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# Selective localization of Bcl-2 to the inner mitochondrial and smooth endoplasmic reticulum membranes in mammalian cells

T Gotow[1,5], M Shibata[2,5], S Kanamori[2,5], O Tokuno[2], Y Ohsawa[2], N Sato[2], K Isahara[2], Y Yayoi[2], T Watanabe[2], JF Leterrier[3], M Linden[3], E Kominami[4] and Y Uchiyama*[,2]

[1] Laboratory of Cell Biology, College of Nutrition, Koshien University, Hyogo, Japan
[2] Department of Cell Biology and Neuroscience, Osaka University Graduate School of Medicine, Osaka, Japan
[3] UMR CNRS 6558 Laboratoire des Biomembranes et Signalisation Cellulaire, Groupe de Biologie des Interactions Cellulaires, Bat. de Biologie, Poitiers, France
[4] Department of Biochemistry, Juntendo University School of Medicine, Tokyo, Japan
[5] First three authors contributed equally to this work
* Corresponding author: Yasuo Uchiyama, Department of Cell Biology and Neuroscience, Osaka University Graduate School of Medicine (A1), 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel: +81-6-6879-3120; Fax: +81-6-6879-3129; E-mail: uchiyama@anat1.med.osaka-u.ac.jp

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## Abstract

**Bcl-2, an anti-apoptotic protein, is believed to be localized in the outer mitochondrial membrane, endoplasmic reticulum, and nuclear envelope. However, Bcl-2 has also been suggested as playing a role in the maintenance of mitochondrial membrane potential, indicating its possible association with the inner mitochondrial membrane. We therefore further examined the exact localization of Bcl-2 in mitochondria purified from wild-type and *bcl-2*-transfected PC12 cells and pre- and postnatal rat brains. Double immunostaining demonstrated that Bcl-2 was co-localized with subunit $\beta$ of $F_1F_0$ATPase in the inner mitochondrial membrane. Biochemical analysis of isolated mitochondria using digitonin and trypsin suggests an association of Bcl-2 with the inner mitochondrial membrane. More interestingly, the majority of Bcl-2 disappeared from the inner membrane of mitochondria when cultured under serum deprivation. These results suggest that Bcl-2 acts as an anti-apoptotic regulator by localizing mainly to the inner mitochondrial and smooth ER membranes.** *Cell Death and Differentiation* (2000) **7,** 666–674.

**Keywords:** apoptosis; Bcl-2; mitochondria; smooth endoplasmic reticulum; PC12 cells; rat brains

**Abbreviations:** ER, endoplasmic reticulum; wt, wild-type

## Introduction

The protooncogene *bcl-2* was discovered as a result of its involvement in the t(14;18) chromosomal breakpoint in human follicular B-cell lymphoma.[1–3] Bcl-2, a mammalian homologue of CED-9 in *Caenorhabditis elegans*, prevents cell death following the withdrawal of growth factor or up-regulation of p53,[4] whereas its prevention of cell death triggered by apoptotic signals originating from Fas is dependent on cell types.[5] The Bcl-2 protein possesses a hydrophobic transmembrane domain located near its carboxy-terminal end, and is considered to be associated with intracellular membrane components with concern to its own functions related to the cell death program.[6–8] Indeed, it has been suggested that Bcl-2 is associated with the outer mitochondrial membrane,[9] the endoplasmic reticulum (ER)[10] and the nuclear envelope[11] on the basis of biochemical analyses, which are consistent with immunoelectron microscopic results.[12,13] Moreover, it has also been proposed that the Bcl-2 family of proteins bind to porin, a voltage-dependent anion channel in order to regulate the mitochondrial membrane potential and the release of cytochrome c during apoptosis.[14] Since Bcl-2 interacts with its related proteins, Raf-1, BAG-1, P53 binding protein and calcineurin,[11,15–19] it is considered to be a multifunctional protein.[20]

However, it has also been suggested that Bcl-2-like proteins function to retain cytochrome c in the mitochondria.[21,22] In fact, Bcl-2 has been shown to be localized predominantly in the inner mitochondrial membrane by the cryo-thin section immunogold method,[23] while Bcl-$x_L$, a Bcl-2 family protein, has been demonstrated to bind specifically to cytochrome c and is suggested to be localized within mitochondria.[24] Moreover, biochemical analysis has also shown that Bcl-2 is localized in the inner mitochondrial membrane,[25] and that it inhibits cell death induced by chemical hypoxia with respiratory chain inhibitors through the maintenance of the mitochondrial membrane potential.[26,27] Taken together, it still remains controversial as to which membrane site, inner or outer membrane, of the mitochondria, Bcl-2 is localized in.

The present study examined the localization of Bcl-2 in wild-type (wt) and human *bcl-2* gene-transfected PC12 cells, and nerve cells of young and adult rat brains. Our data attained through both immunocytochemistry using the cryo-thin section immunogold method and biochemical approaches using purified mitochondria demonstrated preferential localization of Bcl-2 in the inner, but not outer mitochondrial membrane.

