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Biologically distinct conformations of Bcl-x can be resolved using 2D isoelectric focusing

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Abstract

Bcl-x, a potent regulator of cellular decisions of life and death, has multiple survival-enhancing activities that rely on distinct protein regions. Evidence suggests that depending on the local environment and the binding of protein or peptide partners, Bcl-x can take on several conformations that expose different protein regions. However, biological occurrence of conformational forms has been very difficult to study, because structure determination techniques use large quantities of protein, purified under conditions that change Bcl-x conformation. We show here that standard 2D isoelectric focusing techniques can be used to distinguish conformationally distinct forms of Bcl-x in cell lysates. Conformational isoelectric forms were manipulated through the use of detergents and buffers of differing pH. Our data indicate that post-translational modifications are not needed for or associated with conformational changes, distinguishing the dominant isoelectric forms of Bcl-x. We found that Bcl-x conformational isoelectric forms have preferred subcellular localization patterns. Moreover, conformational forms are differently regulated in certain locations during cytokine starvation of IL-3 dependant cells. Therefore, we provide evidence that 2DIEF can be used to view biologically distinct conformational differences in Bcl-x on minute quantities of unpurified protein from cells or lysates.

Keywords

isoelectric focusing; conformation; Bcl-x

INTRODUCTION

Bcl-x_L, a member of the Bcl-2 family of proteins, regulates the multi-staged process of cell suicide known as apoptosis and can also delay cell cycle progression. Bcl-x has multiple conformations that differ in exposure of key protein regions, including the Bcl-2 homology domains (BH). For example, dimerization of Bcl-x depends on conformational flexibility that allows the conserved BH 1 and 2 domains of Bcl-x to form a hydrophobic face to bind BH3 regions, described by Muchmore *et al.* (1997), Aritomi *et al.* (1997) and Sattler *et al.* (1997). Alternatively, the BH3 domain can turn to form an exposed ligand-like domain, described by Conus *et al.* (2000). The overall conformation of Bcl-x in aqueous solution differs greatly from

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that described by Losonczi *et al.* (2000) for Bcl-x imbedded in lipid miscells in the lengths and positions of its alpha helices, including the N-terminal alpha helix, reported by Shimizu *et al.* (2000) to associate with VDAC. The work published by Thuduppathy *et al.* provides further evidence of secondary and tertiary structure differences between soluble, membrane-anchored, and membrane-inserted Bcl-x forms and detailed evidence supporting an electrostatic mechanism of membrane insertion for truncated recombinant Bcl-x (Thuduppathy, 2006). Detergents can also influence the conformation of Bcl-x (Hsu, 1998), and this has led to some confusion in interpretation of Bcl-x protein interaction data from some immunoprecipitation experiments.

Bcl-x function differs depending on location due to the presence of local protein partners and regulators. For example, Stegh *et al.* (2002), Zhang *et al.* (2000), and Shimizu *et al.* (1999) describe Bcl-x:BAR complexes that regulate caspases at the mitochondria, while ER Bcl-x:VDAC complexes are reported to regulate mitochondrial permeability (Shimizu *et al.*, 2000 and Nguyen *et al.*, 2000) Bcl-x:Bap31 complexes in the ER help inhibit calcium sensitization to apoptosis (Breckenridge *et al.*, 2003, Tsuruta, 2002, and Mund *et al.*, 2003). The activities of Bcl-x family members are regulated, in part, through localization, and these proteins change distribution during apoptosis signaling from the cytosolic to membrane compartments of the cell (Hsu, 1997, and Jia *et al.*, 1999).

Several types of post-translational modification have been described in Bcl-x. Phosphorylation of Bcl-x on serine, threonine, and tyrosine residues has been reported. Tyrosine phosphorylation of Bcl-x has been shown to enable the cell cycle delay effects of Bcl-x (Huang *et al.* 1997). Microtubule-targeting drugs induce the formation of multiple phosphorylated Bcl-x forms, the formation of which is reduced by JNK1 and 2 depletion, (Poruchynsky *et al.* 1998, Fan *et al.* 2000). Some JNK-mediated phosphorylation is location-specific. The phosphorylation of Bcl-x threonines 47 and 115 by JNK in response to ionizing radiation occurs after translocation of cytosolic Bcl-x to the mitochondria and negatively regulates Bcl-x function (Kharbala *et al.* 2000). Bcl-x is permanently modified by caspase cleavage, which produces a fragment capable of inducing cell death (Clem *et al.* 1998). Bcl-x deamidation occurs at two residues in the unstructured loop region at the mitochondria in response to DNA damage, a modification, which alters the flexibility of the protein and negatively regulates its function (Deverman *et al.* 2003, Takehara and Takahashi 2003, and Johnstone, 2002).

