Membrane Topology of the Bcl-2 Proto-oncogenic Protein Demonstrated in Vitro*

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The Bcl-2 oncogenic protein was synthesized in vitro and shown to post-translationally integrate asymmetrically into microsomal membranes with no requirement for an amino-terminal signal sequence. Instead, a carboxyl-terminal hydrophobic domain of Bcl-2 served as an insertion sequence essential for membrane assembly since a Bcl-2 mutant lacking this domain completely lost its ability to associate with microsomal membranes. The data demonstrate that Bcl-2 is tightly associated with the lipid bilayer with the nature of an integral membrane protein. The membrane orientation of Bcl-2 was determined using a protease protection assay, which showed that it is predominantly localized to the cytoplasmic face of membranes. A similar type of membrane processing has been shown for cytchrome b$_5$ and also suggested for the viral oncogenic protein polyoma middle-T antigen.

The bcl-2 gene was isolated and analyzed due to its consistent involvement by t(14;18) chromosomal translocations commonly observed in human B-cell malignancies (Bakhshi et al., 1985, Cleary and Sklar, 1985, Cleary et al., 1986a, 1986b, Tsujimoto and Croce, 1986). The bcl-2 gene is juxtaposed with the immunoglobulin heavy chain gene following t(14;18) translocations resulting in its transcriptional deregulation and the accumulation of abnormally high levels of bcl-2 mRNA and protein in t(14;18)-carrying cells (Chen-Levy et al., 1985; Cleary et al., 1986b; Graninger et al., 1987; Ngan et al., 1988). Recent gene transfer studies have demonstrated that Bcl-2 has unique biological properties that confer a prolonged survival advantage on both lymphoid cells and fibroblasts (McDonnell et al., 1989; Reed et al., 1988; Tsujimoto, 1989; Vaux et al., 1988). These and other observations suggest that aberrant expression of the Bcl-2 protein is pathogenetically important in the development of a large proportion of human B-cell tumors.

We have shown previously (Chen-Levy et al., 1989) that Bcl-2 in t(14;18)-carrying cells is tightly integrated with cellular membranes and is predominantly localized to the perinuclear endoplasmic reticulum with a small fraction at the plasma membrane. Based on our biochemical findings and on theoretical analyses of the predicted Bcl-2 amino acid sequence, we proposed that Bcl-2 may integrate with the membrane bilayer via its carboxyl-terminal end that bears the single putative Bcl-2 transmembrane domain. We also speculated that the protein is oriented toward the cytoplasmic side of the membrane rather than to the lumenal or extracellular space (Chen-Levy et al., 1989). However, the actual membrane topology of Bcl-2 and the protein domain involved in its membrane association were never verified experimentally.

To demonstrate the membrane orientation of Bcl-2 and study the process of its membrane assembly, we employed an in vitro synthesis system using two bcl-2 cDNA constructs. One of these directed synthesis of the normal full-length Bcl-2 protein; the other was an engineered mutant that produced a truncated Bcl-2 lacking the COOH-terminal hydrophobic domain. The results reported here clearly demonstrate that Bcl-2 post-translationally integrates into membranes by means of a carboxyl-terminal insertion sequence to assume a characteristic topology such that the protein is predominantly localized along the cytoplasmic face of cellular membranes.

EXPERIMENTAL PROCEDURES

In Vitro Transcription and Translation—DNA fragments were cloned into the pSP65 expression vector. Following linearization, the plasmids served as templates for in vitro transcription of RNA using SP6 RNA polymerase (Promega (Kvieg and Melton, 1984)). RNA produced (0.5 µg) were translated in vitro using a rabbit reticulocyte lysate cell-free translation system (Promega) at 30 °C for 45-60 min in the presence of 1 mCi/ml [35S]methionine, as previously described (Jackson and Hult, 1983). Translation was terminated by the addition of cycloheximide at a final concentration of 20 µg/ml.