## Results

### Bcl-2 is mainly localized to the inner mitochondrial and smooth ER membranes in PC12 cells and neurons of rat brains

Laser scanning microscopic observations confirmed that Bcl-2 was associated with mitochondria when double immunostaining of subunit $\beta$ of mitochondrial $F_1F_0$ATPase (subunit $\beta$) and Bcl-2 was performed in wt or human *bcl-2* gene transfected PC12 cells. Immunodeposits for Bcl-2 were diffuse or in some parts fine-granular in the cytoplasm of *bcl-2*-transfected PC12 cells, while granular staining of subunit $\beta$ was co-localized well with immunoreactivity for Bcl-2 (data not shown). By the cryo-thin section immunogold method, Bcl-2 labeling was shown to be localized mostly in the inner mitochondrial membrane of wt and *bcl-2*-transfected PC12 cells but rarely in the outer membrane (Figure 1A), although the labeling was much less dense in the wt cells (Figure 1B). Immunogold labeling of Bcl-2 was intensely associated with the smooth ER (sER) in these cells but not with the rough ER (rER) and nuclear envelope (Figure 1). We also examined the localization of Bcl-2 in nerve cells of embryonic, postnatal and adult rat brains; immunogold particles showing Bcl-2 were associated with the inner but not outer mitochondrial membrane and sER/vesicular structures even in the adult brain (Figure 1C). This localization pattern of Bcl-2 in neuronal cells did not change depending on the antibodies used, although labeling densities differed. Approximately 30 and 40% of total particles were detected in the inner mitochondrial membrane of wt and transfected PC12 cells, respectively, while the labeling percentage in sER/vesicular structures was around 40% in both cells (Figure 2). Immunogold particles appearing in the rER, nuclear envelope and outer mitochondrial membrane amounted to less than 5%. Immunohisto/cytochemical studies suggest that Bcl-2 is mainly localized in the inner mitochondrial and sER/vesicular membranes of the cells.

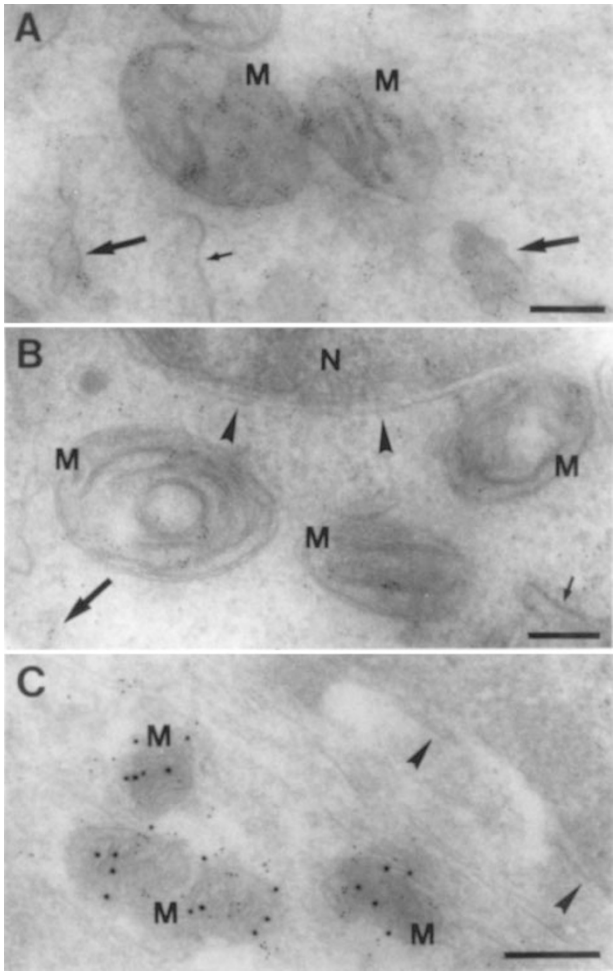


**Figure 1** Immunoelectron micrographs of Bcl-2 in PC12 cells and a nerve cell of the rat cerebral cortex. Cryo-thin sections were immunolabeled with monoclonal antibodies specific for human (**A**) and rat (**B** and **C**) Bcl-2. (**A**), immunogold particles (5 nm in diameter) showing the presence of Bcl-2 are densely detected in the inner membrane of mitochondria (M) and sER/vesicular membranes (thick arrows) in a human bcl-2-transfected PC12 cell, but not in rER (thin arrows). (**B**), the particles in a wt PC12 cell are much less dense but detectable in the inner membrane of mitochondria (M) and sER, but not in the rER and nuclear envelope. (**C**), double immunostaining of Bcl-2 (5 nm in diameter) and subunit $\beta$ (15 nm in diameter) in a nerve cell of the rat cerebral cortex obtained at postnatal day 5. Small immunogold particles indicating Bcl-2 are co-localized with large particles showing subunit $\beta$ in the inner mitochondrial membrane. Small particles are also seen in the sER/vesicular structures, but not in the nuclear envelope (arrowheads). N, nucleus. Scale bars, 0.2 $\mu$m

### Bcl-2 in purified mitochondria is not solubilized by the treatment with digitonin

To verify the localization of Bcl-2 in the inner mitochondrial membrane, we then treated purified mitochondria from wt PC12 cells, and from those expressing Bcl-2 or FLAG-tagged Bcl-2 using digitonin to disrupt the outer mitochondrial membrane. After treatment with 0.5 mg digitonin/mg mitochondrial protein, only a small portion of porin, but neither Bcl-2 nor subunit $\beta$ were detectable in the outer membrane fraction (Figure 3A–C). When treated with 1 mg digitonin, over 40% of the total porin protein was shifted to the outer membrane fraction in each cell with a small amount being moved to the intermembrane fraction, whereas subunit $\beta$ and Bcl-2 were detected only in the inner membrane fraction (Figure 3A–C). Digitonin treatment was also applied to the purified mitochondria from rat brains at postnatal day 5 and adult stage, and the results were similar to those obtained from PC12 cells (Figure 3D).

Using mitochondria treated with digitonin, we examined the localization of Bcl-2 by the cryo-thin section immunogold method. As shown in Figures 4A,B, immunogold particles indicating Bcl-2 were associated with the inner membrane of non-treated mitochondria or intact mitoplasts even after treatment with 1 mg digitonin, while those showing porin were deposited in the outer most membrane of the mitoplasts where debris of the outer membrane were partly seen (Figure 4C). It is therefore likely that Bcl-2 is preferentially localized in the inner membrane of mitochondria in PC12 cells and the neurons of rat brains, even in purified mitochondria.