In this study, we used two dimensional isoelectric focusing (2DIEF) gels to separate charge variants of Bcl-x derived from different subcellular fractions, in order to test whether we could identify localization-specific post-translational modifications. We observed multiple isoelectric forms of Bcl-x in cells and identified hyperacidic, phosphorylated Bcl-x forms, as well as deamidated Bcl-x forms. Striking differences were seen between the isoelectric form profiles of Bcl-x derived from different subcellular fractions. Acidic isoelectric forms of Bcl-x dominated in the cytosolic fraction, while neutral forms dominated in the membrane fractions. Surprisingly, we found that the acidic and neutral isoelectric forms of Bcl-x differ in conformation, rather than chemical modification, and are influenced by pH and detergent. Therefore, the location-specific pools of Bcl-x, which differ in biological behavior, vary in conformation, rather than chemical modification.

EXPERIMENTAL PROCEDURES

Vectors

Murine *Bcl-x* was subcloned by PCR from the pSFFV-neo-Bcl-x expression vector, provided by Ameeta Kelekar and Gabriel Nunez (Gonzalez-Garcia, 1994), into the MSCV-IRES-GFP vector, a gift from Naomi Rosenberg (Hawley *et al.*, 1994). An empty vector served as a control. DNA from selected clones was sequenced. *Generation of Retroviral Particles--* Infectious

virus was produced by using Superfectamine (Qiagen) to co-transfect the MSCV retroviral construct and the pSV- ψ -Eco-MLV packaging DNA (Muller *et al*, 1991) into 293T cells, generating virus capable of infecting murine, but not human, cells.

Cells

FL5.12 cells were grown in RPMI (Gibco), supplemented with 10% FCS and were maintained and assayed for viability, as described by Nunez *et al* (1990). Supernatant from X63Ag8-653 IL-3-secreting cells, a gift from Fritz Melchers, described in Karasuyama and Melchers (1988), was used at a 1:3000 dilution, which consistently protected FL5.12 cells from apoptosis and allowed their proliferation, but did not saturate their growth response. 293T cells were grown in RPMI containing 10% FCS for virus production. Cells were analyzed by flow cytometry using FACSCalibur (Becton Dickinson) and sorted for GFP expression using a MoFlow (Cytomation).

IL-3 starvation assays

FL5.12 cells die through apoptosis when deprived of IL-3, and degrade their DNA during the process. For the IL-3 starvation assays, FL5.12 cells were washed twice in media lacking IL-3, then resuspended at 1×10^5 cells/ml in media with or without IL-3. Cells in media lacking IL-3 were plated as triplicate wells on multiple plates, one plate for each day of the experiment. Cells in media with IL-3 were divided every day or two as needed. Standard protocols for hypotonic propidium iodide staining and flow cytometry were used to detect apoptotic cells with fragmented DNA. Briefly, cells were harvested and washed twice in PBS with .02% Sodium Azide, then swelled in a hypotonic staining buffer (1.0g/L Sodium Citrate, 1ml/L triton-X-100) containing 50 μ g/ml of the DNA stain Propidium Iodide (PI). Cells were incubated in the staining buffer for more than 4 hours, during which time DNA fragments generated during apoptosis diffused out of the cells. The samples were then analyzed for PI staining intensity (a measure of DNA content) through Flow cytometry. In histograms of PI intensity, apoptotic nuclei having less than normal DNA content formed a broad sub-G1 peak clearly distinguishable from the narrow peaks from DNA of non-apoptotic G1, S, and G2-M phase cells.

Protein Analysis of FL5.12 Cell Extracts

FL5.12 cells were harvested at the indicated times by centrifugation at 1500 rpm. Cells were counted using a hemocytometer, washed with PBS/.02% sodium azide, and frozen immediately on dry ice. Cell pellets were kept frozen until buffers containing protease inhibitors were added.

Cell Fractionation

A standard detergent-free cell fractionation technique was adapted from Hsu *et al*. (1997). Cells were resuspended at a concentration of 5×10^7 cells/ml in detergent-free ice-cold hypotonic lysis buffer, consisting of 10 mM HEPES, 38 mM NaCl, with mini-complete protease inhibitor pellets (Roche). Cells were allowed to swell on ice for ten minutes then lysed by 40 strokes of a Dounce homogenizer, lysing cells as well as mitochondria. Nuclei were centrifuged at $900 \times g$ for 30 minutes. Pellets were resuspended in 100 μ l of lysis buffer. Supernatants and resuspended pellets were re-centrifuged at $900 \times g$ for 30 minutes. The respective pellets and supernatants were pooled. Post-nuclear supernatant was centrifuged at $15,000 \times g$ for 15 minutes to enrich for mitochondria. The pellet was resuspended in 50 μ l of lysis buffer and re-centrifuged. The supernatant was re-centrifuged, and the pellets were pooled. Post-mitochondrial supernatants were centrifuged at $100,000 \times g$ for 1 h to enrich for light membranes. Supernatants were removed, and pellets were flash frozen to aid resuspension. Protein concentration was estimated with Micro BCA analysis reagents (Pierce), using 2 to 5 μ l aliquots of the experimental samples and a standard curve of BSA dilutions.

