Multiple Subcellular Localization of bcl-2: Detection in Nuclear Outer Membrane, Endoplasmic Reticulum Membrane, and Mitochondrial Membranes

Yukihiro Akao, Yoshinori Otsuki, Seiko Kataoka, Yuko Ito, and Yoshihide Tsujimoto

Department of Anatomy and Biology, Osaka Medical College, Takatsuki, 569, Osaka [Y. A., Y. O., Y. I.], and Biomedical Research Center, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565 [S. K., Y. T.], Japan

ABSTRACT

The use of biochemical fractionation, immunofluorescence laser-scanning confocal microscopy, and immunoelectron microscopy with mouse anti-human bcl-2 monoclonal antibody to analyze the subcellular localization of the bcl-2 gene product revealed the protein prominently in the nuclear envelope, endoplasmic reticulum membrane, and mitochondrial membranes. Electron microscopy at high magnification more precisely localized bcl-2 to the nuclear outer membrane as confirmed by the biochemical fractionation, as well as to mitochondrial outer and, to a lesser degree, inner membrane. This multisite membrane distribution of bcl-2 suggests an important role for this protein in several different membrane compartments.

INTRODUCTION

The bcl-2 gene was originally identified by studies of the t(14;18) chromosomal translocation associated with human follicular B-cell lymphoma (1–3). bcl-2 is categorically distinct from classical oncogenes in that its overexpression prolongs survival of hematopoietic (4–7) and neuronal (8) cells cultured in the absence of required growth factors. This death-sparing capacity has been linked to an ability of bcl-2 to block apoptosis (7, 9), a mechanism of programmed cell death. The bcl-2 product does not share sequence homology with any proteins with known biochemical functions, and the biochemical mechanism of bcl-2 function has remained unclear. Previous efforts to establish the subcellular localization of the bcl-2 protein as a step toward understanding bcl-2 function have yielded variable results with respect to its presence in the nuclear envelope, endoplasmic reticulum, or mitochondrial membrane, specifically the inner membrane (9–12).

Using immunoelectron microscopy together with laser-scanning confocal microscopy and biochemical fractionation procedures, we demonstrated here that the bcl-2 protein is present in multiple locations, prominently in nuclear membrane, specifically the outer membrane, endoplasmic reticulum membrane, and mitochondrial membranes.

MATERIALS AND METHODS

Cell Line. The EB-4A cell line was established by transforming B-lymphocytes derived from a healthy donor with Epstein-Barr virus.

Subcellular Fractionation. Cells were washed with PBS and suspended in hypotonic solution (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-10 mM MgCl2-42 mM KC1) for 5 min on ice. Cells were passed through a 30-gauge needle and centrifuged at 600 x g for 10 min to collect crude nuclei that were further purified as described below. The supernatant was centrifuged at 10,000 x g for 10 min. The pellet was recovered as heavy membrane fraction. The supernatant was further centrifuged at 100,000 x g for 90 min. The pellet and supernatant were used as light membrane and cytoplasmic fractions, respectively. The crude nuclear fraction was passed through a needle several times, extensively washed with PBS, and centrifuged through a 2 M sucrose cushion at 150,000 x g for 60 min. The pellet was used as a purified nuclear fraction. Under the condition, nuclei were considerably fragmented but membrane integrity remained intact as judged by nearly quantitative recovery of lamin B (see Fig. 1). Pellet fractions were dissolved in 2% PBS (1 x PBS: 137 mM NaCl-2.7 mM KCl-5 mM Na2HPO4-1.5 mM KH2PO4), 1% NP40, 0.5% deoxycholate, and 0.1% sodium dodecyl sulfate. NP40-treated nuclei were prepared by lysing cells suspended in the hypotonic solution using 0.5% NP40. The integrity of the nuclei was checked under a light microscope.

Western Blotting Analysis. Proteins were size fractionated in a 12.5% polyacrylamide/sodium dodecyl sulfate gel and transferred to a nylon membrane. The bcl-2 protein was detected by sequential binding of a specific first antibody, biotin-labeled anti-mouse immunoglobulin goat antibody and horseradish peroxidase-conjugated streptavidin. The immunocomplex was visualized by chemiluminescence (Amersham). Specific first antibodies used were anti-human bcl-2 monoclonal antibody (13), anti-human MnSOD rabbit polyclonal antibody (14), and anti-rat lamin B rabbit polyclonal antibody with cross-reactivity to human lamin B.