### Bcl-2 in purified mitochondria is resistant to the digestion by trypsin

We further examined the association of Bcl-2 with the inner mitochondrial membrane by digesting purified mitochondria

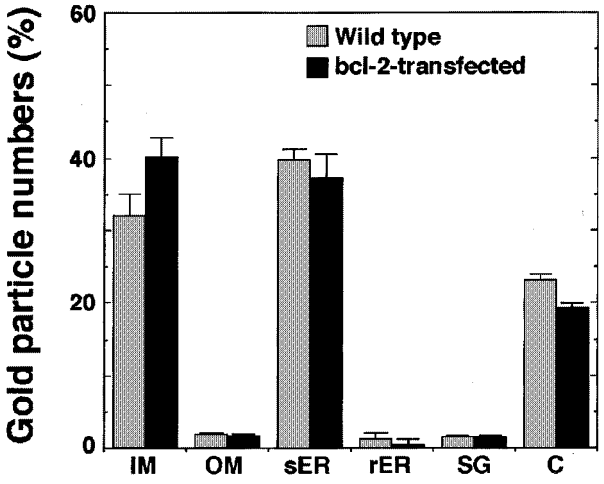


**Figure 2** Histogram of ratios indicating the association of immunogold particles for human and rat Bcl-2 with various organelles in wt and *bcl-2*-transfected cells. Gold particles are most frequently associated with the inner mitochondrial membrane (IM), then with ER and vesicles (sER). The sER counted had tubular, ellipsoid and irregular-shaped profiles. C indicates cytosol, OM outer mitochondrial membrane, rER rough endoplasmic reticulum and SG secretory vesicles

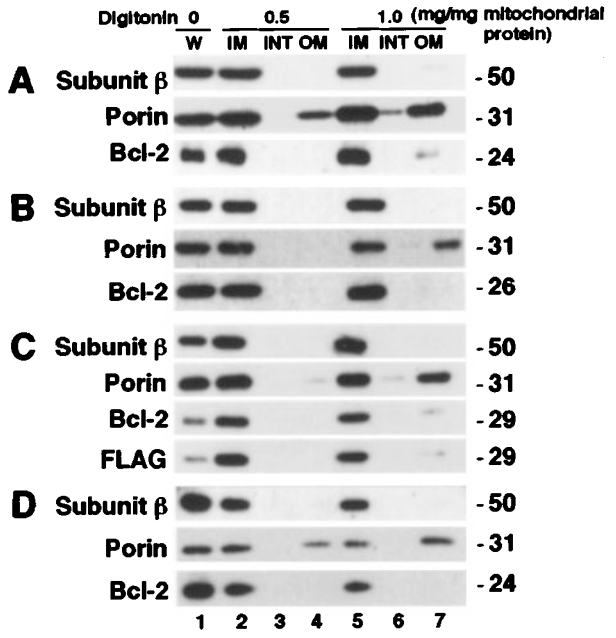


**Figure 3** Solubility of subunit $\beta$, porin, and Bcl-2 by treatment with digitonin from the purified mitochondria obtained from wt (**A**), *bcl-2*- (**B**) and *bcl-2*-FLAG- (C) transfected PC12 cells, and brain tissue at postnatal day 5 (**D**). After treatment with digitonin at concentrations of 0 (W, lane 1), 0.5 (lanes 2–4), and 1.0 (lanes 5–7) mg/mg mitochondrial protein, the samples were fractionated into the inner membrane (IM, lanes 2 and 5), intermembrane (INT, lanes 3 and 6) and outer membrane (OM, lanes 4 and 7) fractions. After treatment with 0.5 mg digitonin porin is solubilized into the outer membrane fraction, especially in samples from wt PC12 cells and postnatal day 5 brain tissue (**A** and **D**). Porin is highly solubilized after treatment with 1.0 mg digitonin in each sample, whereas Bcl-2 and subunit $\beta$ are mostly detected in the inner membrane fraction and only insignificant amounts of them are detectable in the outer membrane fraction (**A**–**D**). Note that the solubility of Bcl-2-FLAG after treatment with 0.5 or 1.0 mg digitonin is mostly detected in the inner mitochondrial fraction, even when detected by anti-FLAG (**C**). Molecular weights of each protein are indicated at the right side

with trypsin. As a marker protein of the outer mitochondrial membrane, we used a mitochondrial import receptor protein, Tom20.[28] When purified mitochondria from wt PC12 cells and adult rat brain tissue were treated with increasing concentrations of trypsin, Bcl-2 and subunit $\beta$ were resistant to the digestion up to a concentration of 250 $\mu$g/ml, whereas Tom20 completely disappeared at a concentration of 100 $\mu$g/ml (Figure 5). The results again suggest the association of Bcl-2 with the inner mitochondrial membrane.

## Fate of Bcl-2 in wt and *bcl-2*-transfected PC12 cells when cultured under serum deprivation

To investigate the behavior of Bcl-2 in the apoptotic process, we examined wt and *bcl-2*-transfected PC12 cells, cultured under serum deprivation, by immunocytochemistry. Using confocal laser microscopy, as stated above, immunoreactivity for Bcl-2 was found to co-localize well with that for subunit $\beta$ in wt PC12 cells before the beginning of culturing under serum deprivation (Figure 6A–C). When examined 24 h after the beginning of serum-free cultures, immunolabeling of Bcl-2 became largely different from that of subunit $\beta$ in the same cells (Figure 6D–F). In *bcl-2*-transfected PC12 cells, co-staining of Bcl-2 and subunit $\beta$ was distinctly detected before and 24 h after the start of serum-free cultures, but mitochondrial localization of Bcl-2 became weak after apoptotic stimulation (Figure 6G–I).

When wt PC12 cells obtained before serum-deprived culturing were applied to immunoelectron microscopy, the immunolabeling of Bcl-2 was weak but well co-localized with that of subunit $\beta$ in the inner mitochondrial membrane (Figure 7A). Immunogold particles detected in the inner mitochondrial membrane became much smaller or rarer in wt PC12 cells obtained 24 h after the beginning of serum-free cultures, while the particles were intensely deposited in ER membranes (Figure 7B).