Immunoblot Analysis

Cell lysates and fractions, as well as focused IEF strips, were separated by SDS-PAGE, using 12.5% polyacrylamide gels with a standard stacking gel. Some pre-cast BioRad 4–15% gradient gels were also used for 1D gels. Proteins were transferred onto PVDF membranes (Immobilon-P, Fisher Scientific Co, pore size 0.45 micron). PVDF membranes were blocked in 0.2% I Block (Tropix) in PBS plus 0.05% Tween 20. Membranes were incubated overnight at 4°C with primary antibody at 1:3000 to 1: 10,000 in I Block, washed in PBST (PBS plus 0.05% Tween 20) three times, and then incubated for 1 h at room temperature in appropriate secondary antibody at 1:10,000 in I Block, then visualized.

Wild type Bcl-x was detected using the rabbit polyclonal antibodies, Bcl-xa1-18 (sc-634, Santa Cruz) and Bcl-x (B22630 BD/Pharmigen.). Vector-derived Bcl-x was detected using FLAG M2 (F3165, Sigma). Samples were probed for Phosphoserine (Zymed), Phosphotyrosine (SC-508, Santa Cruz) and Nitro-tyrosine (A21285, Molecular Probes, Eugene OR.).

5 µg of each fraction was separated using SDS-PAGE and western blotted for control proteins of known distribution. Nuclear fractions were positive for PARP (sc-7150, Santa Cruz), but negative for mitochondrial VDAC (Ab-5, Oncogene Research Products) and ANT-1 (Ab-1, Oncogene), ER calnexin (sc-6465, Santa Cruz), and cytosolic GFP (8372, Clontech) and Apaf-1 (8339, Santa Cruz). Mitochondrial fractions were negative for PARP, positive for ANT-1 and VDAC, and negative for calnexin and GFP. Light membrane fractions were negative for PARP, VDAC and ANT-1, positive for calnexin, and negative for GFP. Soluble fractions were negative for PARP and positive for GFP and Apaf-1.

Anti-FLAG Immunoprecipitation of Vector-Derived Bcl-x

Cell lysates were immunoprecipitated for FLAG-Bcl-x, using anti-FLAG M2 antibodies conjugated to agarose beads (Sigma). 1×10^6 cells per 100 µl lysis buffer were immunoprecipitated with 10 µl of packed beads. The composition of the lysis buffer was 50 mM Tris-HCL 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100. The wash buffer was 1X TBE: 0.05 mM Tris HCl, 0.15 mM NaCl plus 2X Protease inhibitor cocktail (Complete Mini, Roche, Mannheim, Germany). Cells were suspended in lysis buffer, mixed with anti-FLAG-conjugated beads and incubated overnight at 4°C. Empty vector control cells lacking FLAG-Bcl-x were used as a negative control. The beads were washed five times with the wash buffer and then frozen as dry pellets.

CIP Treatment of Immunoprecipitated Bcl-x

Bcl-x protein, purified by anti-Flag immunoprecipitation, was treated with Calf Intestinal Phosphatase (CIP). 15 µl of anti-FLAG-conjugated beads were incubated with 50 µl of 1x CIP buffer plus enzyme and protease inhibitors overnight at 37°C. A reaction lacking CIP was performed as a control.

pH and Deamidation Treatment of Immunoprecipitated Bcl-x

Anti-FLAG immunoprecipitates were treated overnight in 20 mM sodium phosphate buffers plus 0.2% Triton X-100 and protease inhibitors at 37°C. Buffers of pH 4, 7, and 9 were prepared by mixing mono-basic and di-basic 20 mM sodium phosphate solutions to obtain the desired pH. 15 µl of beads were treated in 50 µl of buffer (adapted from Deverman *et al*, 2002.).

Detergent Treatment of Immunoprecipitated Bcl-x

Anti-FLAG immunoprecipitates were resuspended in 20 mM sodium phosphate buffer, pH 7, plus protease inhibitors. Detergents were added to certain samples to obtain concentrations of 0.2% CHAPS or 0.2% Triton X-100. Samples were incubated overnight at 37°C.

Isoelectric Focusing (IEF) Analysis

2D IEF gel analysis was performed using Pharmacia 2D equipment, including the EPS 3500XL power supply, Multiphor II 2D platform, and Multitemp III waterbath. IEF strips of pH 4–7 were used (Amersham Pharmacia). Drystrip fluid (Pharmacia) and Paraffin oil (Fisher) were used to overlay samples. Samples were prepared and IEF gels were run according to Pharmacia/Multiphor instructions. The pH gradients on the isoelectric focusing strips varied slightly between lots; thus, exact migration distances from the acidic end of the gel could only be compared within experiments run on the same batch of strips.