Co-translational and Post-translational Membrane Processing—Microsomal vesicles (Promega), at a concentration of 3.9 eq/0.3 µg of initial RNA in a 25-µl translation reaction, were either included at the beginning (pre-translation) or added at the end (after termination with cycloheximide) of the reticulocyte translation reaction, and samples were incubated for an additional 30 min at 30 °C. At this point, a membrane-stabilizing agent, tetracaine (Scheele, 1983), was added to a final concentration of 2 mM, followed by 5 min of incubation at 24 °C. All subsequent steps were carried out at 4 °C to prevent sedimentation of the membrane vesicles was performed as described (Gilmore and Blobel, 1985) by layering the reaction mixture over a 0.5 M sucrose cushion and centrifuging (100,000 × g) for 3 h at 30 °C. The entire supernatant solution (including the sucrose cushion) was removed, the membrane pellet was resuspended into the same volume, and protein denaturation was performed with SDS sample buffer and heating at 80 °C (5-10 min).

Alkaline Extraction—Alkaline extraction of microsomal membrane proteins was based on previously described methods (Anderson et al., 1985; Fujiki et al., 1982; Mayers et al., 1989). The translation reaction containing microsomal membranes was adjusted to pH 11.5 with 1 M NaOH at the end of translation. The mixture was incubated for 10 min on ice and separated into supernatant and membrane-pellet fractions as described above, except that the sucrose cushion was 0.2 M and was also adjusted to pH 11.5 with sodium hydroxide.

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The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
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proteins resulting from complete Bcl-2 open reading frame were fractionated by SDS-PAGE and subjected to autoradiography for 18-24 h. Lanes: I, total translation reaction; 2, Sepharose-anti-Bcl-2-coupled fraction (ppt) following immunoprecipitation of the translation mixture (imm.); 3, uncoupled supernatant fraction (sup) from immune precipitation; 4, the Sepharose-coupled fraction (ppt) following immunoprecipitation using preimmune rabbit serum (pre-imm.); 5, the uncoupled supernatant (sup) from preimmune precipitation. Size standards are indicated in kilodaltons.

RESULTS AND DISCUSSION

In Vitro Translation of Bcl-2—A fragment of bcl-2 cDNA containing the complete open reading frame was fractionated by SDS-PAGE and subjected to autoradiography for 18-24 h. Lanes: 1, total translation reaction; 2, Sepharose-anti-Bcl-2-coupled fraction (ppt) following immunoprecipitation of the translation mixture (imm.); 3, uncoupled supernatant fraction (sup) from immune precipitation; 4, the Sepharose-coupled fraction (ppt) following immunoprecipitation using preimmune rabbit serum (pre-imm.); 5, the uncoupled supernatant (sup) from preimmune precipitation. Size standards are indicated in kilodaltons.

Site-directed in Vitro Mutagenesis—Mutagenesis was carried out according to the Amersham oligonucleotide-directed in vitro mutagenesis system based on a published method (Nakamuye and Eckstein, 1986).

Protection Assay—RNAs were translated and processed as above using an in vitro translation system to which microsomal vesicles were added at the initiation. Following incubation with the membrane stabilizer tetracaine, trypsin was added to a final concentration of 50 ug/ml, and the samples were further incubated for 30 min at 4 °C. Membrane and supernatant fractions were separated by centrifugation and analyzed by SDS-PAGE.

Post-translational Membrane Integration of Bcl-2—Membrane processing of the in vitro-translated Bcl-2 (and, as a control, that of the Escherichia coli β-lactamase protein) was studied using canine pancreatic microsomal vesicles as an in vitro model for cellular protein processing at the rough endoplasmic reticulum (Walter and Blobel, 1983).

