Investigation of the Subcellular Distribution of the \textit{bcl-2} Oncoprotein: Residence in the Nuclear Envelope, Endoplasmic Reticulum, and Outer Mitochondrial Membranes

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\textbf{ABSTRACT}

A multidisciplinary approach was taken to investigate the intracellular locations of the 26-kDa integral membrane protein encoded by the \textit{bcl-2} gene. Subcellular fractionation analysis of a t(14;18)-containing lymphoma cell line revealed the presence of Bcl-2 protein in nuclear, heavy-membrane, and light-membrane fractions but not in cytosol. Sedimentation of heavy-membrane fractions in Nycozenz and Percoll continuous gradients demonstrated comigration of p26-Bcl-2 with mitochondrial but not other organelle-associated proteins. Fractionation of light-membrane fractions using discontinuous sucrose-gradients revealed association of Bcl-2 protein primarily with lighter-density microsomes (smooth endoplasmic reticulum) as opposed to heavy-density microsomes (rough endoplasmic reticulum). Immune microscopy studies using laser-scanning microscopy, pre- and postembedding electron microscopic methods, and six different anti-Bcl-2 antibodies demonstrated Bcl-2 immunoreactivity in the nuclear envelope and outer mitochondrial membrane in a patchy distribution. Furthermore, anti-Bcl-2 antibody immunoreactivity generally appeared to directly overlie the nuclear envelope in high magnification electron microscopic studies, reminiscent of nuclear pore complexes. Addition of \textit{in vitro} translated p26-Bcl-2 to isolated translocation-competent mitochondria revealed transmembrane domain-dependent association of Bcl-2 protein with mitochondria but provided no evidence for import into a protease-resistant compartment, consistent with immunomicroscopic localization to the outer mitochondrial membrane. Taken together, the findings demonstrate that p26-Bcl-2 resides primarily in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membrane in a nonuniform distribution suggestive of participation in protein complexes perhaps involved in some aspect of transport.

\textbf{INTRODUCTION}

The \textit{bcl-2} gene was first discovered because of its involvement in the t(14;18) chromosomal translocations commonly found in non-Hodgkin's lymphomas (1). This cytogenetic abnormality places the \textit{bcl-2} gene from chromosome 18 into juxtaposition with the transcrip tionally active immunoglobulin heavy-chain locus on chromosome 14, resulting in inappropriately high levels of \textit{bcl-2} gene expression in malignant B-lymphocytes (reviewed in Ref. 2). The protein encoded by this gene is structurally and functionally unique in that it bears little or no significant homology with other known cellular proteins (3, 4) and because it contributes to malignant cell expansion primarily by prolonging cell survival rather than by increasing the rate of cellular proliferation (5). The specific mechanism by which the Bcl-2 protein extends cell survival remains enigmatic, inasmuch as no biochemical activity has thus far been ascribed to this oncoprotein, but high levels of Bcl-2 protein have been shown to delay or block programmed cell death ("apoptosis") in a variety of circumstances (6–9).

Apoptosis is an active form of cell death that often requires new RNA and protein synthesis (10). One of the hallmarks of programmed cell death is degradation of the nuclear DNA of the cell into oligonucleosomal-length fragments, presumably because of the activation of endonucleases that occurs relatively early in the apoptotic process. Although apoptosis plays an important role in normal physiological situations where it helps to ensure that the rate of new cell production in proliferative tissues is offset by a commensurate rate of cell death, it is also important clinically because many chemotherapeutic drugs appear to ultimately kill cancer cells by activating undefined biochemical pathways leading to programmed cell death (reviewed in Ref. 11). In this regard, high levels of Bcl-2 protein production have been shown to protect lymphoid cells from death and DNA fragmentation induced by a wide variety of antineoplastic drugs (12, 13).

The human Bcl-2 protein is an intracellular, integral membrane protein with a molecular mass of ~26 kDa (14). The ability of p26-Bcl-2 to associate with membranes has been attributed to a hydrophobic stretch of amino acids located near its carboxyl terminus (15). The topic of the intracellular membranes in which p26-Bcl-2 resides, however, is controversial. Previously, Hockenbery \textit{et al.} (6) reported that Bcl-2 is found primarily in the inner mitochondrial membrane, based on subcellular fractionation experiments and immunofluorescence confocal microscopy. In this regard, however, the Bcl-2 protein does not contain a positively charged amphiphilic, \(\alpha\)-helical NH\(_2\)-terminal leader sequence typical of many nuclear-encoded proteins that undergo transport into mitochondria (16, 17). In another study, Chen-Levy \textit{et al.} (14) used crude subcellular fractionation methods and conventional immunofluorescence microscopy and reported that Bcl-2 resides mostly in perinuclear membranes, with about one-half of the protein being associated with nuclei and the rest mostly with other intracellular membranes. Bcl-2 is also found primarily in association with the nuclear fraction when expressed in \(\alpha\)9 insect cells using the baculovirus system (18). Liu \textit{et al.} (19) examined the issue using two-color immunofluorescence confocal microscopy and reported that about one-half of the immunodetectable Bcl-2 protein is found in association with mitochondria; the remainder of the protein apparently resided in an undefined nonnuclear compartment. Hamilton \textit{et al.} (20) reached the same conclusion using immunofluorescence confocal microscopy but relied on only single-color methods. Recently, Jacobson \textit{et al.} (21) obtained evidence from 2-color immunofluorescence confocal microscopy suggesting that Bcl-2 protein can enter the membranes of the endoplasmic reticulum, nuclear envelope, and mitochondria when overexpressed in fibroblast-like cells. In a separate study involving primarily immunoelectromicroscopic methods, Monaghan \textit{et al.} (22) reported that Bcl-2 resides in the nuclear envelope and outer mitochondrial membranes.

Taken together, the currently available data concerning the intracellular locations of the Bcl-2 protein paint a confusing picture. There are several potential reasons for the disparate results obtained by various groups. For example, in most of these reports the data were based upon observations obtained with a single antibody reagent or...
with antibodies directed against a single region of the Bcl-2 protein. Ideally, one would like to see equivalent results obtained using at least two different antibodies that recognize distinct nonoverlapping epitopes on the Bcl-2 protein. Another potential factor of course is the particular experimental methods employed, since various techniques have different limitations and advantages.