To prepare samples for 2D IEF, 5 to 10 µg protein per sample was resuspended in 180 µl 8.5 M urea reswelling buffer (RSB, 5 ml: 2.4 g urea (Ultrapure USB), 0.1 g CHAPS (SIGMA), 100 µl IPG buffer 4–7 (Pharmacia), 15 mg DTT, trace Bromophenol blue (BPB), and re-swollen into the strips for 10–16 h. Current was run through the strips to separate the proteins by charge (Step 1: 300 V, 1 mA, 5 W, 0.01 h, 1 Vhr; Step 2: 300 V, 1 mA, 5 W, 6 h, 1800 Vhrs; Step 3: 3500 V, 1 mA, 5 W, 5 h, 9500 Vhrs; Step 4: 3500 V, 1 mA, 5 W, 5.5 h or more, minimum of 9250 Vhrs). The IEF strips were equilibrated for 5 min in equilibration buffer (500 ml Eq buffer: 180 g urea, 150 g glycerol, 10 g SDS; 10 mg/ml DTT, added just prior to use.) Equilibrated strips were loaded onto 2nd D gels. IEF strip acidic ends marker paper, marker bands and BPB front were traced. The membranes were blocked and probed for Bcl-x, as described above.

RESULTS

Bcl-x has multiple Isoelectric Forms

FL5.12 cells were infected with MSCV-IRES-GFP or MSCV-IRES-Bcl-x-GFP and sorted for GFP expression into pools using Fluorescence activated cell sorting (FACS). Pools of sorted, GFP⁺ FL5.12 cells were tested for apoptosis following IL-3 starvation. Recombinant Bcl-x expression was verified in the pooled, sorted cells by western blot analysis. As expected, the MSCV-Bcl-x-IRES-GFP vector protected FL5.12 cells from apoptosis induced by IL-3-starvation, whereas the empty vector MSCV-IRES-GFP did not, as seen in Figure 1.

Cell lysates were separated by IEF and SDS-PAGE, then immunoblotted for Bcl-x. As can be seen in Figure 2, at least three isoelectric forms of Bcl-x can be discerned. The endogenous Bcl-x isoelectric form pattern (A) and the vector-derived Bcl-x isoelectric form pattern (B) were very similar, but vector-derived Bcl-x was slightly more acidic than endogenous Bcl-x due to the presence of the vector-encoded FLAG-tag at the N-terminus. These results indicate that gross regulation of Bcl-x isoelectric forms is similar for the over-expressed vector-derived Bcl-x and for the endogenous Bcl-x. Multiple isoelectric forms were observed that were clustered in three groups. The dominant form was the most neutral of the three forms. The central of the three forms appeared to be similar in molecular weight to neutral form, but was more acidic. The hyperacidic form at the left of the gel was slightly delayed in mobility compared to the other Bcl-x isoelectric forms. This delay in mobility could be explained either by an actual increase in molecular weight due to chemical modification or by differences in the migration of the different forms through the gel.

Each of the three dominant forms had beside it a less abundant isoelectric form that was slightly heavier and slightly more acidic than the dominant form. These secondary isoelectric forms

were more visible with dark film exposures than with light exposures. The isoelectric form pattern did not change with differences in the concentration of urea used in the sample buffers (between 6.5 M and 8.5 M) or with the time of incubation in those buffers (Data not shown).

Phosphorylation of Bcl-x

Phosphorylation has been described on several sites in Bcl-x, including tyrosine, serine and threonine residues. Phosphorylation renders the isoelectric point of a protein more acidic and increases the weight of the protein slightly. CIP treatment was used to remove phosphate groups from immunoprecipitated Bcl-x. The 2D gel pattern of CIP treated Bcl-x was then compared to the pattern of a control sample that was treated only with buffer (Figure 3A). The samples treated with CIP lost the hyperacidic, but not the acidic or neutral forms of Bcl-x. This experiment was repeated three times. The results indicate that only the hyperacidic forms of Bcl-x have phosphorylation modifications.

The loss of hyperacidic forms caused by CIP treatment was consistent, but their isoelectric points were more variable than those of the other forms. The hyperacidic Bcl-x forms were difficult to study, because their isoelectric points were near the limit of the isoelectric focusing strips used in these experiments. However, it is also possible that the observed variability may be evidence of phosphorylation-associated conformational differences.