Under our conditions, 90% of Bcl-2 in the lysate sedimented with the microsomal membranes (Fig. 2A) whether they were added before (lanes 3 and 4) or after (lanes 5 and 6) in vitro translation. Bcl-2 migrated with a molecular mass corresponding to 24 kilodaltons both in the absence (lanes 1 and 2) and presence (lanes 4 and 6) of membranes, indicating that it had not been altered by “processing” following addition of membranes. The observations suggest that signal peptide cleavage is not involved in Bcl-2 membrane association. The migration

Fig. 1. In vitro translation of Bcl-2. [35S]Metionine-labeled proteins resulting from in vitro translation of RNA containing the complete Bcl-2 open reading frame were fractionated by SDS-PAGE and subjected to autoradiography for 18-24 h. Lanes: I, total translation reaction; 2, Sepharose-anti-Bcl-2-coupled fraction (ppt) following immunoprecipitation of the translation mixture (imm.); 3, uncoupled supernatant fraction (sup) from immune precipitation; 4, the Sepharose-coupled fraction (ppt) following immunoprecipitation using preimmune rabbit serum (pre-imm.); 5, the uncoupled supernatant (sup) from preimmune precipitation. Size standards are indicated in kilodaltons.

Fig. 2. Post-translational membrane integration of Bcl-2. A, autoradiography was performed following SDS-PAGE separation of [35S]methionine-labeled in vitro-translated Bcl-2 (lanes 1–6) and β-lactamase (lanes 7–10). Reticulocyte lysates were incubated in the presence (M) or absence (−) of canine microsomal membranes either during translation (co-) or following termination of translation reaction (post-). Translation products were separated by centrifugation into membrane (P) or soluble supernatant fractions (S). The precursor and processed forms of β-lactamase (lac) are denoted by upper and lower arrows, respectively. Lanes: 1 and 2, Bcl-2 translation without added membranes; 3 and 4, Bcl-2 translated in the presence of microsomal membranes added at the initiation of translation; 5 and 6, as in 3 and 4 but membranes added post-translationally; 7 and 8, membrane co-translational processing of β-lactamase; 9 and 10, translated β-lactamase to which membranes were added post-translationally. Size standards are indicated in kilodaltons. B, alkaline extraction of microsomal membranes. Membrane pellets of in vitro-translated Bcl-2 (lanes 1 and 2) and β-lactamase (lanes 3 and 4) after normal translation conditions (lanes 1 and 3) and after exposure to pH 11.5 (lanes 2 and 4).
of Bcl-2 was artifically shifted downward in some, but not all, supernatant fractions (those containing high amounts of the lysate, Fig. 2A, lanes 3 and 5) due to comigration with endogenous lysate protein (possibly globin). This was not observed in the corresponding pellet fractions since, unlike Bcl-2, the interfering endogenous protein did not sediment with microsomes.

For comparison, Fig. 2A also presents membrane processing of $\beta$-lactamase, which served as a control for these studies. $\beta$-Lactamase is a secreted E. coli protein which, when synthesized in vitro, can be translocated across the membrane of microsomal vesicles into their lumen via an NH$_2$-terminal signal peptide (Muller et al., 1982). $\beta$-Lactamase synthesized under identical conditions as used for Bcl-2, either in the absence of membranes or when microsomal vesicles were added following translation, migrated as the unprocessed precursor form. The $\beta$-lactamase precursor also remained in the supernatant solution (lanes 9 and 10) unasociated with the sedimented microsomal membranes. However, when canine pancreatic microsomal vesicles were present during translation, the major translation product was a smaller $\beta$-lactamase protein that sedimented with the membranes (lane 8) as a result of its co-translational translocation into the microsomal lumen and subsequent maturation (Muller et al., 1982).

To study the nature of Bcl-2 membrane association, we exposed the microsomal bound Bcl-2 to high alkaline pH to resistance to alkaline extraction is a commonly used criterion for integral membrane proteins (Fujiki et al., 1982) and was also applied to study the microsomal membrane-binding properties of some de novo-synthesized polypeptides (Anderson et al., 1982). Fig. 2B shows that most of the Bcl-2 remained membrane-associated in both regular and high pH conditions (lanes 1 and 2), whereas the processed $\beta$-lactamase (which is a soluble luminal protein) was almost completely removed from the membranes when they were exposed to alkaline conditions (lanes 4 versus 2). These results indicate that Bcl-2 interacts strongly and hydrophobically with the lipid bilayer. They are consistent with previous studies showing that Bcl-2 in lymphoid cells is completely membrane-associated (no soluble cytosolic Bcl-2 could be detected) and that it has the nature of an integral transmembrane protein (Chen-Levy et al., 1989).