In an effort to clarify some of the present controversies surrounding the subcellular locations of the Bcl-2 oncoprotein, we undertook a multidisciplinary investigation of this issue. For these studies, we utilized four polyclonal and two monoclonal antibody reagents raised against synthetic peptides corresponding to two nonoverlapping segments of the human Bcl-2 protein. Among the experimental approaches that were used to examine the location of the Bcl-2 protein were: (a) immunomicroscopy using laser-scanning and electron microscopic methods; (b) subcellular fractionation; (c) studies of Bcl-2 protein interaction in vitro with intact freshly isolated mitochondria; and (d) gene transfer to produce the human Bcl-2 protein in cellular backgrounds in which it is not normally found. On the basis of these investigations, we conclude that the Bcl-2 protein resides primarily in 3 intracellular membrane compartments: nuclear envelope; endoplasmic reticulum; and outer mitochondrial membrane. The implications of these findings are discussed below.

**MATERIALS AND METHODS**

**Cells and Culture Methods**

Cell lines used for these studies included the t(14;18)-containing B-cell lymphoma lines RS11846 and SU-DHL-4 (23, 24). These cells were maintained at 10^5-10^6 cells/ml in RPMI 1640 with 10% (v/v) heat-inactivated fetal bovine serum at 37°C in 5% CO_2/95% air. 32D-NEO and 32D-BCL-2 cells are subclone derivatives of the human Bcl-2 protein (17) and were used for immunoprecipitations under conditions that released p26-Bcl-2 from cells (26). Similar results were obtained with both nuclei and cytosolic fractions. The supernatant resulting from the initial low-speed centrifugation was resuspended in 1% Triton-containing lysis buffer (26) and either analyzed directly or the HM fraction was resuspended in 0.2-0.3 ml of MES buffer and centrifuged at 100,000 × g for 60 min. The resulting membrane pellet was solubilized in 1% Triton X-100-containing lysis buffer and Bcl-2 protein was immunoprecipitated using polyclonal rabbit antisera (26). Proteins remaining in the lysates after pre-clearing of p26-Bcl-2 were then evaluated by immunoblot assays using mouse monoclonal antibodies to mitochondrial and ER proteins.

**Preparation of Subcellular Fractions**

RS11846 cells (1-10 × 10^6 cells) were washed twice with ice-cold Dulbecco’s phosphate-buffered saline (pH 7.4) ( Gibco, Inc.) and resuspended in 0.5-1.5 ml of MES 5 mM buffer containing protease inhibitors as described previously (26). After homogenization for 10-20 strokes with a Dounce homogenizer and centrifugation at 10,000 × g (average) for 15 min at 4°C to obtain the heavy-membrane fraction (pellet), and this supernatant was then centrifuged for 60 min at 150,000 × g (SW50.1) to obtain the light-membrane (pellet) and cytosolic (supernatant) fractions. The HM and LM fractions were resuspended in 1% Triton-containing lysis buffer (26) and either analyzed immediately for enzyme activities or frozen at -80°C for subsequent immunoblot assays.

**Separation of Organelles by Density Gradient Centrifugation**

Sucrose, Percoll, and Nycodenz gradients were prepared as described previously and used to separate organelles based on buoyant density (28-30) utilizing either the HM fraction or the postnuclear low-speed supernatants derived from 5-10 × 10^6 cells. Briefly, for sucrose, the postnuclear low-speed supernatants or the HM fraction was resuspended in 0.2-0.3 ml of MES buffer and layered onto 4 ml of a 1-2 M linear sucrose gradient and centrifuged at 110,000 × g (SW50.1) for 3 h at 4°C. For Nycodenz, a 9-35% w/v linear gradient was used and centrifugation was at 140,000 × g for 2 h. Percoll gradients were formed in situ by resuspending cell fractions in 30% Percoll (final concentration) containing 0.25 M sucrose, 42 mM KCl, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), and 5 mM MgCl₂ and centrifuging at 48,000 × g for 60 min at 4°C. Protease inhibitors were included in all solutions. Sequential fractions of 0.2 ml were collected from the gradients and either assayed immediately for enzyme activities or mixed with an equal volume of 2% Triton lysis buffer (26) and stored at -80°C.

**Isolation of Microsomal Membranes**

Microsomal membranes were prepared from 10^6 RS11846 cells by centrifugation of the LM fraction on discontinuous sucrose step gradients by the method of Meyer and Dobberstein (31). The material recovered from the 0.8-1.2 M and the 1.23-1.31 M sucrose interfaces was pooled (smooth ER). The heavier-density microsomes were recovered from the 1.31-2.0 M interface (rough ER). Microsomal membranes were mixed with 3 volumes of 50 mM Tris (pH 7.5)-5 mM MgCl₂ and centrifuged at 105,000 × g for 60 min. The resulting membrane pellet was solubilized in 1.0% Triton X-100-containing lysis buffer and Bcl-2 protein was immunoprecipitated using polyclonal rabbit antisera (26). Proteins remaining in the lysates after pre-clearing of p26-Bcl-2 were then evaluated by immunoblot assays using mouse monoclonal antibodies to mitochondrial and ER proteins.

**Cellular Uptake and Assay for Horseradish Peroxidase**

RS11846 cells were cultured for 16-18 h at 0.25-1.0 × 10^6 cells/ml in RPMI 1640 containing 10% fetal bovine serum, antibiotics, 1 mM L-glutamine, and 100 μg/ml HRPase to label the lysosomal compartment (32). The cells were then washed 3 times with Dulbecco’s phosphate-buffered saline to remove HRPase that had not entered cells by fluid-phase pinocytosis, and the heavy-membrane fraction was prepared for loading onto Nycodenz and Percoll gradients. Fractions collected from gradients were then assayed for HRPase activity by the method of Steinman and Cohn (33), using diaminobenzidine as the substrate and measuring the absorbance at 450 nm after 3 min.