In order to confirm the phosphorylation of the hyperacidic IEF forms of Bcl-x, anti-phosphoserine antibodies were used to probe 2D western blots of immunoprecipitated Bcl-x (Figure 3B). Whole-cell-lysate was loaded beside the isoelectric focusing gel strip in the second dimension gel to serve as a positive control. The phospho-serine antibody recognized a cluster of spots at the acidic end of the gel. The membrane was then stripped and re-probed for Bcl-x. The Bcl-x hyperacidic forms aligned precisely with the phosphoserine signal, indicating the presence of phosphoserine in the hyperphosphorylated Bcl-x isoelectric form cluster. This experiment was repeated twice, with similar results. The presence of Triton-X 100 in the immunoprecipitation resulted in the change in isoelectric form pattern between lysate samples shown in Figure 2 and the immunoprecipitated samples shown in this figure, an effect explored further in Figure 5. The evidence of phosphorylation of Bcl-x hyperacidic forms in FL5.12 cells is consistent with our results, which showed that the hyperacidic Bcl-x forms appeared to be phosphorylated in ^{32}P -labeled HT-2 T-cells (data not shown).

Deamination of Bcl-x

Deamidation occurs spontaneously at a rate determined by the local molecular environment and increasing with increasing pH. Deamidation of Asparagines 52 and 66 of Bcl-x has been shown to negatively regulate Bcl-x function, as described by Deverman *et al.* (2002), Takehara, (2003), and Johnstone, (2002). Recently, Deverman *et al.* (2002) reported that deamidation of Bcl-x at two residues in the loop domain occurs during incubation of Bcl-x under basic conditions. This paper reported that compared to unmodified Bcl-x, the deamidated forms were delayed in mobility by SDS-PAGE, a difference due to conformational rigidity introduced by the modification, rather than by the minute difference in molecular weight between modified and unmodified forms.

To determine if the deamidated forms could be detected using the 2D western system, Bcl-x samples were treated according to the methods described by Deverman *et al.* (2002), using 20 mM pH 9 sodium phosphate buffer plus 0.2% Triton X-100 and incubating overnight at 37° C. Samples were also treated in 20 mM sodium phosphate buffers of pH 7 and pH 4. The results of these experiments, shown in Figure 4, demonstrate that deamidation can be detected in base-treated samples and reveal an unexpected pH dependence of the acidic and neutral IEF forms of Bcl-x. The basic conditions increased the appearance of slightly acidic, delayed mobility

forms consistent with deamidated Bcl-x. The forms created under these conditions resemble the subforms seen in whole-cell lysates of control cells and Bcl-x-infected cells. In fact, these sub-forms increased in any Bcl-x sample incubated for prolonged periods at 37°C, such as during CIP treatment. Acidic pH enhanced acidic isoelectric forms of Bcl-x, while basic conditions enhanced neutral isoelectric forms. Chemical modifications did not seem to explain the differences in the profile of isoelectric forms seen at different pH. Therefore, conformation was considered as a possible basis for these differences.

Conformational Changes of Bcl-x

To determine whether Bcl-x IEF forms differ by conformation, rather than by chemical modification, we tested the influence of detergents on the isoelectric form patterns. We first performed a preliminary experiment to see if isolated IEF forms might be interconverted by detergents. Using the ideas of Lutter *et al.* (2001), the gel regions corresponding to the acidic and neutral clusters of Bcl-x isoelectric forms were isolated and incubated overnight at 37°C in 20 mM Sodium Phosphate pH 7 plus either 0.2% CHAPS or 0.2% Triton X-100. The detergent conditions were chosen based on their impact on the structure of Bcl-x relatives (Hsu, 1998). Triton X-100 provides a non-ionic lipid environment, whereas CHAPS presents a zwitterionic environment of balanced positive and negative charges. Upon re-analysis of the samples by 2D western blotting, we observed that the isolated acidic IEF forms could be converted into a mixture of acidic and neutral IEF forms by incubation in Triton X-100. The neutral spot was unchanged by incubation in Triton X-100. The samples incubated in CHAPS and re-analyzed by 2DIEF focused poorly (Data not shown). The differences in isoelectric point are reversible and the gel-isolated forms are interconvertable; properties that rule out numerous modifications, including cleavage, glycosylation, and enzymatic modifications. The acidic IEF forms are not decreased by treatment at pH 4, ruling out tyrosine-O-sulfate modification. The acidic and neutral IEF forms do not differ by deamidation, although deamidated forms of the acidic and neutral IEF forms were identified. The acidic and neutral IEF forms appear to differ by more than four charges, based on shifts in charge seen for the phosphorylated and deamidated Bcl-x forms. These preliminary data support the hypothesis that the acidic and neutral Bcl-x isoelectric forms differ only by conformation, but not by chemical modification. Since conformational differences are generally removed by prolonged incubation in high concentration of urea, such as those used in the 2D IEF protocols, this was a surprising finding. However, the proteins of this family have been reported to have pH-dependent conformational flexibility.