To confirm the behavior of Bcl-2 during apoptosis, we further performed subcellular fractionation of postnuclear supernatants (PNS), using a 15% Percoll gradient. For this we used *bcl-2*-transfected PC12 cells before and 24 h after the beginning of serum-deprived cultures, since it was hard to obtain and fractionate PNS from wt PC12 cells 24 h after the start of serum-deprived cultures. When intact cells were examined, a mitochondrial marker protein, subunit $\beta$, was detected in relatively heavy fractions, where Bcl-2 was recovered, although Bcl-2 also appeared in the membrane fractions corresponding to ER/vesicular fractions (Figure 8A). In the cells obtained 24 h after the beginning of serum-free cultures, the distribution of subunit $\beta$ was similar to that in the intact cells, whereas that of Bcl-2 markedly decreased in the mitochondrial fractions, compared to that in the membrane fractions (Figure 8B). In this situation, however, Bcl-2 still remained in the mitochondrial fractions (Figure 8B), and its localization was confirmed in the inner membrane of the mitochondria when examined by immunocytochemistry (Figure 8C).

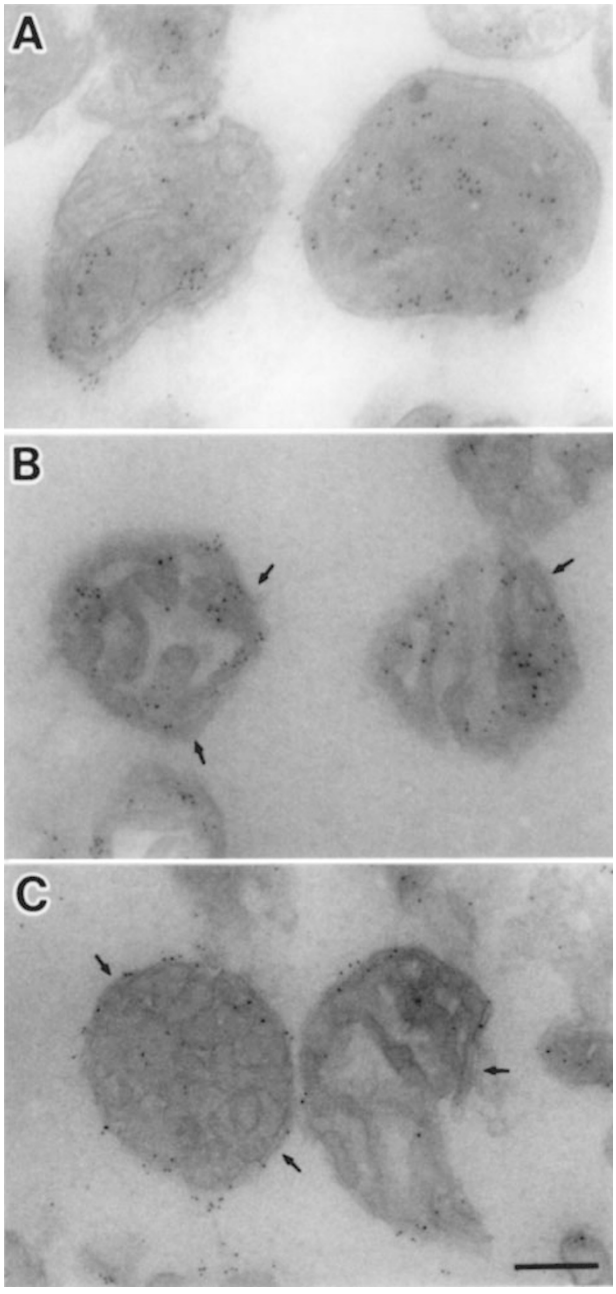


**Figure 4** Immunostaining of Bcl-2 in non-treated mitochondria (**A**), and Bcl-2 (**B**) and porin (**C**) in digitonin-treated mitochondria. The cryothin-section immunogold method was applied to purified mitochondria which were obtained from adult rat brains, and *bcl-2*-transfected PC12 cells, and PC12 cells treated with digitonin at a concentration of 1 mg/mg mitochondrial protein. (**A**), immunolabeling of Bcl-2 is distinctly localized to the inner mitochondrial membrane. (**B**) and (**C**), immunogold particles indicating porin are localized to the inner membrane located in the outer most part of the treated mitochondria, whereas those showing Bcl-2 are distinctly associated with the membrane located in the inner part. In some parts of the mitochondria, fragments of the outer membrane can be seen (arrows). Scale bar, 0.2 $\mu$m

## Discussion

The main purposes of the present study were to accurately define the intracellular localization of Bcl-2. The present

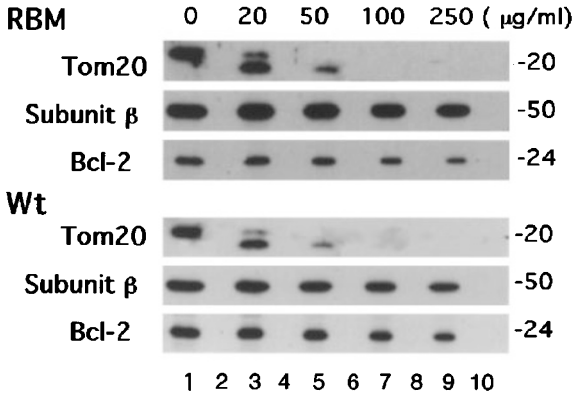


**Figure 5** Digestion of purified mitochondria from rat brain tissue at postnatal day 5 (RBM) and wt PC12 cells (Wt) with trypsin. Immunoblotting of subunit $\beta$, Bcl-2 and Tom20 in pellets (lanes 1, 3, 5, 7 and 9) and supernatants (lanes 2, 4, 6, 7 and 10) of purified mitochondria after treatment with trypsin at concentrations of 0, 20, 50, 100 and 250 $\mu$g/ml for 20 min on ice. In both samples, Tom20 is completely digested by 100 $\mu$g/ml trypsin, whereas Bcl-2 and subunit $\beta$ are resistant to trypsin even at concentrations of 250 $\mu$g/ml

immunocytochemical as well as biochemical studies demonstrated that Bcl-2 was preferentially associated with the inner mitochondrial and ER/vesicular membrane compartments. These results differ strongly from a recent consensus on the subcellular localization of Bcl-2 with mitochondria, which was considered to be solely associated with the cytoplasmic face of the outer mitochondrial membrane.[8,12,29–31]

