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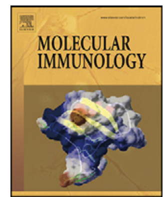

Biologically distinct conformations of Bcl-x can be resolved using 2D isoelectric focusing

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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-dependent 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.

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1. 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. (1996), 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 that described by Losonczi et al. (2000) for Bcl-x imbedded in lipid miscells in the lengths and positions of its alpha helixes, 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 et al., 2006). Detergents can also influence the conformation of Bcl-x (Hsu and Youle, 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; Nguyen et al., 2000) Bcl-x:Bap31 complexes in the ER help inhibit calcium sensitization to apoptosis (Breckenridge et al., 2003; Tsuruta et al., 2002; 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 et al., 1997; 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. Tryosine 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

Abbreviations: 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 phosphatase.

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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 (Kharbanda 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; 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.

2. Experimental procedures

2.1. 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 et al., 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.

2.2. 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.

2.3. 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).

2.4. 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 (1.0 g/L Sodium Citrate, 1 ml/L triton-X-100) containing 50 μgs/ml of the DNA stain Propidium Iodide (PI). Cells were incubated in the staining buffer for more than 4 h, 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.

2.5. 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.

2.6. 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 10 min then lysed by 40 strokes of a Dounce homogenizer, lysing cells as well as mitochondria. Nuclei were centrifuged at $900 \times g$ for 30 min. Pellets were resuspended in 100 μl of lysis buffer. Supernatants and resuspended pellets were re-centrifuged at $900 \times g$ for 30 min. The respective pellets and supernatants were pooled. Post-nuclear supernatant was centrifuged at $15,000 \times g$ for 15 min to enrich for mitochondria. The pellet was resuspended in 50 μl 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 2–5 μl aliquots of the experimental samples and a standard curve of BSA dilutions.

2.7. 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 1:3000–1:10,000 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-xaa1-18 (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 frac-

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