this activity. The cleavage-induced increase in pore-forming activity likely accounts for the apoptosis-inducing function of cleaved Bcl-x<sub>L</sub> through the permeabilization of mitochondrial membranes to release apoptogenic intermembrane proteins.
Post-transcriptional modifications can affect the function of Bcl-2 family proteins by modulating their subcellular localization (1, 3). The pro-apoptotic activity of Bcl-xL elicited by proteolytic cleavage could be due, at least in part, to an increased affinity of the cleaved fragments for mitochondrial membranes, as shown for tBID (13-16). However, Bcl-xL and its C-terminal cleavage products bind similarly to LUV. Furthermore, in situ cleavage of membrane-bound Bcl-xL increased its pore-forming activity to levels similar to those obtained with ΔN76Bcl-xL. Bcl-xL, but not Bid, contains a hydrophobic C-terminal domain that has been proposed to function as a membrane-anchoring domain (1-3). Although deletion of this hydrophobic domain greatly impaired the association of Bcl-xL to LUV, efficient binding of full-length Bcl-xL, ΔN76Bcl-xL, and ΔN61Bcl-xL to LUV required the presence of anionic lipids in the liposomal membrane. Because Bcl-xL has a biased charge distribution with basic residues being concentrated in one region of the molecule (56), this positively charged region might contribute to the membrane line tension.

FIG. 7. Size-exclusion chromatography of Bcl-xL, ΔN76Bcl-xL, and ΔC20Bcl-xL in the absence and in the presence of LUV. Representative migration patterns of Bcl-xL proteins (2 μM) in solution (a) and after incubation with DOPC/DOPG (6/4) LUV (1 mM) for 30 min, followed by solubilization of membranes with 2% (w/v) CHAPS (b). Similar results were obtained at 200 mM protein concentration. Samples containing the indicated proteins were loaded onto a Superose 200 column, elution fractions of 2 ml were collected, and protein migration profiles were determined by immunoblotting. Arrows indicate the elution peaks of standard proteins: thyroglobulin (670 kDa), gamma globulin (158 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa).

The capacity of Bax and Bak to induce permeabilization of pure lipid bilayers and mitochondrial membranes appears to be related to their ability to multimerize (39, 40, 52-54, 60-62). Similarly, pro-apoptotic Bcl-xL cleavage products may oligomerize as one step in a process of large pore formation in lipid bilayer membranes. In agreement with this idea, gel filtration chromatography studies showed that ΔN76Bcl-xL formed multimers composed of two to five protein molecules in CHAPS-solubilized LUV. However, Bcl-xL oligomers in a manner similar to ΔN76Bcl-xL, despite forming pores much smaller than ΔN76Bcl-xL. Perhaps the oligomers of ΔN76Bcl-xL penetrate the membrane to a different extent than the Bcl-xL oligomers. Interestingly, a recent study showed that Bcl-xL exists as membrane-integrated large molecular weight complexes in CHAPS-solubilized mi-
toochondrial extracts of HeLa cells (61). Although the exact composition of such intramembranous Bcl-xL complexes remains to be determined, this finding raises the possibility that Bcl-xL may also exist in a multimeric form in vivo, at least under certain conditions. Future studies are needed to determine the exact status of endogenous Bcl-xL and cleaved Bcl-xL, as well as their relation to the apoptotic permeabilization of mitochondrial membranes.

Pore formation by Bcl-2 family proteins is likely to be a multistep process coordinated through a set of conformational changes in these molecules. Such conformational changes of Bcl-2 family members may modulate not only protein-protein interactions, but also their effect in the surrounding membrane lipid environment. We have previously proposed that Bax may form pores together with lipid molecules (37). The highly variable membrane conductance changes and decreased membrane lipid stability induced by ∆N61Bcl-xL and ∆N76Bcl-xL in planar lipid bilayer membranes suggest that pro-apoptotic Bcl-xL cleavage products may form lipid-containing pores as well. According to a general model for such pores, the energy $E$ of a pore of radius $r$ can be described as,

$$E = 2\pi r \gamma - 4\pi r^2$$

(Eq. 2)

where $\gamma$ is the line tension of the membrane that creates a barrier against pore opening due to the hydrophobic nature of lipid molecules, and $s$ is the surface tension of the membrane that tends to open the pore (55). Because $\gamma$ can originate from the energetic cost to curve lipid molecules in the edge of the pore and because Bax, ∆N61Bcl-xL, and ∆N76Bcl-xL reduce $\gamma$ in a concentration-dependent manner (37, and present work), pro-apoptotic Bcl-2 proteins may form pores through lipid monolayer bending. From this perspective, because the energetic cost to induce lipid monolayer bending is relatively high (63), this process is more likely to proceed through the concerted action of several protein molecules, rather than through changes at the level of isolated protein monomers. In other words, oligomerization of pro-apoptotic proteins may assist in the formation of lipidic pores, as previously proposed for a number of bacterial toxins, membranolytic peptides, and membrane fusion proteins (64–71).

In summary, our results show that the autonomous pore-forming function of cleaved Bcl-xL is sufficient to induce efflux of apoptogenic proteins entrapped within the mitochondrial intermembrane space. The necessity and redundancy of this mechanism for physiological apoptosis, as well as the exact structure of the pore formed by pro-apoptotic proteins in mitochondrial membranes, await further studies.

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Pro-apoptotic Cleavage Products of Bcl-xL Form Cytochrome c-conducting Pores in Pure Lipid Membranes

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