**Enzyme Assays for LDH, β-Hexosaminidase, and Galactosyltransferase**

The lysosomal marker β-hexosaminidase was assayed by measuring the hydrolysis of p-nitrophenyl-β-N-acetylgalactosamine to yield free p-nitrophenol (34). The Golgi marker UDP-glucosamine:Galactosyltransferase was assayed by the method of Morré (35) using UDP-[14C]galactose from Amersham, Inc. (CFB129) and measuring by liquid scintillation counting the amount of [14C]lactose generated in 20 or 30 min at room temperature, where dehydrolyzed substrate was removed from an anion exchange resin (AG1-X2/Cl-form; Bio-Rad). LDH activity was measured using a kit from Sigma (No. 500). Data from enzyme assays were normalized for total protein content where indicated.

**Antibodies, Immunoblot, and Immunoprecipitation Assays**

Polyclonal rabbit antisera specific for the human Bcl-2 protein were raised against synthetic peptides corresponding to amino acids 41-54 or 61-76 of p26-Bcl-2 and were used for immunoprecipitations under conditions that resulted in recovery of essentially all immunodetectable Bcl-2 protein from cell
lysates (26). In some cases, 5 μg of competing peptide were added to lysates prior to antibody, to block binding to p26-Bcl-2. Samples were normalized for total protein content using the bicinchoninic acid method (36) where indicated. Antibodies used for immunoblot assays included the polyclonal rabbit antisera against the 41-54 peptide described above (9716-10, 1631-8); (b) two antisera raised against the 41-54 peptide (purchased from DAKO, Inc.); and (d) ascites from a mouse monoclonal antibody which identifies an unchar-acterized 60-kDa endoplasmic reticulum protein (0.025%); 80-61-76 peptide (9718-8; 1633-8); (c) Mab 124, a murine monoclonal antibody against the 61-76 peptide (9718-8); and mouse monoclonal antibodies included 7H8.2C12, specific for cytochrome c (ascites at 0.1%); 80-61-76 peptide (9718-8) and a antibody specific for the p70-α-docking protein of the rough ER (41). SDS-PAGE and electroblotting to nitrocellulose filters were performed as described previously (26, 42), except when assessing mammone 6-phosphate receptor, in which case proteins were not reduced to preserve immunoreactivity. Antibodies were detected with biotinylated goat anti-rabbit IgG serum (HRPase-avidin-biotin complex reagent (Vector Laboratories, Inc.) and 3-amino-9-ethylcarbazole; and detection of mouse monoclonals was accomplished with alkaline phosphatase-conjugated rabbit anti-mouse IgG serum (Dako, Inc.) followed by the chemiluminescent substrate Lumiphos (Bohringer-Mannheim, Inc.) according to the manufacturer’s recommendations.

**In Vitro Transcription, Translation, and Mitochondriai Transport Assays**

Bcl-2α and Bcl-2β complementary DNAs, cloned into the pBluescript (Stratagene, Inc.) vector pSK-II (43), were transcribed in vitro using T3 or T7 RNA polymerase, respectively, and a Riboprobe kit from Promega according to the supplier’s instructions. Approximately 2 μg of the resulting RNAs were used without capping for in vitro translation with a rabbit reticulocyte lysate translation kit (Promega) supplemented with [35S]methionine according to the supplier’s protocol. The translation products were used directly in the mitochondrial transport experiments.

Mitochondria were prepared from the livers of male Sprague-Dawley rats according to the procedure of Conboy and Rosenberg (44). They were sus-pended at a protein concentration of 20 mg/ml in 220 mM mannitol-70 mM sucrose-2 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (pH 7.4; 4°C) for use in transport experiments. Mitochondrial transport was assessed as described previously (45), except that 3 volumes of translated protein mixture were combined with 2 volumes of mitochondria. After a 20-min incubation at 27°C, the mitochondria were recovered by a 3-min centrifugation in an Ep-pendorf microcentrifuge. The mitochondria were resuspended in an equal volume of isolation buffer (above), and an aliquot was treated with trypsin at a final concentration of 10 μg/ml for 20 min at 4°C. An aliquot of the supernatant was treated with the same concentration of trypsin. The reactions were terminated with SDS-PAGE loading buffer, the samples were heated at 95°C for 5 min, and the proteins were separated on a SDS-10% polyacrylamide gel. The gels were fixed, treated with Autofluor (National Diagnostics), and exposed to XAR-5 film at ~70°C. Similar results were obtained when import assays were performed at 37°C and for longer times (up to 60 min), except that in this case translation of intramitochondrial RNAs occurred, resulting in the products of additional [35S]labeled proteins the appearance of which could be blocked by addition of chloramphenicol.

**Microscopy**

Immune staining of cells was accomplished using a variety of anti-Bcl-2 antibody reagents, including: (a) two different rabbit antisera against the 41–54 peptide described above (9716-10, 1631-8); (b) two antisera raised against the 61–76 peptide (9718-8; 1633-8); (c) Mab 124, a murine monoclonal antibody against the 41–54 peptide (purchased from DAKO, Inc.); and (d) ascites from the hybridoma 4D7 which produces a monoclonal antibody directed against the 61–76 peptide (46). All data presented were reproduced with a minimum of 2 different antibody reagents directed against nonoverlapping epitopes on Bcl-2.

**Laser-scanning Microscopy.** Immunostaining of cells for evaluation by laser-scanning confocal microscopy was accomplished with an argon ion laser (488 nm) and transmitted bright-field mode, similar to the method of Itoh et al. (47). A Planapochromat (X63) objective lens with a numerical aperture of 1.4 was used. The images were derived through electronic processing methods, which enhance the positive reaction signals by a combination of contrast normalization and scaling density adjustments. Final images were printed or photograped using a Sony UP-D7000 Digital Color Printer or a Lasergraphics FR Mark II film graphics recorder. These data were representative of findings obtained by routine light microscopy with regard to relative signal intensities.

**Electron Microscopy.** For preembedding EM studies, cells were prepared as exactly described above. For postembedding EM studies, cells were fixed in glutaraldehyde as above, immobilized in 1.5% agarose, postfixed in 1% OsO4, and washed extensively with water prior to incubating for 1 h in 1% uranyl acetate on ice in the dark. Cells in agarose were then dehydrated by sequential 0.5–1 h incubations in 30, 50, 70, 80, 96, and 100% ethanol followed by three 0.5–1 h incubations in propylene oxide, several h in propylene oxide:plastic resin [25% (v/v) TAAB, 25% Epon 812, 30% dodecyensuccinic anhydride, 20% natic methyl anhydride, and 1.5% (v/v) DMP-30 (1:1)]. Sections 1 μm thick were mounted on poly-l-lysine-coated slides.