In order to further test the hypothesis that conformation plays a role in determining Bcl-x isoelectric points, immunoprecipitated Bcl-x was incubated in buffer with the detergents CHAPS or Triton X-100, or buffer alone overnight at 37°C, then analyzed by 2D western. The results of a representative experiment are shown in Fig. 4. Bcl-x immunoprecipitate treated with CHAPS yields an increase in the acidic cluster of isoelectric forms relative to the pH 7-treated control. In contrast, Triton X-100-treated samples show increased neutral cluster of isoelectric forms relative to CHAPS-treated samples, but largely resemble the buffer-treated Immunoprecipitate, because the immunoprecipitations had been performed in the presence of Triton X-100. Incubation with Triton X-100 influences the isoelectric point of Bcl-x and thereby increases the ratio of neutral to acidic isoelectric forms. In contrast, incubation with CHAPS decreases the ratio of the neutral to acidic isoelectric forms. Remarkably, these results confirmed that conformation, rather than modification, differentiates the acidic and neutral isoelectric forms of Bcl-x, and that this conformational difference is stable during prolonged incubation in high concentrations of urea.

Summary of Modifications Identified in the Bcl-x isoelectric Form Pattern

The results of these experiments explain the differences between all of the isoelectric forms of Bcl-x that are visible on the 2D western blots of cell lysates. The most neutral isoelectric form is a lipid conformation of Bcl-x, while the acidic form is a soluble conformation of Bcl-x and the hyperacidic forms are phosphorylated. Variability in the pattern of spots in this hyperacidic cluster suggests that a conformational change contributes to the small difference in their isoelectric points. The minor species to the left of the acidic and neutral isoelectric forms appear to be deamidated. Thus, the isoelectric forms of Bcl-x have been identified, and the various forms can be characterized as to their differences in biological behavior.

Bcl-x IEF forms have different distributions in FL5.12 Cells and different Fates during IL-3 Starvation

The major isoelectric forms of Bcl-x, once identified, were then characterized by determining their subcellular distribution in IL-3 treated and IL-3 starved FL5.12 cells. Cells were fractionated to enrich for cellular components of interest, using a standard detergent-free centrifuge fractionation procedure. Cell lysates were separated into four fractions: nuclei, heavy membranes including mitochondria, light membranes including ER membranes, and soluble cytosolic proteins. Fractionation was verified by western blotting for control proteins, shown in representative western blots in figure 6.

Figure 7A shows the distribution of Bcl-x and actin in each fraction of the cell. Bcl-x was lost from the soluble fraction of IL-3 starved FL5.12 cells, compared to those treated with IL-3. However, the Bcl-x levels in the nucleus, heavy membrane and light membrane fractions were not reproducibly different between these conditions. The localization-specific depletion of Bcl-x demonstrates that the various subcellular pools of Bcl-x are independently regulated during IL-3 starvation.

Post-translational modification, chemical or conformational, might mark pools of Bcl-x in specific subcellular locations and/or influence the regulation of Bcl-x in specific locations. Thus, the distribution of each of the various isoelectric forms of Bcl-x within the cell was determined. In addition, experiments were performed to examine the effects of IL-3 starvation on the localization of each of the isoelectric forms of Bcl-x. The film exposures shown in Figure 7B were chosen to best show the differences in predominance of the major isoelectric forms within each sample, but do not show relative intensities of Bcl-x between samples.

The Bcl-x isoelectric forms display specific localization patterns and different fates following cytokine starvation. The two dominant isoelectric form clusters could be seen in all fractions of the IL-3 treated cells. The neutral cluster predominated in nuclear, heavy membrane and light membrane fractions, while the acidic form predominated in soluble fractions. The Bcl-x isoelectric form distribution in IL-3 starved cells resembled those of IL-3 treated cells in many ways, but substantial differences existed as well. The most noticeable change was seen in the soluble fraction that was specifically depleted of the neutral isoelectric form cluster. Four independent experiments were performed with paired lysate fractions from IL-3 treated and IL-3-starved cells. In each of these experiments, the neutral cluster predominated in the nuclei, heavy membranes, and light membranes of IL-3 treated cells, while the acidic form dominated in the soluble fraction. The ratio of neutral to acidic Bcl-x isoelectric forms differed between experiments and cell groups, but the preferential localization was consistent.

These results demonstrate that the isoelectric forms of Bcl-x differ in their sub-cellular distribution and regulation during cytokine starvation. The neutral isoelectric form of Bcl-x that is enhanced by non-ionic detergents is also the predominant form present in membrane

fractions of FL5.12 cells. Thus, the conformational forms of Bcl-x can be detected in subcellular fractions.