Immunofluorescent Staining. Cells were fixed in 4% paraformaldehyde for 10 min, washed with PBS three times, and permeabilized with 0.1% Triton X-100 in PBS for 20 min. Following incubation with PBS containing 10% NGS and 1% BSA for 30 min, cells were further incubated with mouse anti-human bcl-2 monoclonal antibody (IgG1) (culture supernatant of the hybridoma) diluted 1:5 with PBS supplemented with 1% NGS and 1% BSA (PBS/NGS/BSA) for 30 min. After cells were washed three times with PBS/NGS/BSA, the monoclonal antibody was labeled with fluorescein-conjugated goat anti-mouse IgG (TAGO Corp., Burlingame, CA) at a dilution of 1:50 with PBS/NGS/BSA for 30 min. After three washings with PBS, cells were mounted on polyl-lysine-coated slides and examined using a Zeiss laser scanning microscope. All procedures were carried out at room temperature; control cells were incubated with PBS/NGS/BSA and examined for nonspecific staining.

Immunoelectron Microscopy. More than 2 x 10⁶ cells were fixed with 4% paraformaldehyde in PBS for 20 min, centrifuged at 200 x g for 5 min, washed three times with 5 ml of PBS, and stained using the two-step indirect immunoperoxidase method detailed elsewhere (15). Briefly, cells were treated with PBS containing 10% NGS and 1% BSA and incubated for 30 min with 100 μl of mouse anti-human bcl-2 monoclonal antibody (IgG1) diluted 1:5 with PBS/NGS/BSA. As a negative control, cells were stained with mouse anti-human CD3 monoclonal antibody (IgG1) (Cosmo Bio. Corp., Tokyo, Japan). After three washings, cells were further incubated for 30 min with 100 μl of goat anti-mouse IgG conjugated to horseradish peroxidase (DAKO Japan Co. Ltd., Kyoto, Japan) diluted 1:100 with PBS/NGS/BSA. The peroxidase reaction was developed by exposing cells to diaminobenzidine (0.05%) and hydrogen peroxide (0.01%) for 5–10 min. Following two washes, cells were fixed with 2% glutaraldehyde in PBS for 30 min and postfixed with 1% osmium tetroxide in PBS. After two washes, cells were stained with 1% uranyl acetate in PBS for 10 min, dehydrated, and embedded in Epon 812 according to routine procedures. Ultrathin sections (60–80 nm) were prepared and examined in a Hitachi H 7000 electron microscope. All procedures were carried out at room temperature.

RESULTS

Biochemical fractionation was used initially to approximate the subcellular distribution of the bcl-2 protein. Epstein-Barr virus-trans-
formed human B cells (EB-4A) were fractionated into crude nuclear, heavy membrane (containing mitochondria), light membrane (containing microsomes), and cytoplasmic fractions. Since crude nuclear fraction was likely contaminated with other cellular components, it was further purified as described in "Materials and Methods." Each fraction was analyzed for the presence of bcl-2 protein by Western blot with anti-human bcl-2 monoclonal antibody as described in "Materials and Methods." Approximately one-third of the bcl-2 protein was recovered in the purified nuclear fraction, and the remainder was recovered in the heavy membrane fraction. A trace amount of bcl-2 protein was also detected in the light membrane and cytoplasmic fractions (Fig. 1). To ensure appropriate fractionation, the same fractions were analyzed for the presence of MnSOD (a mitochondrial protein) and lamin B (a nuclear lamin protein) (Fig. 1). When treated with 0.5% NP40, the crude nuclear fraction lost virtually all bcl-2 protein (Fig. 1, N'), suggesting that bcl-2 is associated with nuclear envelope, specifically outer membrane, but not with nucleoplasm, since the outer nuclear membrane is mostly removed under the condition (Ref. 16 and data not shown). The presence of a considerable amount of bcl-2 in nuclear fraction suggested that the bcl-2 protein is not exclusively mitochondrial.

Analysis by laser-scanning confocal microscopy, carried out as described in "Materials and Methods" with mouse monoclonal anti-bcl-2 antibody, revealed the bcl-2 protein throughout the cytoplasm distributed in a heterogeneous pattern and in the perinuclear but not the nucleus (Fig. 2), consistent with the results from biochemical fractionation procedures. Immunoelectron microscopy (15) was carried out to localize the bcl-2 protein more precisely as described in "Materials and Methods." EB-4A cells possess abundant mitochondria, rough and smooth endoplasmic reticulum, granules, multiple vesicular bodies, and free ribosomes (Fig. 3, A and B), unlike normal human B-lymphocytes in which intracellular organelles are sparse. Immunoreactivity with the anti-bcl-2 antibody was detected prominently in the nuclear envelope, mitochondria, smooth and rough endoplasmic reticulum membrane, and multiple vesicular bodies but not in granules or nucleoplasm (Fig. 3). At higher magnification (×75,000), immunoreactivity was detected in the outer but not the inner membrane of the nuclear envelope and in the outer and, to a lesser extent, the inner mitochondrial membrane (Fig. 3, A, C, E, and F). Control cells stained with isotype-matched mouse anti-CD3 monoclonal antibody all failed to exhibit specific staining (Fig. 3, B and D). Staining of myometrial smooth muscle cells at late secretory phase that were shown to be bcl-2 negative by immunohistochemistry (data not shown) did not reveal specific staining (Fig. 3G). These results were in good agreement with those obtained by laser-scanning confocal immunofluorescence microscopy and biochemical fractionation. The virtually identical immunoelectron microscopic result was obtained with mouse pre-B-cell stained with anti-mouse bcl-2 rabbit polyclonal antibodies (data not shown).

