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# THE BCL-2 PROTEIN IS INSERTED INTO THE OUTER MEMBRANE BUT NOT INTO THE INNER MEMBRANE OF RAT LIVER MITOCHONDRIA *IN VITRO*

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**SUMMARY :** The *bcl-2* gene encodes a 26 kDa protein, overexpression of which blocks cell apoptosis. Since conflicting data have been reported concerning the localization of the Bcl-2 protein with mitochondrial inner and outer membranes, we analyzed *in vitro* import of the human Bcl-2 protein into isolated rat liver mitochondria. The Bcl-2 protein translated in rabbit reticulocyte lysate was efficiently inserted into the mitochondrial outer rather than the inner membrane in a membrane potential-independent manner. The carboxyl-terminal hydrophobic stretch is essential for the Bcl-2 protein to integrate into the outer membrane. Binding of the Bcl-2 protein to the mitochondria was not affected by pretreatment of the mitochondria with trypsin, indicating that the Bcl-2 protein does not require protease-sensitive mitochondrial surface components for its membrane insertion. 

The *bcl-2* gene was originally discovered in follicular B-cell lymphomas in association with the t(14;18) chromosomal translocation, which leads to deregulated expression of the *bcl-2* gene and synthesis of high levels of the Bcl-2 protein (1, 2). Overexpression of the Bcl-2 protein results in inhibition of apoptosis without stimulation of cell division (3-5), but the molecular mechanism of this block of apoptosis remains unclear. An important clue to understanding the mechanism of the Bcl-2 function could be obtained by establishing the subcellular location of the Bcl-2 protein.

The *bcl-2* gene encodes a 26 kDa protein, which has no obvious signal or membrane-anchoring sequence at the amino terminus but instead contains a stretch of hydrophobic or apolar amino acid residues near the carboxyl terminus. Although the Bcl-2 protein is membrane-associated, its subcellular location is in dispute (6-10). Earlier studies showed that the Bcl-2 protein was associated with the plasma membrane or perinuclear endoplasmic

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reticulum (6, 7). Korsmeyer and co-workers reported that the Bcl-2 protein is an inner mitochondrial membrane protein since it co-segregated with marker enzymes for the inner mitochondrial membrane on a sucrose density gradient after lysis of mitochondria (8). However, Monaghan *et al.* recently reported on the basis of immunocytochemical observations that the Bcl-2 protein associated preferentially with the outer mitochondrial membrane instead of the inner membrane (10).

In the present study, we have analyzed *in vitro* import of the *in vitro*-synthesized human Bcl-2 protein into isolated rat liver mitochondria. Our results indicate that the Bcl-2 protein is predominantly integrated into the outer mitochondrial membrane rather than the inner membrane *in vitro*.

## MATERIALS AND METHODS

**Preparation of Rat Liver Mitochondria.** Mitochondria were prepared from the liver of male Wistar rats of about 200 g according to a published procedure (11). The purified mitochondria were suspended in 20 mM Hepes-KOH, pH 7.4, 0.25 M sucrose (buffer A).

***In vitro* Transcription and Translation.** The following plasmids were used for the cell-free transcription/translation: pCI-MAS70/SP encoding MAS70, a yeast mitochondrial outer membrane protein, was constructed by inserting the *MAS70* gene into pGEM-1 (Promega); pCI-COX IV/SP encoding subunit IV of yeast mitochondrial cytochrome *c* oxidase was constructed by inserting the gene into pGEM-2 (Promega); spbcl-2 encoding the human *bcl-2* was a pSP65 (Promega) derivative. The pGEM-bcl2(M16L) containing the mutated *bcl-2* gene with the nucleotide substitution of CTT leucine codon at nucleotide position +46 for the 2nd ATG methionine codon was constructed by inserting the mutated *bcl-2* gene, which was constructed by polymerase chain reaction, into pGEM-4Z (Promega). The pGEM-Δbcl2 was constructed by modifying spbcl-2 to encode a truncated Bcl-2 protein (ΔBcl-2 protein) in which the carboxyl-terminal 20 amino acid residues are replaced by Asp-Pro-Gly. The $^{35}$S-labeled precursor proteins were synthesized in nuclease-treated reticulocyte lysate by coupled transcription/translation according to the methods by Stueber *et al.* (12) except that SP6 RNA polymerase was used for the transcription.