Laser-scanning microscopy was performed using a Zeiss inverted LSM-410 laser-scanning confocal microscope equipped with an argon ion laser (488 nm) and transmitted bright-field mode, similar to the method of Itoh et al. (47). A Planapochromat (X63) objective lens with a numerical aperture of 1.4 was used. The images were derived through electronic processing methods, which enhance the positive reaction signals by a combination of contrast normalization and scaling density adjustments. Final images were printed or photograped using a Sony UP-D7000 Digital Color Printer or a Lasergraphics FR Mark II film graphics recorder. These data were representative of findings obtained by routine light microscopy with regard to relative signal intensities.

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RESULTS

Subcellular Fractionation Analysis of Bcl-2 Protein Distribution

Evidence for Association of p26-Bcl-2 with Mitochondria. The RS11846 cell line contains a t[14;18] translocation involving bcl-2 and was established from a patient with non-Hodgkin’s B-cell lymphoma. Crude fractionation of these cells to yield nuclei, heavy membranes, light membranes, and cytosol revealed p26-Bcl-2 protein primarily in the nuclear and HM fractions, although a small amount of 26-kDa material reacting with anti-Bcl-2 antibodies was detected also in light membranes during these immunoblot assays where subcellular fractions were normalized for total protein content (Fig. 1). Similar results were obtained using two different anti-Bcl-2 antisera directed against nonoverlapping epitopes (not shown). The presence of Bcl-2 protein in the nuclear compartment could not be attributable to residual nonlysed cells, based on LDH measurements, and persisted despite centrifugation of nuclei twice through 2.0–2.3 M sucrose. Despite attempts at rigorous purification, however, nuclear preparations from these malignant B-lymphocytes frequently appeared to be contaminated with mitochondria, since p50-F1-β-ATPase and p12-cytochrome c were also detected by immunoblotting using specific antibodies (Fig. 1). However, we often observed disproportionate amounts of p26-Bcl-2 in the nuclear fraction relative to mitochondrial markers such as F1-β-ATPase and cytochrome c (Fig. 1), suggesting that mitochondrial contamination cannot entirely account for the pool of Bcl-2 found in this fraction.

Because the HM fraction typically contains not only mitochondria but also lysosomes, Golgi, and some of the rough endoplasmic reticulum, we next subjected HM material obtained from RS11846 cells to centrifugation in continuous Nycodenz and Percoll gradients to achieve separation of various organelles based on differences in their buoyant density. Analysis of fractions recovered from these gradients was accomplished by either immunoblotting [using antibodies specific for p26-Bcl-2, mitochondrial p50-F1-β-ATPase, and p12-cytochrome c or an uncharacterized 60-kDa protein found only in the ER (antibody 2H1)] or by performing standard enzymatic assays for lysosomal β-hexosaminidase and Golgi galactosyltransferase. In some experiments, RS11846 cells were cultured with HRPase to allow pinocytotic marking of the lysosomal compartment (32).

In all experiments in which HM material was used, p26-Bcl-2 cosedimented in gradients with mitochondrial proteins but not with lysosomal, Golgi, or ER markers. Fig. 2A, for example, shows the results of an experiment where heavy membranes were centrifuged in a 9–30% Nycodenz gradient. The Bcl-2 protein cosedimented exactly with the mitochondrial proteins F1-β-ATPase and p12-cytochrome c but not with the ER marker 2H1 (Fig. 2). Fig. 2B shows typical results from an experiment using a continuous Percoll gradient and HRPase-containing RS11846 cells. In Percoll gradients, Bcl-2 again cosedimented with mitochondrial proteins F1-β-ATPase and cytochrome c but was clearly separated from the lysosomal enzymes β-hexosaminidase and HRPase which sedimented at lighter densities than the mitochondria (Fig. 2A and D). The second peak of higher-density material containing lysosomal markers (Fractions 18–20) can be attributed to HRPase-induced aggregation of lysosomes (48). The results of experiments in which HM preparations were subjected to centrifugation in these density gradients thus are consistent with a mitochondrial location for p26-Bcl-2.

Evidence for a Nonmitochondrial Pool of p26-Bcl-2 in RS11846 Lymphoma Cells. In Fig. 1, a faint band corresponding to p26-Bcl-2 was seen during immunoblot analysis of LM material. Therefore, to compare the relative amounts of p26-Bcl-2 in the light- and heavy-density compartments, HM and LM fractions were prepared from 10^7 RS11846 cells and p26-Bcl-2 was immunoprecipitated from the detergent-solubilized proteins under conditions previously shown to recover nearly 100% of immunodetectable Bcl-2 protein (26). Under these circumstances where samples were not normalized for total protein content prior to immunoprecipitation (unlike Fig. 1), substantial amounts of p26-Bcl-2 were detected in the LM fraction, representing 15–25% of the postnuclear pool of this protein (Fig. 3). Peptide competition experiments confirmed the specificity of these anti-Bcl-2 antibodies for immunoprecipitation of the 26-kDa Bcl-2 protein from both HM and LM preparations (Fig. 3). No mitochondrial marker proteins were found in the light-membrane preparation (not shown), indicating that the presence of p26-Bcl-2 in the lower-density compartment cannot be attributed to mitochondrial contamination.