DISCUSSION

The studies reported here examined post-translational modification of Bcl-x using 2D IEF. Unexpectedly, conformation, in addition to chemical modification, was discovered to cause differences in the isoelectric point of Bcl-x. Mass spectrometry would be necessary to conclusively prove that the acidic and neutral IEF forms of Bcl-x differ only by conformation, but the evidence presented here strongly indicates that conformation differentiates these IEF forms. We were able to manipulate the IEF forms of Bcl-x with detergents and pH, and the forms were interconvertable. The detergents used were chosen to allow comparison with previously described Bcl-x conformational studies. The acidic and neutral conformational variants of Bcl-x reported here are consistent with the structures determined by others for purified Bcl-x in acidic and in non-ionic lipid environments. These conformationally distinct charge variants of Bcl-x differed in subcellular distribution and regulation in IL-3-starved cells.

Our results demonstrate that non-ionic detergent enhances the form of Bcl-x that occurs preferentially in membrane fractions of the cell. Charged, zwitterionic detergent, in contrast, enhanced the form of Bcl-x that occurs preferentially in the soluble fraction of the cells. The published reports on the effect of pH and detergent treatment on Bcl-x require further explanation. Acidic pH was demonstrated by Xie *et al* (1998) to cause spontaneous membrane insertion and to enhance heterodimerization of Bcl-x by delaying the dissociation of dimers. Likewise, Triton X-100 was shown to enhance heterodimerization, while CHAPS was reported by Hsu (1998) to inhibit dimerization. However, in the experiments presented here, acid pH and CHAPS treatment enhanced the same isoelectric form, as seen in Figures 4 and 5. Hydrophobic regions of a protein will be repelled by a charged and polar environment, but attracted to non-ionic lipids. Conversely, charged regions of a protein will be repelled by a non-ionic environment and attracted to an aqueous environment. Accordingly, thermodynamic forces will incline the negative charges in the Bcl-x protein, including those in the pore-channel region, to associate with a positively charged environment, but to be repelled from a negatively charged environment. According to this model, the basic environment would favor the same conformation of Bcl-x as the membrane environment. The conformation of Bcl-x in negatively charged or in non-ionic surroundings would tend to minimize contact between the negative charges of the residues in Bcl-x and the thermodynamically unfavorable environment.

The conformational isoelectric forms identified by our study are consistent with the forms previously described by others using different techniques and purified Bcl-x protein. The membrane conformation of Bcl-x that was described by Losonczi (2000) is consistent with the neutral conformation of Bcl-x seen here in samples treated with the non-ionic detergent Triton X-100 (Figure 5). We also observed this neutral form when the isolated acidic form was incubated with Triton X-100 (Figure 5). This membrane conformation appears to dominate in the nuclear, heavy membrane and light membrane fractions of FL5.12 cells (Figure 7). The soluble conformation reported by Muchmore (1996) appears to correspond to the acidic isoelectric forms that were enhanced in our CHAPS-incubated immunoprecipitates (Figure 5). These acidic forms dominate in the soluble cell fraction (Figure 7). Non-ionic detergents, like Triton X-100, have been reported to allow heterodimerization and homodimerization of Bax and Bcl-x and to permit Bcl-x to assume the membrane conformation. Charged detergents, like Tween 20 and CHAPS, do not allow heterodimerization and appear to maintain Bcl-x in soluble structure.

Most methods of determining Bcl-x conformation, such as NMR and circular dichroism, are relatively complicated, difficult to perform, and require large quantities of purified protein.

During purification, proteins are subjected to conditions that can alter Bcl-x conformation, such as varying pH (Xie, 1998), incubation with detergents, (Hsu, 1998) and proteins (Huang 2000). The results presented here show that IEF provides a new way to study Bcl-x that yields information on the conformational state of Bcl-x in unpurified cell lysates and subcellular fractions. Exact quantitation of Bcl-x isoelectric forms was not possible with the detection system in use when these experiments were performed, because neither the light output, nor the detection were linear. Additionally, quantities of the isoelectric forms varied within and between samples, and light from abundant forms could obscure less abundant forms nearby. However, improvements in detection methods and technology and improved IEF markers more recently available would now allow quantitation of Bcl-x isoelectric forms detected using 2DIEF. The discovery that IEF can be used to separate conformationally distinct forms of Bcl-x opens new horizons for future research, allowing rapid screening of reagents and Bcl-x mutations for the capacity to alter conformational dynamics of Bcl-x. Indeed, Bcl-x mutants D61A, S62A, and D76A, deserve further attention for altered conformation, relocalization and/or degradation (data not shown). The potential to find Bcl-x mutations that differ in conformation will allow the identification of conformation-dependent binding partners and regulators.