***In vitro* Import into Isolated Mitochondria.** Each import reaction (200 μl) contained 200 μg of mitochondrial protein, 5 μl of radiolabeled precursor, 0.25 M sucrose, 30 mM Hepes-KOH, pH7.4, 120 mM potassium acetate, 1 mM Mg-acetate, 2 mM dithiothreitol, 0.02 % bovine serum albumin, 1 mM ATP, 5 mM phosphoenol pyruvate, 10 mM succinate, 10 mM malate, 5 mM NADH, 0.2 mg/ml methionine, 8 μg/ml pyruvate kinase (Sigma P1560) and, where indicated, 10 μg/ml of valinomycin. After 60 min at 30°C, import was stopped by addition of valinomycin to 10 μg/ml and transfer to 0°C. For proteinase K digestion, mitochondrial suspension after import was incubated for 30 min at 0°C with 250 μg/ml protease, and then 1 mM phenylmethylsulfonyl fluoride (PMSF) was added. The mitochondria with or without proteinase K treatment were recovered by centrifugation at 12,000 x g for 10 min, washed once with 500 μl of buffer A, and subjected to SDS-PAGE followed by fluorography.

**Protease and Alkali Treatments of Mitochondria.** For alkali extraction of mitochondria, reisolated mitochondria after import were suspended in 400 μl of 0.1 M $Na_2CO_3$ and incubated for 15 min at 0°C, and pellet and soluble fractions were obtained by centrifugation at 100,000 x g for 10 min. For pretreatment of mitochondria with trypsin, mitochondria were suspended to 10 mg protein/ml in buffer A containing various amounts of trypsin (80, 200, or 400 μg/ml) and incubated for 20 min at 0°C. The reaction was stopped by adding soybean trypsin inhibitor (2 mg/ml as a final concentration) and incubating for another 20 min at 0°C. The mitochondria were sedimented by centrifugation and subjected to import reactions (with 1 mM PMSF and 2 mg/ml soybean trypsin inhibitor).

## RESULTS

***In vitro* Synthesis of the Human Bcl-2 Protein.** Transcription and translation of the human *bcl-2* gene in the presence of [$^{35}$S]methionine yielded a major polypeptide of 26 kDa and

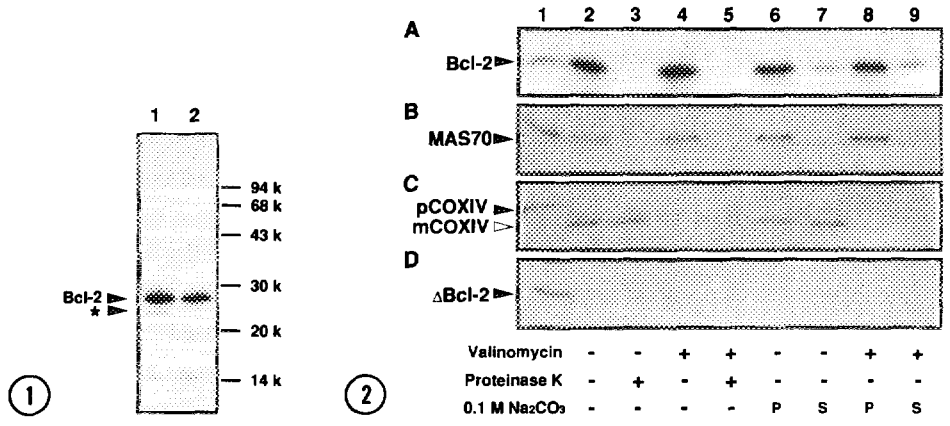


**Fig. 1. *In vitro* translation of the *bcl-2* mRNA.** *In vitro* transcription and translation with reticulocyte lysate were performed with either spbcl-2 encoding the Bcl-2 protein (lane 1) or pGEM-bcl2(M16L) encoding the mutant Bcl-2 protein, which has Leu-16 instead of Met-16, as template DNA. The translation products were analyzed by SDS-PAGE and fluorography. The asterisk (lane 1) indicates the minor translation product of 23 kDa.