The findings in Fig. 3 prompted us to further examine the distribution of p26-Bcl-2 in postnuclear lysates by density gradient centrifugation methods. In contrast to experiments using HM preparations where a single peak of Bcl-2 was produced in gradients and where Bcl-2 invariably cosedimented with mitochondrial proteins, postnuclear lysates which contain cytosol, HM, and LM fractions produced two peaks of Bcl-2 protein in continuous Nycodenz gradients (Fig. 4). Only the higher-density fraction of Bcl-2 cosedimented with mitochondrial proteins in this case. Furthermore, as shown in Fig. 4, the lighter density fraction of p26-Bcl-2 did not cosediment with the 50-kDa IgG heavy-chain, which resides primarily in the plasma membrane of these B-cells, or with the 210-kDa M6PR which resides in the cis-Golgi, plasma membrane, and endosomal compartments (39). The
SUBCELLULAR DISTRIBUTION OF Bcl-2 ONCOPROTEIN

Fig. 2. Density gradient subfraction of heavy-membrane material from RS11846 cells. HM preparations from RS11846 cells were subjected to centrifugation in either 9–30% preformed Nycodenz gradients (A) or Percoll gradients formed in situ (B). In A, the lysosomal compartment of the cells was marked with HRPase. Fractions of 250 μl each were collected and numbered from top to bottom (1–18 or 1–20). A 50-μl aliquot of each fraction from the gradients was then subjected to SDS-PAGE and subsequent immunoblot assay using antibodies specific for p60-2H1 (ER protein), p50-F1-β-ATPase, p26-Bcl-2, or p12-cytochrome c. In B, a portion of each aliquot was assayed for β-hexosaminidase or HRPase activity. kD, kilodaltons.

Data in Fig. 4 showing the relative amounts of total protein in each fraction recovered from a Nycodenz gradient also suggest a possible explanation for the failure to detect a significant portion of Bcl-2 protein in the LM fraction in Fig. 1. Namely, because samples were previously normalized for protein content prior to analysis, the LM fraction was underrepresented.

To confirm that the 26-kDa protein detected in the lighter-density portion of Nycodenz gradients by immunoblotting was truly p26-Bcl-2, Fractions 3–7 (low density) and Fractions 12–17 (high density) of the experiment shown in Fig. 4 were independently pooled and subjected to immunoprecipitation using anti-Bcl-2 antibodies directed either against amino acids 41–54 or 61–76 of the human Bcl-2 protein. The immunoprecipitated proteins were then subjected to SDS-PAGE and immunoblotting analysis using the opposite anti-Bcl-2 antibody. As shown in Fig. 5, the 26-kDa material sedimenting at lower-density in the Nycodenz gradient was in fact p26-Bcl-2, based on this

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were prepared from RS11846 cells using discontinuous sucrose step 
methods.

et al. of p26-Bcl-2 in isolated low-density microsomes (Fig. 6 and data not 
contrast to whole-cell and heavy-membrane preparations, thus demon-
strating that mitochondrial contamination cannot explain the presence 
of the total cellular Bcl-2 residing in the smooth ER. Immunoblot analysis of the same microsomal extracts after pre-clearing 
p26-Bcl-2 demonstrated the presence of the p70 α-docking protein 
in the heavy but not the-light density microsomes, confirming effective 
separation of smooth and rough ER (41). No p50-F1-β-ATPase or 
p12-cytochrome c was detected in the microsomal fractions, in con-
trast to whole-cell and heavy-membrane preparations, thus demonstrat-
ing that mitochondrial contamination cannot explain the presence 
of p26-Bcl-2 in isolated low-density microsomes (Fig. 6 and data not 
shown).

Immunomicroscopic Evaluation of Bcl-2 Protein Location

The consistent presence of Bcl-2 protein in nuclear fractions 
coupled with the difficulty in routinely removing contaminating mi-
tochondria from nuclear preparations prompted us to evaluate the 
issue microscopically using laser-scanning and electron microscopic 
methods.

Laser-scanning Microscopy. Lymphoma cell lines containing a 
t(14;18) were stained with anti-Bcl-2 antibodies and examined using a 
laser-scanning microscope thus permitting optical tomographic images 
to be obtained at various depths into the cells as described by Itoh 
et al. (47). Both pre- and postembedding methods were used and 
similar results were obtained with all 6 anti-Bcl-2 antibody reagents.

two-step assay using two independent anti-Bcl-2 antibody reagents.

To further characterize the pool of p26-Bcl-2 associated with LM 
fractions, heavy (rough) and light (smooth) microsomal membranes 
were prepared from RS11846 cells using discontinuous sucrose step 
gradients (31). These membranes were then detergent solubilized and 
p26-Bcl-2 was immunoprecipitated and subjected to SDS-PAGE and 
immunoblot analysis. As shown in Fig. 6, p26-Bcl-2 was found in the 
lighter-density microsomal membrane fraction but not in the heavier-
density membranes. Comparisons with the relative amounts of p26-
Bcl-2 extracted from an equal number of whole, intact cells suggested that (~20%) of the total cellular Bcl-2 resided in the smooth ER. 
Immunoblot analysis of the same microsomal membranes after pre-clearing 
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trast to whole-cell and heavy-membrane preparations, thus demonstrat-
ing that mitochondrial contamination cannot explain the presence 
of p26-Bcl-2 in isolated low-density microsomes (Fig. 6 and data not 
shown).

As shown in Fig. 7A, intense immunostaining was clearly evident in 
the nuclear envelope of SU-DHL-4 cells that had been incubated with 
polyclonal antibodies directed against the 41–54 region of Bcl-2. 
Granular staining of the cytosol was also seen. Similar results were
obtained for SU-DHL-4 and other lymphoma cell lines using other anti-Bcl-2 antibodies. Fig. 7D, for example, shows the results obtained when SU-DHL-4 cells were stained using a monoclonal anti-Bcl-2 antibody directed against the 61–76 region of Bcl-2. Interestingly, in some lymphoma cell lines such as RS11846 that contain large mitochondria (confirmed by EM), intense anti-Bcl-2 antibody immunostaining was observed on the surface of cytoplasmic organelles resembling mitochondria, when cross-sectional images were obtained (Fig. 7B). Preabsorption of anti-Bcl-2 antibodies with competing peptide markedly diminished the immunoreactivity in all areas of the cells (Figs. 7, C and E), but residual areas of nuclear reactivity suggested that this represented nonspecific staining. Again, the specificity of these results was confirmed by peptide competition experiments (Fig. 8A). Furthermore, in some cells where the plane of sectioning occurred more tangentially, the immunostaining of the nuclear envelope appeared as two closely spaced concentric circles around the nucleus, suggestive of inner and outer nuclear membranes (Fig. 8B). In contrast, 32D-NEO cells which harbor a control virus lacking human Bcl-2 had been stably transfected with control or human Bcl-2-producing plasmid (Fig. 8A), suggestive of nuclear core complexes. The specificity of this result was confirmed by peptide competition (Fig. 8F).