Our results clearly demonstrate that in vitro-synthesized Bcl-2 is tightly associated with the membrane fraction whenever microsomal vesicles are present and that this association is not followed by a change in its apparent molecular mass. It appears that Bcl-2 is post-translationally integrated into the rough endoplasmic reticulum membrane, probably by means of an internal hydrophobic domain rather than an amino-terminal signal peptide.

A Carboxyl-terminal Hydrophobic Region Is Essential for Bcl-2 Membrane Assembly—To test the possibility that the carboxyl-terminal hydrophobic region of Bcl-2 serves as the membrane anchor, we constructed a bcl-2 transcription template that codes for a Bcl-2 protein lacking this domain. Using site-directed in vitro mutagenesis, a single base mutation was inserted at nucleotide 642 of the bcl-2 open reading frame. This converted tryptophan codon 214 (TGG) to a stop codon (TGA) resulting in premature termination of the protein and synthesis of a shorter peptide lacking the COOH-terminal 26 amino acids (including the 23-amino acid fragment of the potential hydrophobic domain (Chen-Levy et al., 1989; Fig. 3A). Transcription, translation, and co-translational processing of the mutant construct (mut1.bcl-2) were carried out as described above and the resulting protein fractions analyzed on 11.5% SDS-PAGE (Fig. 3B). The mut1.Bcl-2 protein migrated faster than Bcl-2 at an apparent molecular mass of about 22 kilodaltons (lanes 5 and 7), as expected for its predicted sequence. In the presence of microsomal vesicles, mut1.Bcl-2 appeared in the supernatant fraction (lane 7) indicating that it was not associated with nor translocated across microsomal membranes. Bcl-2, however, sedimented mostly with the membrane fraction (lane 4) as was shown and discussed above (Fig. 2A). Also, the gel migration of mut1.Bcl-2 was not influenced by membrane addition (compare lanes 5 and 7) since, like the native Bcl-2, it does not have a signal peptide.

The processing difference between Bcl-2 and mut1.Bcl-2 demonstrates the role of the COOH-terminal 26 amino acids in membrane association. Their absence rendered the protein completely membrane-nonassociated while their presence facilitated integration of the protein into microsomal membranes. These observations, together with the above results showing the tight association of Bcl-2 with cellular membranes, indicate that the hydrophobic carboxyl-terminal segment of Bcl-2 serves as the post-translational insertion signal and is also responsible for the permanent association of the protein with rough endoplasmic reticulum membranes.

Bcl-2 Is Oriented Toward the Cytoplasmic Face of Cellular Membranes—We have shown above that Bcl-2 is post-translationally integrated into microsomal membranes and that the region responsible for integration lies at the COOH-terminal end of the protein. To determine the membrane orientation of Bcl-2 we employed a “protection assay” system as described by others to study the orientation and membrane assembly of several secreted and integral membrane proteins (Mayero et al., 1988; Muller et al., 1982; Scheele, 1983; Watanabe et al., 1986). In such a protection assay, segments of the protein which have been translocated into the microsomal lumen or inserted across the membrane are protected from proteolytic degradation. However, parts of the protein that are exposed at the microsomal surface corresponding to the cytoplasmic surface of the membrane should be completely degraded.

For our studies, in vitro-translated Bcl-2, mut1.Bcl-2, and the control protein $\beta$-lactamase were allowed to assemble on canine pancreatic microsomal vesicles, and then a proteolytic enzyme, trypsin, was used to determine the protein’s orientation with respect to the lipid bilayer. The results are presented in Fig. 4. The mature $\beta$-lactamase polypeptide, which had translocated into the microsomal lumen, remained intact (compare lanes 6 and 8), whereas Bcl-2, as well as the precursor untranslocated form of $\beta$-lactamase, were completely degraded after trypsin addition (compare lanes 2–4 and upper band in lane 5 versus 7).