**Fig. 2. Import of the human Bcl-2 protein into rat liver mitochondria.** The translation products containing the $^{35}$S-labeled Bcl-2 protein (A), MAS70 (B), pCOX IV (C), or ΔBcl-2 protein (D) were incubated with isolated rat liver mitochondria for 60 min at 30°C in the absence (lanes 2, 3, 6, and 7) or presence (lanes 4, 5, 8, and 9) of 10 μg/ml valinomycin. The mitochondria with (lanes 3 and 5) or without (lanes 2 and 4) proteinase K digestion were recovered by centrifugation and the imported proteins were analyzed by SDS-PAGE and fluorography. The alkali-treated mitochondria were fractionated into pellet and supernatant and the proteins in each fraction were analyzed by SDS-PAGE and fluorography (lanes 6 and 8 for the pellet and lanes 7 and 9 for the supernatant). Lanes 1 contained 20% of the amounts of the *in vitro* translated proteins added to the mitochondria.

a minor, smaller one of 23 kDa (Fig. 1, lane 1). To confirm that the 26 kDa protein but not the 23 kDa form was the full-length Bcl-2 protein, a mutant *bcl-2* gene which has CTT leucine codon instead of the 2nd ATG codon at position +46 was constructed. *In vitro* transcription/translation of the mutant *bcl-2* gene resulted in a single protein product of 26 kDa and no detectable 23 kDa protein (Fig. 1, lane 2). The major translation product of 26 kDa (Fig. 1, lane 1) thus corresponds to the full-length Bcl-2 protein.

**The Human Bcl-2 Protein Is Inserted into the Outer Membrane of Rat Liver Mitochondria.** Next we performed *in vitro* import of the human Bcl-2 protein, yeast MAS70 (an outer membrane protein), and the precursor to yeast cytochrome *c* oxidase subunit IV (pCOX IV, an inner membrane protein targeted to the mitochondrial matrix) into isolated rat liver mitochondria (Fig. 2). pCOX IV was cleaved to a mature-sized protein, which was protected from externally added protease, in a membrane potential-dependent manner (Fig. 2C) as expected for a protein targeted to the matrix. On the other hand, MAS70 (Fig. 2B) bound to both energized and deenergized mitochondria without being cleaved to a smaller size and remained accessible to externally added protease. The human Bcl-2 protein (Fig. 2A) behaved in a similar manner to the outer membrane protein, MAS70; it bound to the mitochondrial surface without any detectable change in the apparent molecular size upon incubation with both energized and deenergized mitochondria (lanes 2 and 4) and the bound species was accessible to externally added protease (lanes 3 and 5). The Bcl-2 protein and MAS70 that bound to

mitochondria were minimally or not extracted by $Na_2CO_3$ at pH 11.5 (Fig. 2A and B, lanes 6 and 7), suggesting that they were tightly associated with the outer membrane primarily through protein-lipid interactions (13).

Localization of the Bcl-2 protein to the mitochondrial outer membrane might merely reflect a possible *in vitro* artifact that the Bcl-2 protein was converted to an import-incompetent state, e.g. a tightly folded state or aggregated form, after synthesis in a cell-free translation system. Some mitochondrial precursor proteins that have lost their ability to be imported into mitochondria can regain import competence after denaturation with urea (14). We thus tested import ability of the urea-denatured Bcl-2 protein, which was precipitated from the translation mixture with ammonium sulfate and subsequently dissolved in 8M urea. However, even the urea-denatured Bcl-2 protein remained accessible to externally added protease after binding to mitochondria (data not shown). Alternatively, the efficiency of protein import into mitochondria may depend on the species from which the mitochondria were isolated (15). However, the results of *in vitro* import experiments were not affected when we used yeast mitochondria instead of rat liver mitochondria (data not shown). These observations show that Bcl-2 is predominantly inserted into the mitochondrial outer membrane and does not reach the inner membrane *in vitro*.

**Insertion of the Bcl-2 Protein into the Mitochondrial Outer Membrane Requires the Carboxyl-Terminal Hydrophobic Stretch.** Since the Bcl-2 protein contains a cluster of hydrophobic or apolar amino acid residues at the carboxyl terminus, this region may serve as the membrane anchor. To test this possibility, we followed the import of the ΔBcl-2 protein lacking this region into rat liver mitochondria (Fig. 2D). After incubation with mitochondria, the truncated ΔBcl-2 protein was not recovered with mitochondria at all while the wild-type Bcl-2 protein efficiently associated with mitochondria. This indicates that the carboxyl-terminal hydrophobic stretch is essential for Bcl-2 to integrate into the mitochondrial outer membrane.