**Electron Microscopy (EM).** The presence of Bcl-2 in the nuclear envelope of lymphoma cells was also confirmed at the EM level. For these experiments, immunostaining was performed by both preembedding and postembedding methods to minimize risks of artifactual immunolocalization results. Fig. 9 shows some typical results obtained by the preembedding method where anti-Bcl-2 antibody detection was accomplished using HRP/3,3′-diaminobenzidine. At the higher magnification afforded by EM, it was appreciated that the immunostaining of the nuclear envelope occurred in a patchy, nonuniform distribution. Furthermore, the immunoreactivity appeared to directly overlie the nuclear envelope in many cases (Fig. 9, D and E), reminiscent of nuclear pore complexes. In some views, membrane structures resembling tubes were evident in the cytosol of cells, particularly towards the periphery of the cell (see Fig. 9, C and D). In these membranes structures, anti-Bcl-2 immunostaining again occurred in discrete patches and appeared to span the lumen between these membranes (Fig. 9D). In fact, in some cells these membrane structures occurred in parallel sheets of flattened membrane cisternae (Fig. 9D), resembling the annulate lamellae (reviewed in Ref. 50) which are known to contain densely packed nuclear pore complexes (51).

These immunoelectron microscopic data also provided evidence that the Bcl-2 protein is located exclusively in the outer membrane of mitochondria (Fig. 9, A, C, and F), consistent with a previous report (22). The immunoreactivity seen in association with the mitochondrial outer membrane also occurred in a nonuniform distribution (Fig. 9A). Patches of antibody reactivity were also seen in the cytosol (Fig. 9C), perhaps representing the pool of Bcl-2 associated with the ER. In Fig. 9F, for example, a loop of ER that appears to be contiguous with the nuclear envelope can be seen winding into the cytosol. Patches of intense anti-Bcl-2 antibody immunoreactivity were found associated with this membrane structure. Peptide competition, use of control antibodies, and evaluations of the 32D-NEO and 32D-BCL-2 cell lines described above confirmed the specificity of these results. In Fig. 9, A and B, for example, where whole-cell views are provided, comparisons of cells stained with anti-Bcl-2 antibody with and without peptide preabsorption revealed essentially complete abrogation of specific immunoreactivity when competing peptide was included.

When postembedding methods were used in conjunction with immunogold detection of anti-Bcl-2 antibodies, again the majority of the immunoreactivity was associated with the nuclear envelope and outer mitochondrial membranes (Fig. 10). Fig. 10B, for example, shows gold particles localized along the nuclear envelope and the circumference of a mitochondrion in SU-DHL-4 cells. Gold particles were also seen in the cytosol, perhaps reflecting Bcl-2-associated with ER or annulate lamella, but there was little deposition of particles over the nucleus. No clear evidence for association of Bcl-2 with the plasma membrane or with lysosomes or other intracellular organelles was obtained. Fig. 10C shows a high-power image of a mitochondrion, revealing two areas of gold particle deposition over the outer membrane. In several high-power images, the gold particles localized to the nuclear envelope appeared to cluster directly over nuclear membranes (Fig. 10D), suggestive of nuclear core complexes. The specificity of these results was confirmed by peptide competition experiments (Fig. 10A), use of preimmune serum, and evaluations of 32D-NEO and 32D-BCL-2 cells, as well as in preliminary experiments involving cryo-EM methods (not shown).

**Studies of in Vitro Association of Bcl-2 Protein with Isolated Mitochondria**

To further investigate the association of Bcl-2 protein with mitochondria, we performed experiments in which the in vitro-translated Bcl-2 product was added to intact, isolated mitochondria under conditions where typical mitochondrial proteins will undergo import into this organelle (45). As shown in Fig. 11A, when 35S-labeled p26-Bcl-2 protein (referred to as Bcl-2x) was added to mitochondria, most of the protein became associated with mitochondria as determined by its cosedimentation with these organelles upon centrifugation (compare Lanes 2 and 4). Essentially all of the mitochondria-associated Bcl-2 protein, however, appeared to remain on the surface of the organelle,
since treatment with trypsin completely digested the protein to smaller fragments (Lane 5).

To explore the molecular basis for the association of the Bcl-2α protein with mitochondria, experiments were also performed using the 35S-labeled Bcl-2β in vitro translation product. The Bcl-2β protein can potentially arise through an alternative splicing mechanism (3) and, unlike Bcl-2α, lacks a hydrophobic stretch of amino acids for membrane insertion. As shown in Fig. 11A (Lanes 6–10), the Bcl-2β protein remained largely in the supernatant when mitochondria were pelleted by centrifugation (compare Lanes 7 and 9) and was completely sensitive to digestion by trypsin (Lanes 8 and 10). Thus, the presence of a transmembrane domain presumably is required for association of Bcl-2 proteins with mitochondria.

Fig. 11B shows the import data derived with a classical intramitochondrial protein, the Rieske Fe/S subunit of IUCR, which resides in the inner mitochondrial membrane (52). Like Bcl-2α, when the 35S-labeled, in vitro-translated IUCR precursor protein was added to mitochondria, much of the protein associated with mitochondria based on its cosedimentation with these organelles upon centrifugation (Lane 3). Unlike Bcl-2α, however, the IUCR underwent physiological proteolytic cleavage to a mature 22-kDa polypeptide concomitant with its translocation into a trypsin-resistant intramitochondrial compartment (compare Lanes 1, 3, and 4). Residual p25-IUCR precursor protein that remained in the supernatant or on the surface of the mitochondria was digested with trypsin (compare Lane 1 with Lane 2 and Lane 3 with Lane 4), proving that the protease was indeed active...
Fig. 8. Laser-scanning microscopic analysis of 32D cells. Thin sections of formalin-fixed, paraffin-embedded murine 32D cells were immunostained using a polyclonal antiserum 9716-10 directed against the 41-54 region of the human Bcl-2 protein and examined by laser-scanning confocal microscopy. A, 32D-BCL-2 cells at x 4000; B, the same cell as seen in upper left of A, at x 7000 original magnification; C, 32D-NEO cells at x 5800; D and E, 32D-BCL-2 cells at x 5800; and F, 32D-BCL-2 cells where competing 41–54 peptide was included x 5800. In C and F, contrast settings were increased above the other images so that the cells would be visible. In B, the double leaflet of nuclear envelope was reminiscent of tangential sections seen by EM where a large distance can be seen between membranes that are normally closely apposed.

during these experiments. The behavior of the Bcl-2α protein therefore clearly differs from a classical inner membrane protein in this in vitro import assay and thus corroborates the immunoelectron microscopic data where Bcl-2 protein was found exclusively in association with the outer mitochondrial membrane (Figs. 9 and 10).