In summary, our results demonstrate the ability of the 2D IEF technique to detect biologically distinct conformations of Bcl-x. This evidence provides long-awaited support to the hypothesized occurrence in cells of the different conformations of Bcl-x, which have been described previously using purified proteins. Furthermore, these studies show that the various conformations of Bcl-x which can be detected by 2D IEF differ in their cellular localization and regulation.

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Abbreviations Footnote

2DIEF	two dimensional isoelectric focusing
IEF	isoelectric focusing
ER	endoplasmic reticulum
PBS	Phosphate buffered saline
Memb	Membrane fraction
GFP	Green fluorescent protein
CIP	Calf intestinal Alkaline phosphatase

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Figure 1. Infection of FL5.12 cells with Bcl-x vector, but not empty vector, aids survival during IL-3 withdrawal

Infected GFP-positive sorted cell pools (containing MSCV-Bcl-x-IRES-GFP or MSCV-IRES-GFP retrovirus) were starved of IL-3 and assayed for viability every day. FACS analysis and hypotonic propidium iodide staining were used to detect cells with apoptotic nuclei. Error bars represent the standard deviations of triplicate wells. The data are representative of more than six experiments.

Figure 2. Bcl-x has multiple isoelectric forms in FL5.12 cells

Lysates from FL5.12 cells expressing MSCV-IRES-GFP or MSCV-BCL-X-IRES-GFP were analyzed in 2D western blots by charge separation using IEF, then separating by size using SDS-PAGE and visualization by western blotting for Bcl-x. The region of the gradient pH 4 to 5.5 is shown, with the acidic end of the gel aligned with 0 cm of a ruler. These data are representative of at least six independent experiments. A, endogenous Bcl-x isoelectric forms in FL5.12 cells infected with empty vector MSCV-IRES-GFP. B, vector-derived Bcl-x isoelectric forms in FL5.12 cells infected with MSCV-Bcl-x-IRES-GFP.

Figure 3. Hyperacidic Bcl-x is phosphorylated

MSCV-Bcl-x-IRES-GFP vector-derived Bcl-x was immunoprecipitated from infected, sorted FL5.12 cells using anti-FLAG antibody. A, hyperacidic Bcl-x vanishes with CIP treatment. Bcl-x immunoprecipitates were treated with CIP or with buffer only. Samples were analyzed by 2D western blot probed for Bcl-x. Three repetitions were performed. B, phosphoserine antibody binds to Bcl-x hyperacidic forms. Samples were analyzed using 2D western blotting for phosphoserine. The blot was then stripped and re-probed for Bcl-x. Two repetitions were performed. The immunoprecipitated Bcl-x hyperacidic forms reacted in western blots with anti-phosphoserine antibody.

Figure 4. pH induced isoelectric form changes in Bcl-x

MSCV-Bcl-x-IRES-GFP vector-derived Bcl-x was immunoprecipitated from infected, sorted FL5.12 cells using anti-FLAG antibody. Samples were treated overnight at 37°C in 20 mM sodium phosphate buffers of either pH 4, 7 or 9. Samples were then analyzed using 2D western blotting for Bcl-x. Four repetitions of this experiment were analyzed with pH 7 and 9, and two that included pH 4. IEF forms consistent with deamidated Bcl-x are marked.

Figure 5. Detergents induce isoelectric form changes in Bcl-x

MSCV-Bcl-x-IRES-GFP vector-derived Bcl-x was immunoprecipitated from infected, sorted FL5.12 cells using anti-FLAG antibody. Samples were treated overnight at 37°C in 20 mM sodium phosphate buffer alone or in buffer containing 0.2% CHAPS or 0.2% Triton X-100. Samples were then analyzed using 2D western blotting for Bcl-x. Three repetitions of this experiment were analyzed.

Figure 6. Centrifugation procedure enriches for subcellular fractions

FL5.12 cells were infected with MSCV-Bcl-x-IRES-GFP and sorted for GFP. Cells were treated in IL-3 or starved of IL-3 for one day. Cells were harvested, homogenized and then fractionated using detergent free centrifugation-based fractionation. Fractions were verified by analyzing 5 μ g of protein by western blot for control proteins, including PARP, VDAC, calnexin, APAF, GFP, and β -actin. Representative examples of various fractionation control western blots are shown.

A: 1D analysis of Bcl-x distribution throughout the cell.

B: 2D analysis of Bcl-x distribution throughout the cell.

Figure 7. Intracellular localization of Bcl-x

A, western blot analysis of cell fractions from Bcl-x-infected cells shows Bcl-x loss from the soluble fraction in IL-3 starved cells. Fractions were analyzed for Bcl-x and for β -actin, a control. B, IEF analysis showed differential Bcl-x isoelectric form localization. 5 μ g of each fraction was then analyzed using 2D western blotting for Bcl-x. Two repetitions using only IL-3+ conditions and four repetitions in paired IL-3 +/- experiments were analyzed.