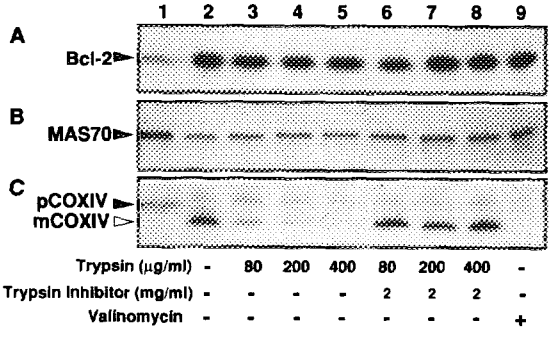


**Fig. 3. Integration of the Bcl-2 protein into the trypsin-pretreated mitochondria.** Mitochondria were pretreated with the indicated amounts of trypsin as described in MATERIALS AND METHODS and subjected to *in vitro* import reaction. After incubation for 60 min at 30°C, the mitochondria were recovered by centrifugation and analyzed directly by SDS-PAGE ((C), pCOX IV protein), or subjected to alkali extraction followed by SDS-PAGE analyses of the pellet fractions ((A), the Bcl-2 protein; (B), MAS70). Where indicated, an excess amount of soybean trypsin inhibitor was added prior to trypsin pretreatment (lanes 6- 8). Lanes 1 contained 20% of the amounts of the *in vitro* translated proteins added to the mitochondria.

**Trypsin Treatment of Rat Liver Mitochondria Does Not Affect Import of the Bcl-2 Protein.** Several proteins of the mitochondrial outer membrane have been reported to facilitate import of precursor proteins into mitochondria (16). Import of many precursor proteins into mitochondria is impaired completely or partially by removal by gene disruption or inactivation with specific antibodies or protease digestion of these proteins. We thus examined whether insertion of the Bcl-2 protein into the outer membrane of rat liver mitochondria was affected by pretreatment of the mitochondria with trypsin. As shown in Fig. 3C, import of pCOX IV into rat liver mitochondria was strongly inhibited by pretreatment of intact mitochondria with 80μg/ml trypsin (lanes 2-5). The control experiments (lanes 6-8) eliminated the possibility that the soybean trypsin inhibitor failed to inactivate trypsin and precursor proteins were degraded by the residual trypsin activity. In contrast, binding of the Bcl-2 protein and MAS70 to the mitochondrial outer membrane was not affected at all by pretreatment of mitochondria with even higher concentration (400 μg/ml or 40 μg trypsin / mg mitochondrial protein) of trypsin. These results suggest that integration of the Bcl-2 protein into the mitochondrial outer membrane probably takes place by a different route from that for pCOX IV and does not require protease-sensitive surface components of the mitochondrial outer membrane *in vitro.*

## DISCUSSION

The present results have shown that the human Bcl-2 protein inserts into the outer membrane but does not reach the inner membrane of rat liver mitochondria *in vitro.* Like all the mitochondrial outer membrane proteins investigated so far, insertion of the Bcl-2 protein into the outer membrane did not require an electrochemical potential across the inner membrane, and the protein was not proteolytically processed. A significant fraction of the Bcl-2 protein that bound to the mitochondria was accessible to externally added protease but resistant to alkali extraction. This means that the Bcl-2 protein is integrated into the lipid bilayer of the outer membrane but exposes at least a part of the polypeptide to the cytosolic side. A similar topological association of the Bcl-2 protein with microsomal membranes has been demonstrated by Chen-Levy and Cleary (17).

The present results are consistent with the previous observation of the immunoelectron microscopy that the Bcl-2 protein showed a peripheral association with the mitochondrial outer membrane (10). On the other hand, our results are not readily compatible with the suggestion from sucrose density gradient fractionation of the disrupted mitochondria that the Bcl-2 protein is a mitochondrial inner membrane protein (8). A possible explanation for this discrepancy is that the Bcl-2 protein is localized in specialized regions of the mitochondrial outer membrane (contact sites), where the outer and inner membranes are in close apposition; upon subfractionation of the mitochondria by centrifugation through a sucrose density gradient, the submitochondrial vesicles containing contact sites often do not behave like the outer membrane vesicles but cofractionate with the inner membrane vesicles (18, 19). The localization of the Bcl-2 protein in the outer but not the inner mitochondrial membrane renders the involvement of the mitochondrial respiration in the Bcl-2 function unlikely, and this is consistent with the recent

observation that the Bcl-2 protein blocks apoptosis in cells that lack mitochondrial DNA and therefore do not have a functional respiratory chain (20).