DISCUSSION

It is well established that bcl-2 is a integral membrane protein (10, 17). The signals for membrane localization are unknown, although the COOH-terminal portion with a stretch of hydrophobic amino acid residues has been implicated. Removal of the COOH-terminal hydrophobic stretch of the bcl-2 protein rendered the protein unable to associate with the membrane in an in vitro transport system (18) or a DNA transfection system. The mutant lacking the COOH-terminal portion was unable to protect cells from apoptosis as well as the normal protein (19), suggesting the importance of membrane localization in bcl-2 function.

We have demonstrated, through the combined use of laser-scanning microscopy, biochemical methods, and immunoelectron microscopy, that the bcl-2 membrane protein clearly resides prominently in the nuclear envelope, mitochondria, smooth and rough endoplasmic reticulum, and multiple vesicle body membranes. These data are consistent with a recent study using immunofluorescence laser-scanning microscopy (12). Electron microscopy at high magnification localized the bcl-2 protein more precisely in nuclear outer membrane, as well as mitochondrial outer and, to a lesser extent, inner membrane. Our detection of bcl-2 in several different membrane compartments contrasts with a previous report of the presence of the bcl-2 protein exclusively in mitochondrial inner membrane (9) and is consistent with the results of an immunoelectron microscopic study by Monaghan et al. (20) and a confocal microscopic study by Jacobson et al. (12). The discrepancy does not appear to stem from differences in levels of bcl-2 expression in the cell lines used, since we obtained similar results with several cell lines expressing the bcl-2 protein at a variety of levels. Although the bcl-2 protein resides in different compartments, the actual site(s) at which it exerts its function remains unknown. Recently, Jacobson et al. (12), using cells lacking mitochondrial DNA, have reported that bcl-2 function is not linked to mitochondrial respiration. This finding, together with the multiple distribution of bcl-2, argues against the likelihood that mitochondrial DNA...
MULTIPLE SUBCELLULAR LOCALIZATION OF bcl-2

Fig. 3. Immunoelectron micrographs for bcl-2 distribution in EB-4A cells. (A) Low power view of the EB-4A cell stained with the anti-bcl-2 antibody. Arrows, nucleus bordered by diaminobenzidine reaction products; bar, 1 μm × 10,000. (B) Low power view of the control EB-4A cell stained with an isotype-matched anti-CD3 monoclonal antibody. Arrowheads, no significant staining detected in nuclear envelope; bar, 1 μm × 10,000. (C) High power view of nuclear region. bcl-2 detected in the outer nuclear membrane (arrows) and smooth endoplasmic reticulum (arrowheads) but not in the inner nuclear membrane. Bar, 0.1 μm × 75,000. (D) High power view of the control nuclear region with mitochondria. Bar, 0.1 μm × 75,000. (E) Part of the same cell as shown in A. Mitochondrial outer and, at a lesser intensity, inner membranes (arrowheads) were stained with the antibody as compared to the outer nuclear membrane shown in C. Bar, 0.1 μm × 75,000. (F) Anti-bcl-2 antibody staining in rough endoplasmic reticulum (arrows) and outer nuclear membrane (arrowheads). Bar, 0.1 μm × 75,000. (G) View of the control myometrial smooth muscle cell stained with the anti-bcl-2 antibody. Bar, 0.1 μm.

function per se is involved in the apoptosis-blocking activity of bcl-2. It is possible that bcl-2 functions in membranes of different compartments or that it protects several organelles by the same mechanism. bcl-2 has been suggested to be involved in an antioxidant pathway (19, 21). This idea is consistent with the multiple membrane localization of the bcl-2 protein because reactive oxygens are produced in mitochondria, nuclear envelope, and endoplasmic reticulum (22).

We have begun using a series of bcl-2 mutants to identify domains required for membrane localization and function of the protein. These mutants will be very useful in pinpointing the functional site of the bcl-2 protein by altering its localization through various transport signals and analyzing its apoptosis-blocking activity.

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