### Association of Bcl-2 with ER

It has been suggested that Bcl-2 is associated with ER,[1,5,32] and more specifically with sER.[12] The association of Bcl-2 with sER was demonstrated in this study by cryo-thin section gold labeling in wt and *bcl-2*-transfected PC12 cells and neurons of rat brains. Judging from our data that Bcl-2 is almost unrelated to the rER and nuclear envelope in intact cells, Bcl-2 is considered to be specifically associated with smooth membrane compartments but not with the ribosome-attached membranes of the ER system. The association of Bcl-2 with the nuclear envelope has been proposed by many authors.[1,23,31–33] Since the nuclear envelope is studded with ribosomes, Bcl-2 may not be associated with the nuclear envelope.

The association of Bcl-2 with the ER should be emphasized as much as that with the mitochondria, because Bcl-2 is significantly associated with vesicular profiles including the ER system. The presence of Bcl-2 in sER membranes suggests the essential role of this localization in regulating cell death processes. Indeed, it has been shown in mouse lymphoma cells that overexpression of Bcl-2 reduces an efflux of $Ca^{2+}$ from ER lumen into the cytosol, thereby interfering with $Ca^{2+}$ signaling and preventing apoptosis.[10]

### Association of Bcl-2 with the inner mitochondrial membrane compartment

Hockenbery *et al*.[25] first reported in their elegant study that Bcl-2 is localized to the inner mitochondrial membrane. They

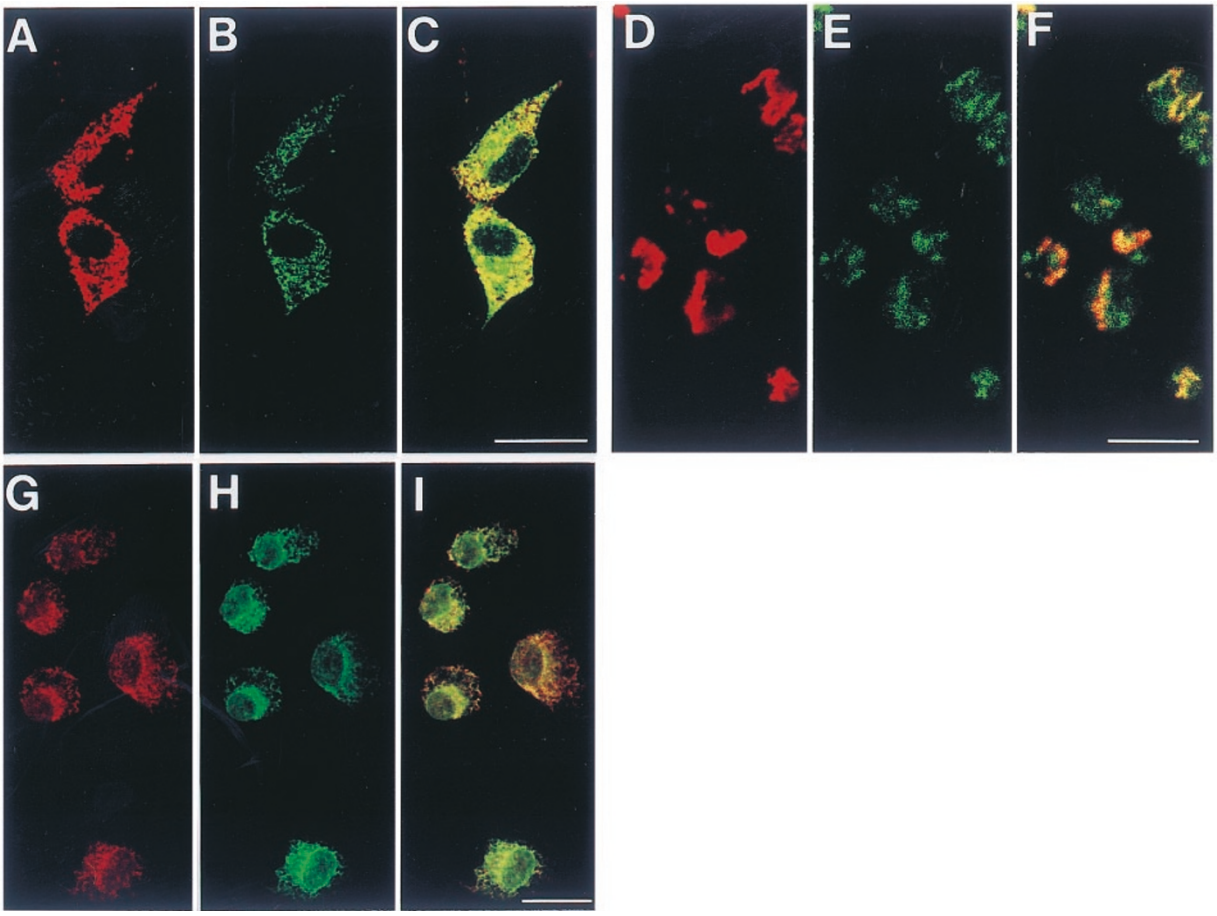


**Figure 6** Double immunostaining of Bcl-2 (FITC-labeled) and subunit $\beta$ (Texas red-labeled) in wt (**A**–**F**) and *bcl-2*-transfected (**G**–**I**) PC12 cells before (**A**–**C**) and 24 h after (**D**–**I**) the start of cultures under serum deprivation. Laser scanning micrographs show that immunolabeling of Bcl-2 (**B**) is weak but clearly co-localized with subunit $\beta$ (**A**) in intact cells (**C**, overlay), whereas the localization of Bcl-2 immunoreactivity (**E**) is mostly different from that of subunit $\beta$ immunoreactivity (**D**) in the cells following serum deprivation (**F**, overlay). In bcl-2-transfected PC12 cells, immunolabeling of Bcl-2 (**H**) is co-localized with that of subunit $\beta$ (**G**), to some extent (**I**, overlay). Scale bars, 20 $\mu$m

separated inner and outer mitochondrial membranes with hypotonic treatment, and demonstrated by Western blotting that Bcl-2 immunoreactivity was associated specifically with the inner membrane fraction but not with the outer membrane.[25] However, further biochemical and/or immunocytochemical studies of the mitochondrial localization of Bcl-2 showed opposite data, concluding the predominant association of Bcl-2 with the outer mitochondrial membrane.[10,12,29–31] In contrast, Akao *et al*.[1] suggested the association of Bcl-2 with both the inner and outer mitochondrial membranes, although the DAB staining for the inner membrane was somewhat difficult to recognize.