There are several integral membrane proteins that have no amino-terminal signal sequence, but instead possess a hydrophobic segment near the carboxyl terminus (21). These proteins have been found in several cellular compartments including the ER and mitochondria and, in some cases, simultaneously in several organelles. They are probably delivered to target membranes by a unique mechanism and inserted into the membranes with their carboxyl-terminal hydrophobic segment; some of them do not require organellar proteins for their membrane insertion *in vitro*. The Bcl-2 protein, which has a hydrophobic segment near the carboxyl terminus and is inserted into the membrane without requirement of protease-sensitive organellar surface components *in vitro*, appears to be a member of this class of the "tail-anchored proteins", like mitochondrial cytochrome $b_5$ (22) and rat liver monoamine oxidase B (23). Indeed, the carboxyl-terminal hydrophobic stretch was shown in the present study to be required for *in vitro* insertion of the Bcl-2 protein into the mitochondrial outer membrane, and Tanaka *et al.* showed that it was necessary for the function and targeting of the Bcl-2 protein *in vivo* (24).

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## REFERENCES

1. Cleary, M. L., Smith, S. D. and Sklar, J. (1986) Cell 47, 19-28.
2. Tsujimoto, Y., Finger, L. R., Yunis, J., Nowell, P. C. and Croce, C. M. (1984) Science 226, 1097-1099.
3. Strasser, A., Harris, A. W. and Cory, S. (1991) Cell 67, 889-899.
4. Sentman, C. L., Shutter, J. R., Hockenbery, D., Kanagawa, O. and Korsmeyer, S. J. (1991) Cell 67, 879-888.
5. Garcia, I., Martinou, I., Tsujimoto, Y. and Martinou, J.-C. (1992) Science 258, 302-304.
6. Tsujimoto, Y., Ikegaki, N. and Croce, C. M. (1987) Oncogene 2, 3-7.
7. Chen-Levy, Z., Nourse, J. and Cleary, M. L. (1989) Molec. Cell Biol. 9, 701-710.
8. Hockenbery, D., Nuñez, G., Milliman, C., Schreiber, R. D. and Korsmeyer, S. J. (1990) Nature 348, 334-336.
9. Alnemri, E. S., Robertson, N. M., Fernandes, T. F., Croce, C. M. and Litwack, G. (1992) Proc. Natl. Acad. Sci. USA,89, 7295-7299.
10. Monaghan, P., Robertson, D., Amos, T. A. S., Dyer, M. J. S., Mason, D. Y. and Greaves, M. F. (1992) J. Histochem. Cytochem. 40, 1819-1825.
11. Pedersen, P. L., Greenawalt, J. W., Reynafarje, B., Hullihen, J., Decker, G. L., Soper, J. W. and Bustamente, E. (1978) Methods Cell Biol. 20, 411-481.
12. Stueber, D., Ibrahimi, I., Cutler, D., Dobberstein, B. and Bujard, H. (1984) EMBO J. 3, 3143-3148.
13. Fujiki, Y., Hubbard, A. L., Fowler, S. and Lazarow, P. B. (1982) J. Cell Biol. 93, 97-102.
14. Becker, K., Guiard, B., Rassow, J., Söllner, T. and Pfanner, N. (1992) J. Biol. Chem. 267, 5637-5643.
15. Zara, V., Palmieri, F., Mahlke, K. and Pfanner, N. (1992) J. Biol. Chem. 267, 12077-12081.
16. Pfanner, N., Söllner, T. and Neupert, W. (1991) Trends Biochem. Sci. 16, 63-67.
17. Chen-Levy, Z. and Cleary, M. L. (1990) J. Biol. Chem. 265, 4929-4933.
18. Pon, L., Moll, T., Vestweber, D., Marshallsay, B. and Schatz, G. (1989) J. Cell Biol. 109, 2603-2616.

19. Ono, H. and Tsuboi, S. (1990) J. Biochem. (Tokyo) 107, 840-845.
20. Jacobson, M. D., Burne, J. F., King, M. P., Miyashita, T., Reed, J. C. and Raff, M. C. (1993) Nature 361, 365-369.
21. Kutay, U., Hartmann, E. and Rapoport, T. A. (1993) Trends Cell Biol. 3, 72-75.
22. Darrigo, A., Manera, E., Longhi, R. and Borgese, N. (1993) J. Biol. Chem. 268, 2802-2808.
23. Mitoma, J. and Ito, A. (1992) J. Biochem. 111, 20-24.
24. Tanaka, S., Saito, K. and Reed, J. C. (1993) J. Biol. Chem. 268, 10920-10926.