The susceptibility of Bcl-2 to trypsin digestion, under the same conditions in which mature $\beta$-lactamase was completely protected, indicated that Bcl-2 was fully exposed on the surface of the microsomal membranes. Protection of the putative membrane-embedded carboxyl-terminal domain of Bcl-2 could not be detected with our system because of its small size (less than 3 kDa). A different set of studies is necessary to demonstrate directly that this segment is actually inserted into the membrane, but this is beyond the scope of the present study. However, we have provided evidence for the tight and hydrophobic interaction of Bcl 2 with the lipid bilayer which supports the notion that the COOH-terminal domain is indeed embedded, rather than just loosely associated, with cellular membranes.

We have demonstrated that the human Bcl-2 protein post-translationally tightly associates with microsomal membranes via a carboxyl-terminal membrane domain to assume an
Fig. 3. The COOH-terminal hydrophobic domain of Bcl-2 is required for membrane association. A, Kyme and Doolittle hydropathy profile of Bcl-2. The bar marks the COOH-terminal hydrophobic domain of Bcl-2 that is deleted in the mutant protein mut1.Bcl-2. B, Bcl-2 (lanes 1–4) and mut1.Bcl-2 (lanes 5–8) were synthesized in vitro in the absence (−) or presence (+) of microsomal vesicles, separated into supernatant (S) and membrane-pellet (P) fractions, and electrophoresed on 11.5% SDS-PAGE. An autoradiogram of the fractionated [35S]methionine-labeled protein products is shown. Arrows denote migration of Bcl-2 and mut1.Bcl-2. Size standards are indicated in kilodaltons.

Fig. 4. Membrane orientation of Bcl-2, a protection assay. Bcl-2 (lanes 1–4) and β-lactamase (lanes 5–8) were synthesized in vitro in the presence of microsomal vesicles. Following in vitro translation and prior to sedimentation of microsomal vesicles, translation products were incubated in the presence (T) and absence (−) of trypsin. S and P refer to supernatant and pellet fractions, respectively, as in Figs. 2 and 3; lac, β-lactamase forms as in Fig. 2. Size standards are indicated in kilodaltons.

Asymmetrical orientation such that it is exposed at the cytoplasmic side of the lipid bilayer. Our data suggest that the integration of Bcl-2 with membranes is mediated by an insertion sequence (Anderson et al., 1983) rather than an uncleaved signal peptide. Thus, its membrane assembly will be expected to be signal recognition particle-independent and may proceed into any exposed cellular membrane. This type of membrane processing, in which a COOH-terminal hydrophobic domain serves as an insertion sequence, is not common but has been well studied for the microsomal protein cytochrome b_{6} (Takagaki et al., 1983) and has also been suggested for the oncogenic protein polyoma middle-T antigen (Ito et al., 1977; Templeton et al., 1984). Further studies are necessary to demonstrate that Bcl-2 insertion is in fact signal recognition particle-independent. It was shown in a previous study that Bcl-2 is found in cellular membranes with a minor fraction present at the plasma membrane (Chen-Levy et al., 1989). Since its membrane orientation is cytoplasmic, the Bcl-2 is localized to the inner side of the plasma membrane resembles the membrane-associated fraction of several cytoplasmic oncogenic proteins, e.g. c-ras (Willumsen et al., 1984), v-src (Buss et al., 1984), and v-erb-B (Privalsky and Bishop, 1984). Bcl-2 differs from these latter proteins in that it does not partially exist in a soluble state but rather exclusively as a single entity, which was shown to be completely membrane-associated (Chen-Levy et al., 1989). This and our present results indicate that the human bcl-2 gene product is always integrated into cellular membranes, and we believe that this membrane disposition is necessary for its physiological function.

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REFERENCES
Membrane Topology of Bcl-2

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