Irrespective of previous biochemical and immunocytochemical data showing the localization of Bcl-2 to the outer mitochondrial membrane, the present results raised strong evidence for the hypothesis of a preferential localization of Bcl-2 in the inner mitochondrial membrane. Such an assumption is based on the following independent experimental results: (1) Quantitative as well as qualitative analyses of immunogold labels in wt and *bcl-2*-transfected PC12 cells and rat brain neurons showed the clear-cut association of Bcl-2 with the inner mitochondrial membrane. (2) Subfractionation of cell homogenates revealed the co-distribution of Bcl-2 with a mitochondrial marker and likely ER membranes. (3) The selective disruption of the outer membrane of purified mitochondria from PC12 cells and rat brain tissues with increasing concentrations of digitonin resulted in almost no release of Bcl-2 into the outer membrane fraction. In these digitonin-treated mitochondria, Bcl-2 was confirmed by immunoelectron microscopy to be localized in the inner membrane. (4) The digestion of purified mitochondria from wt PC12 cells and rat brain tissues with increasing concentrations of trypsin demonstrated the resistance of Bcl-2 as well as subunit $\beta$ of $F_1F_0$ATPase.

To confirm our immunoelectron microscopic findings concerning the localization of Bcl-2 in intact cells, we used various types of polyclonal and monoclonal antibodies against human, rat and mouse Bcl-2. Although labeling densities of immunogold particles indicating Bcl-2 differed from each antibody, they were clearly localized mainly to the inner mitochondrial membrane. The association of Bcl-2 in the inner mitochondrial membrane was also confirmed in PC12 cells expressing FLAG-tagged Bcl-2 by using both anti-FLAG (data not shown) and anti-Bcl-2. Moreover, we

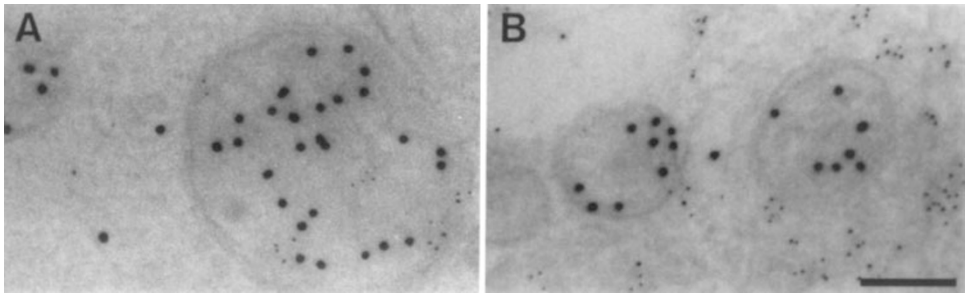


**Figure 7** Immunoelectron micrographs of Bcl-2 (small immunogold particles: 5 nm in diameter) and subunit $\beta$ (large immunogold particles: 15 nm in diameter) in wt PC12 cells obtained before (**A**) and 24 h after (**B**) the beginning of cultures under serum deprivation. (**A**), immunogold particles showing Bcl-2 are weak but well co-localized with those showing subunit $\beta$ in the inner mitochondrial membrane of an intact cell. (**B**), immunolabeling of Bcl-2 is not detected in the inner mitochondrial membrane of a cell 24 h after serum-free culturing. The immunogold particles are mostly seen in the membrane of smooth endoplasmic reticulum and some are localized to the outer membrane of mitochondria, whereas those indicating subunit $\beta$ are localized to the inner membrane

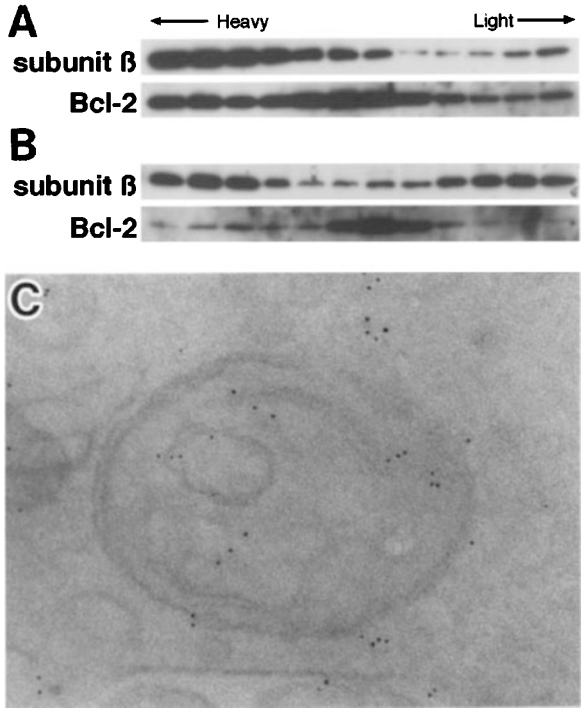


**Figure 8** Effects of serum-deprived culturing on the localization of Bcl-2 in *bcl-2*-transfected PC12 cells. (**A**) and (**B**), subcellular fractionation of PNS from *bcl-2*-transfected PC12 cells obtained before and 24 h after the beginning of serum-deprived cultures was performed on a Percoll gradient. Aliquots of 200 $\mu$l each were collected from the gradient and 10 $\mu$l each was loaded, while they were subjected to SDS–PAGE and finally to immunoblotting by antibodies specific for human Bcl-2 and subunit $\beta$ of mitochondrial ATP synthase. The subcellular distribution of Bcl-2 has two peaks; the heavier fraction coincides with the peak of subunit $\beta$ and the lighter corresponds to that of the ER/vesicular fraction. Cathepsin B appears in the heaviest fraction (data not shown). (**C**), an immunoelectron micrograph of Bcl-2 in a *bcl-2*-transfected cell obtained after serum-deprived culturing for 24 h. Positive signaling is distinctly detected in the inner mitochondrial membrane. Scale bar, 0.2 $\mu$m