DISCUSSION

The Bcl-2 oncoprotein is noteworthy for its ability to block or interfere with apoptosis induced by a wide variety of agents ranging from calcium ionophores and glucocorticosteroids to chemotherapeutic drugs and γ-irradiation (7–9, 12, 13). This finding has suggested that p26-Bcl-2 regulates some undefined step in a final common pathway that controls the initiation of the programmed cell death process. Given the lack of sequence homology between Bcl-2 and other reported cellular proteins, and thus the dearth of clues as to a possible biochemical activity for this protein, resolving the issue of the subcellular location of Bcl-2 could be important for deciphering the mechanism of action of this unique oncoprotein.

Using subcellular fractionation techniques, we obtained evidence that p26-Bcl-2 resides in both mitochondrial and nonmitochondrial pools within a human B-cell lymphoma cell line that contains a t(14;18) translocation involving the bcl-2 gene. When the heavy-membrane fraction was prepared from RS11846 cells and subjected to centrifugation in various density gradient media, p26-Bcl-2 invariably co-sedimented with mitochondrial proteins but not with markers of other organelles. The proportion of Bcl-2 protein associated with mitochondria in RS11846 cells was typically 30–50% of the total cellular Bcl-2 protein (see, e.g., Fig. 6). These subcellular fractionation data regarding association of Bcl-2 with mitochondria were corroborated by the results obtained when Bcl-2 was immunolocalized using laser-scanning and electron microscopy (Figs. 7–10). The most convincing among these microscopic methods were the EM data, showing anti-Bcl-2 immunoreactivity with the outer mitochondrial membrane in a patchy nonuniform distribution (Figs. 9 and 10). The localization of Bcl-2 to the outer mitochondrial membrane was further substantiated through protein import studies using isolated intact mitochondria (Fig. 11). Thus, in contrast to a previous report (6), we were unable to find any clear evidence that Bcl-2 resides in the inner mitochondrial membrane.

In contrast to a previous report where Bcl-2 was said to be associated exclusively with mitochondria based on subcellular fraction analysis (6), we found that about 15–25% of Bcl-2 protein resided in a light-membrane compartment in RS11846 cells. Although it has been reported that damaged mitochondria that lose their outer membrane can sediment at lower densities in gradients (29), it is unlikely that this artifact explains our observations since we utilized the inner mitochondrial membrane protein F₁β-ATPase as a marker for most
Fig. 9. Immunoelectron microscopic analysis of Bcl-2 in lymphoma cells by a preembedding method. RS11846 lymphoma cells were fixed in 2% glutaraldehyde and immunostained in suspension using a polyclonal antiserum specific for the 41-54 region of Bcl-2. Antibodies were detected by a HRP/3,3'-diaminobenzidine method prior to embedding in plastic and preparation of ultrathin sections for EM. A, and B, lymphoma cell at × 10,000 immunostained without or with preadsorption of the antibody with 41-54 competing peptide, respectively. C–F, higher magnification photographs of cells prepared as in A, at × 17,000, × 20,000, × 25,000, and × 25,000 original magnification, respectively. Counterstaining was with 1% osmium tetroxide. In C, the border between nucleus (top) and cytosol (bottom) is shown with the nuclear envelope oriented horizontally. Several mitochondria can be seen in cross-sectional image in the cytosol. A tubular membrane structure is present in the lower right corner of the cell, between a mitochondrion and the plasma membrane. d, a higher magnification photograph of the same cell shown in c. Arrows pointing to electron-dense immune complex/3,3'-diaminobenzidine deposits in the nuclear envelope (NE) are found in...
subcellular distribution of Bcl-2 oncprotein

Fig. 10. Immunoelectron microscopic analysis of Bcl-2 in lymphoma cells by a postembedding method. Lymphoma cells were fixed in 2% glutaraldehyde, counterstained with 1% reduced osmium tetroxide for 2 h, prestained with uranyl acetate, and embedded in resin. Ultrathin sections were then immunostained using anti-Bcl-2 antisera. Antibody detection was accomplished by an immunogold method. a, SU-DHL-4 cells where the 9716-10 antibody (directed against amino acids 41–54 of Bcl-2) was preadsorbed with 41–54 competing peptide (original magnification, × 10,000); b, SU-DHL-4 cells stained with 9716-10 without competing peptide (× 25,000); c, RS11846 cells stained with antisera 1633-8 (directed against amino acids 61–76 of Bcl-2) at × 80,000; d, RS11846 cells prepared as in C at × 100,000; inset, × 160,000 original magnification. In B, the border between nucleus (N) and cytosol (C) is shown, with the nuclear envelope oriented diagonally from the upper left to lower right corners. A mitochondrion (m) is indicated in the lower middle portion. Gold particles overlie both the inner (bold arrows) and outer (fine arrows) membranes of the nuclear envelope and outer mitochondrial membrane (arrowheads) and are scattered through the cytosol but not the nucleus. In C, a mitochondrion is shown, with its longest axis oriented vertically. Two clusters of gold particles can be seen overlying the outer mitochondrial membrane (arrows). In D, the border between nucleus (N) and cytosol (C) is shown, with the nuclear envelope oriented diagonally from lower left to upper right corners. A cluster of gold particles is seen overlying the nuclear envelope (arrow). Inset, gold particles clustered directly over the nuclear envelope, a higher magnification photograph taken from elsewhere in the same cell.