applied the present most reliable immunoelectron microscopic method to *in vivo* and *in vitro* neuronal cells; this method allowed us to explore the subcellular localization of Bcl-2. Indeed, using this technique, Motoyama *et al*.[23] have shown the localization of Bcl-2 to the inner mitochondrial membrane in rat hepatocytes.

We further confirmed the immunocytochemical data of Bcl-2 by treating purified mitochondria with digitonin. The highest concentration of digitonin used in this experiment did not induce a leakage of mitoplasts to which a fraction of porin is associated in cholesterol-poor domains at contact points between the inner and outer membranes specific to brain mitochondria.[34] This particular resistance of porin to digitonin extraction, identical between mitochondria isolated from the neuron-like PC12 cells and rat brain tissues, is in contrast with that of porin under digitonin extraction from pure liver mitochondria.[35] Moreover, even in these digitonin-treated mitochondria, Bcl-2 was clearly associated to the inner mitochondrial membrane when observed by immunocytochemistry. The results, showing that Bcl-2 remained mostly in the mitoplasts of PC12 cells and rat brain tissues, suggest the localization of Bcl-2 to the inner mitochondrial membrane.

In addition to the treatment of isolated mitochondria with digitonin, we performed a digestion study with trypsin, which is considered to be the most important experiment to determine on which sides of mitochondrial membranes, inner or outer, Bcl-2 is localized. Since previous digestion studies showed that Bcl-2 appeared to be more sensitive to trypsin than proteins of the mitochondrial intermembrane space[8] or the inner mitochondrial membrane,[12] we carefully isolated mitochondria from wt PC12 cells and rat brain tissues and digested them with increasing concentrations of trypsin. A mitochondrial import receptor protein, Tom20 was completely digested by 100 $\mu$g/ml trypsin, whereas Bcl-2 as well as subunit $\beta$ were resistant to the treatments with 250 $\mu$g/ml trypsin. These lines of evidence thus argue strongly for the preferential association of Bcl-2 with the inner membrane compartment, even in the purified mitochondria.

## Possible roles of Bcl-2 in mitochondria during apoptosis

How Bcl-2 regulates the processes of cell death through its localization in the inner mitochondrial membrane remains unknown. Bcl-2 and Bcl-$x_L$ have been shown to repress the processes of both apoptosis and some forms of chemical hypoxia-induced necrosis by blocking the mitochondrial membrane potential.[26] During apoptosis, cytochrome c is released from mitochondria and forms the vertebrate

apoptosome with Apaf-1 and procaspase-9, resulting in the activation of caspase-9.[36,37] This release of cytochrome c from mitochondria is prevented by Bcl-2.[22] Bcl-2 is, thus very likely to regulate the cell death processes through the maintaining of the mitochondrial function, and, particularly, its membrane potential. Indeed, release of cytochrome c from the inner mitochondrial membrane is one major consequence of the loss of the mitochondrial potential.[27]

Prevention of apoptosis by Bcl-2 differs depending on its expression level in the cells.[38] As stated above, wt PC12 cells used in the present study expressed Bcl-2, to some extent. It is very interesting that the mitochondrial localization of Bcl-2 was markedly decreased in wt and bcl-2-transfected PC12 cells when they were cultured under serum deprivation. Different from wt PC12 cells, however, the transfected cells possessed a certain level of Bcl-2 in mitochondria even after apoptotic stimulation, resulting in cell survival. At present, it still remains unexplained how Bcl-2 blocks the release of cytochrome c from the mitochondria. These lines of evidence, however, suggest that selective localization of Bcl-2 in the inner mitochondrial membrane plays a key role in the maintenance of physiological function in mitochondria and in the prevention of apoptosis.

## Materials and methods

### Cell culture

PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing high glucose (4.5 g/L) supplemented with 10% heat-inactivated horse serum, and penicillin (100 units/ml) and streptomycin (100 $\mu$g/ml). The human *bcl-2* cDNA-transfected PC12 cells used were the same clone of the transfected cells as have been previously reported.[38] PC12 cells transfected with the human *bcl-2* cDNA linked with the FLAG epitope tag sequence at the site corresponding to the COOH-terminus of the human Bcl-2 proein were also prepared and confirmed to stably express their mRNAs and proteins.

### Antibodies

A series of antibodies against human, mouse and rat Bcl-2 were used. Polyclonal antibodies against human Bcl-2 (two types) and mouse Bcl-2 were raised by immunizing rabbits with partially purified human and mouse GST-Bcl-2 recombinant proteins, respectively, and purified by affinity column cross-linked with each recombinant protein. Specific monoclonal anti-human[39] and anti-rat Bcl-2 and commercially available polyclonal anti-human Bcl-2 (PharMingen) antibodies were also used. Polyclonal antibodies against subunit $\beta$ of mitochondrial ATP synthase,[40] porin[41] and Tom20[28] were used for marker proteins of the inner and outer mitochondrial membranes, and polyclonal antibodies against cathepsin B for a lysosomal enzyme marker. Commercially available monoclonal antibody against the FLAG epitope (M2) (Eastman Chemical) was used for detection of FLAG-tagged Bcl-2.

### Immunohisto/cytochemistry

*Confocal laser scanning microscopy* Wild-type (wt) and bcl-2-transfected PC12 cells plated on Chamber Slides (Nunc), those cultured under serum deprivation for 24 h, and wt PC12 cells cultured in the presence of NGF for several days were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 1 h at room temperature.[42] After washing with PBS they were incubated overnight at 4°C with anti-Bcl-2 antibody diluted in PBS containing 0.05% Tween 20, and then with anti-rabbit or -mouse IgG conjugated with FITC (Biomedia). Some samples were incubated with both monoclonal anti-Bcl-2 and polyclonal anti-subunit $\beta$ or -porin and then with anti-mouse IgG coupled with FITC and anti-rabbit IgG with Texas Red (Biomedia) for colocalization studies. For double staining with Bcl-2 and Rhodamine 123, the cells were incubated first with Rhodamine123 at a concentration of 10 $\mu$g/ml in DME for 30 min at 37°C and rinsed with DME, then fixed before incubation of anti-Bcl-2 antibodies. Control samples were incubated with mixture of the antibody against Bcl-2, Bcl-$x_L$ or Bax and its respective recombinant proteins. The slides were mounted using 90% glycerol containing 0.1% p-phenylendiamine dihydrochloride and viewed with an Olympus laser scanning microscope (LSM-GB200).

*Electron microscopy* Immunocytochemical analysis using electron microscopy was carried out with cryothin-section immunogold labeling,[43] originally designed by Tokuyasu.[44] Wild-type and bcl-2-transfected PC12 cells plated on the dishes and those cultured under serum deprivation for 24 h were fixed first with 0.1% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer for 10 min at room temperature, then with 4% paraformaldehyde alone in the same buffer for 30 min. The fixed cells were washed with the buffer, scraped and centrifuged at 250 g for 5 min. Pelleted cells were suspended with 10% gelatin in 0.1 M phosphate buffer and again centrifuged at $1200 \times g$ for 5 min. Gelatin-embedded cells were immersed in 2.3 M sucrose in 0.1 M phosphate buffer and frozen in liquid nitrogen. The mitochondrial fraction treated with digitonin, described below, was also treated similarly for immunoelectron microscopy on cryothin sections.

Cryothin sections were cut with a ultramicrotome (Reichert-Nissei ULTRACUT S, Nissei Sangyo Co. Ltd.). Sections on grids were treated with 1% BSA in PBS, incubated with anti-Bcl-2 or -mitochondrial marker proteins overnight and with respective secondary antibodies coupled to 5 nm colloidal gold particles (Amersham) for 1 h. For double labeling, some grids were also incubated with both monoclonal anti-Bcl-2 and polyclonal anti-mitochondrial marker proteins, and then with anti-mouse IgG coupled with 15 nm gold particles and anti-rabbit IgG coupled with 5 nm gold particles (Amersham). Controls were incubated with the antibody to Bcl-2 and its corresponding recombinant protein. The grids were then treated with 2% glutaraldehyde, 1% $OsO_4$ and 1% uranyl acetate. They were then dehydrated, embedded in LR white and examined in a JEOL JEM-100CX or Hitachi H-7100 electron microscope.

For comparison, neuronal cells in the central nervous tissue of the rat were treated, as described elsewhere,[43] for cryothin-section gold methods. Adult and postnatal day 5 rats anesthetized with chloral hydrate were perfused with 0.1% glutaraldehyde 4% paraformaldehyde in 0.1 M phosphate buffer for 10 min, and with 4% paraformaldehyde for 30 min. Parts of the brain and spinal cord were immersed in 2.3 M sucrose, cryothin sections were cut and subsequently processed for immunogold labeling.

*Subcellular fractionation* *bcl-2*-transfected PC12 cells obtained before and 24 h after the beginning of serum-deprive culture, were rinsed with PBS after removal of medium, scraped from dishes and centrifuged at $250 \times g$ for 5 min at 4°C, respectively. Pelleted cells were homogenized in 0.25 M sucrose by passing 6 times through 27 gauge needle at 4°C, and centrifuged at $1200 \times g$ for 5 min at 4°C. The

supernatant was used as postnuclear supernatant (PNS). PNS was loaded on a cushion of 15% Percoll in 0.25 M sucrose, centrifuged at 50 000 × *g* for 30 min, and fractionated into 12 fractions from bottom to top. These fractions were then incubated with lysis buffer containing 150 mM NaCl, 50 mM Tris, 1% Triton X-100 including the proteinase inhibitor cocktail (Boehringer Mannheim) and subjected to SDS–PAGE and immunoblotting.

*Purification of mitochondria* Mitochondria were isolated from the PNS of wt, *bcl-2* or *bcl-2*-FLAG-transfected PC12 cells, or adult or postnatal day 5 rat brains by centrifugation at 50 000 × *g* for 1 h, using a hybrid Percoll-metrizamide discontinuous density gradient.[45] After removal of metrizamide by centrifugation at 12 500 × *g* for 15 min, the mitochondrial fraction was suspended in 0.1 M Tris-HCl buffer (pH 7.4), containing 1 mM EGTA, 0.25 M sucrose, and proteinase inhibitors for digitonin treatment or 1 mM EDTA and 0.32 M sucrose for trypsin digestion.

Identical aliquots of mitochondria (3 mg protein/ml) were incubated with digitonin at concentrations of 0, 0.5 or 1 mg/mg mitochondrial protein dissolved in the same buffer for 15 min at 4°C[34] and then the samples were diluted with 3 volumes of the buffer. The inner membrane fraction was obtained as a pellet by centrifugation at 12 500 × *g* for 15 min, and its supernatant was further separated into the outer membrane (pellet) and intermembrane (supernatant) fractions by centrifugation at 100 000 × *g* for 1 h. Each fraction was applied to SDS–PAGE and immunoblotting.

For trypsin digestion, purified mitochondria were treated with trypsin at concentrations of 0, 20, 50, 100, and 250 μg/ml in the reaction buffer for 20 min at 4°C. The samples were centrifuged at 15 000 × *g* for 20 min after stopping the reaction, and pellets and supernatants were applied to SDS–PAGE and immunoblotting.

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