Studies. F1-β-ATPase is an integral membrane protein that we would not expect to be lost from mitochondria even under circumstances where the outer membrane is destroyed (37). Also, since we utilized immunoblotting rather than enzymatic assays to track mitochondrial proteins, it cannot be argued that we failed to detect mitochondrial markers in the low-density fractions because of loss of enzymatic activity. Conversely, although ER membranes can be found in non-specific association with mitochondria (53), it seems unlikely that this explains the association of p26-Bcl-2 with mitochondria, based on the results of immuno-EM studies (Fig. 9) (22). Furthermore, we recently reported the results of studies involving use of 2-color immunofluorescence confocal microscopy, where co-immunolocalization of Bcl-2 and ER marker proteins was seen in a cytosolic reticular pattern characteristic for ER in a human fibroblast cell line that contained high levels of Bcl-2 protein because of gene transfer manipulations (21).

Based on fractionation of LM preparations in discontinuous sucrose gradients, Bcl-2 appears to reside in lighter density microsomal membranes. This lighter-density compartment is most consistent with the smooth ER but further studies are needed to confirm this observation. It is unlikely that the pool of Bcl-2 protein found in LM fractions resides in the plasma membrane or in association with endocytic
vessicles based on the absence of migration of this fraction of Bcl-2 with IgG and M6PR in density gradients (Fig. 4). Furthermore, in the immuno-EM photographs showing patches of anti-Bcl-2 antibody immunoreactivity in the cytosol of cells, Bcl-2 appeared to be associated with membrane structures resembling ER and annulate lamellae but was not found in the plasma membrane, Golgi, or other organelles that typically co-sediment with lighter-density microsomes. The ultrastructural details of our EM findings, however, were not sufficient to directly distinguish rough from smooth ER, and attempts to attain better counterstaining of membranes in cells with heavy-metals compromised our immunodetection methods.

Previously, Chen-Levy et al. (14) reported that a significant fraction of the p26-Bcl-2 is associated with nuclei, and we and others have observed the same when Bcl-2 is produced in Sf9 cells using recombinant baculoviruses (18). The subcellular fractionation data reported here for RS11846 lymphoma cells also suggested that a significant proportion of Bcl-2 protein is associated with nuclei. Given the continuity of the ER and outer nuclear membrane and the frequent difficulty in removing contaminating mitochondria from nuclear preparations (possibly because of their large size in some cells), it is difficult to estimate the proportion of total cellular Bcl-2 protein that resides in this compartment. Our immunocytochemical and laser-scanning microscopy data, however, give the impression that a relatively large proportion of the Bcl-2 protein resides in the nuclear envelope, at least in t(14;18)-containing lymphoma cell lines and 32D cells that express a human bcl-2 complementary DNA (Figs. 7 and 8). Indeed, on occasions when nuclei were obtained relatively free of contaminating mitochondria, up to 50% of p26-Bcl-2 was associated with this compartment (Fig. 6, e.g.). Regardless of the relative proportion of Bcl-2 protein that truly resides in the nuclear envelope, the immuno-EM data derived from cells stained with anti-Bcl-2 antibodies revealed that Bcl-2 is also distributed nonuniformly in the nuclear envelope. Furthermore, in the highest magnification photographs (Figs. 9 and 10), anti-Bcl-2 antibody immunoreactivity appeared to span the space between the inner and outer nuclear membranes, reminiscent of nuclear pore complexes. Given that this nonuniform immunoreactivity was seen in the nuclear envelope and other biological membranes with using 6 different anti-Bcl-2 antibodies and both pre- and postembedding methods it seems highly unlikely that the results can be attributed to artifact.

The findings presented here have important implications for the possible mechanism of action of the Bcl-2 oncoprotein. The previous report of Bcl-2 in the mitochondrial inner membrane (6), for example, raised the possibility that Bcl-2 might regulate cell survival by influencing oxidative phosphorylation and thus controlling cellular energy stores. With evidence that approximately one-half of the Bcl-2 protein resides in nonmitochondrial compartments, however, a role for Bcl-2 in regulating oxidative phosphorylation now seems unlikely. Although it is theoretically possible that the p26-Bcl-2 associated with the ER and nuclear envelope represents a transient pool of newly synthesized Bcl-2 molecules destined for eventual transport to mitochondria, it is without precedent for such a large relative amount of a mitochondrial protein to accumulate in the ER. Furthermore, we have recently obtained evidence that Bcl-2 can protect cells from apoptosis even if they contain no mitochondrial DNA and thus lack some of the electron chain transporters required to execute aerobic glycolysis (21).

The immuno-EM data suggesting that Bcl-2 protein is nonuniformly distributed in membranes are particularly intriguing. This observation raises the possibility that p26-Bcl-2 is targeted to specialized regions in membranes. Since the Bcl-2 protein lacks any obvious organelle-specific targeting sequences, one possibility is that Bcl-2 enters into large protein complexes in these intracellular membranes and is held there through protein-protein interactions. Obvious candidates for such proteins are those associated with the nuclear pore complex and the junctional complexes in mitochondria, both of which are involved in protein transport across membranes (54, 55). Although dual labeling studies will be required to determine whether Bcl-2 actually resides in nuclear pore complexes or mitochondrial junctional complexes, one observation at least consistent with the former was our finding of Bcl-2 in association with membrane structures resembling AL. AL are most commonly observed in the cytoplasm of rapidly dividing cells and appear as flattened membrane cisternae organized

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6 Unpublished observations.
either singly or in stacks of parallel sheets (reviewed in Ref. 50). Typically, AL are located towards the periphery of cells and contain densely spaced nuclear pore complexes. It has been speculated that AL serve as a reservoir of nuclear envelope precursor material that can be mobilized quickly in tumors and other rapidly dividing cells to ensure that their postmitotic chromosomes become enclosed by a nuclear envelope containing functional pore complexes (50, 51).

Although we can only speculate on a function for Bcl-2 based on the data presented here, one potential explanation lies with the Ca2+ mobilized quickly in tumors and other rapidly dividing cells to ensure envelope containing functional pore complexes (50, 51).


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Investigation of the Subcellular Distribution of the \textit{bcl-2} Oncoprotein: Residence in the Nuclear Envelope, Endoplasmic Reticulum, and Outer Mitochondrial Membranes

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