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# Overexpressed full-length human BCL2 extends the survival of baculovirus-infected Sf9 insect cells

(baculovirus expression system/membrane–protein association/apoptosis/subcellular fractionation/immunofluorescence)

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ABSTRACT **Full-length and truncated human BCL2 lacking the entire C-terminal hydrophobic domain have been overexpressed in *Spodoptera frugiperda* insect cells with the baculovirus expression system. Immunoblot analysis with BCL2-specific antibodies revealed that both full-length and truncated BCL2 are expressed as multiple immunoreactive species, suggesting posttranslational modifications. The expression of the full-length but not the truncated BCL2 extended the survival of baculovirus-infected cells by preventing virus-induced DNA cleavage. This result is consistent with the reported protective effect of BCL2 against apoptosis in mammalian lymphocytes and suggests a conserved function in evolution. Subcellular fractionation and indirect immunofluorescence studies in intact cells demonstrated that the recombinant full-length and truncated BCL2 proteins were expressed predominantly as nuclear membrane-associated proteins. These results imply that BCL2 must utilize hydrophobic domains other than the deleted domain for its association with the subcellular membranes. Metabolic labeling of insect cells expressing the full-length and the truncated form of BCL2 with $^{32}P_i$ demonstrated that BCL2 is a phosphoprotein.**

The protooncogene *BCL2* has been isolated and characterized as a result of its involvement with the t(14;18) chromosomal translocation in follicular lymphomas (1–5). The t(14;18) translocation places *BCL2* under the transcriptional regulation of the immunoglobulin heavy-chain locus, causing the deregulation of *BCL2* expression (4, 5). The BCL2 protein is a 26-kDa membrane-associated cytoplasmic protein (6–8). It is different from other protooncogene products in that it seems to function in enhancing the survival of hematopoietic cells of T and B origins rather than promoting their growth (9–15). The ability of *BCL2* to enhance the survival of these cells is related to its ability to block programmed cell death, or apoptosis, initiated by several factors. BCL2 overexpression in pre-B lymphocytes inhibits apoptosis following cytokine deprivation (8, 9, 14) or glucocorticoid treatment (16). Recently, two independent studies (17, 18) have also shown that expression of BCL2 in immature thymocytes of transgenic mice protects them from glucocorticoid-, radiation-, and anti-CD3-induced apoptosis. BCL2 upregulation also inhibits apoptosis of Epstein–Barr virus-infected B-cell lines (19). The mechanism by which BCL2 inhibits apoptosis is not known.

To gain insight into the structure and mechanism of action of BCL2, we overexpressed full-length and truncated BCL2 in insect cells with the baculovirus expression system. Because of the low abundance of BCL2 in lymphocytes, overexpression of this protein in Sf9 insect cells will avail large quantities of protein for structural, functional, and biochemical analysis.

## MATERIALS AND METHODS

**Cell Culture.** Sf9 insect cells were grown in TNM-FH medium (20, 21). Cells were maintained and infected either as monolayers or in suspension in a rotary shaker. When cells were grown in suspension the medium was supplemented with the surfactant Pluronic F-68 (GIBCO) at 0.1% (vol/vol). Cells were routinely infected with 1–4 × $10^8$ plaque-forming units of baculovirus per ml of culture at a density of 1.5–2 × $10^6$ cells (>95% viability) per ml.

**Construction of Recombinant Transfer Vectors and Recombinant Baculovirus.** The recombinant transfer vector pVL1393-BCL-2 was constructed from the transfer vector pVL1393 (22) and the plasmid pB4 (23), which contains the entire *BCL2* cDNA. In brief, the 0.85-kilobase (kb) *Eco*RI fragment of pB4, encompassing the entire *BCL2* sequence from −57 to approximately +800 relative to the ATG start site was inserted into the *Eco*RI site of pVL1393. To construct the baculovirus transfer vector encoding the truncated BCL2 that lacks the C-terminal hydrophobic domain, the DNA sequence between the *Bam*HI sites at −76 and +640 relative to the ATG start site was excised from pVL1393-BCL-2 and subcloned into the *Bam*HI site of the transfer vector pVL1393-hsp500. pVL1393-hsp500 contains a 0.5 kb *Bam*HI–*Eco*RI insert derived from the C-terminal cDNA of the human hsp90 heat shock protein (24). This transfer vector contains a TGA termination codon in frame with the *BCL2* sequence 30 nucleotides downstream of the *Bam*HI site. This TGA termination codon was also part of the unique *Bcl* I restriction enzyme recognition sequence, which made it easier to verify the presence of this sequence in our baculovirus clones. Because of the addition of the 30 nucleotides downstream of the *Bam*HI site, the truncated BCL2 contains the following extra amino acids at its C terminus: PRPTPTASIA. The recombinant baculoviruses AcNPV-BCL-2 and AcNPV-TBCL-2, containing the full-length *BCL2* and the truncated *BCL2* sequences under the transcriptional control of the polyhedrin promoter, were produced by "*in vivo*" homologous recombination between the transfer vector pVL1393 and the wild-type virus (*Autographa californica* nuclear polyhedrosis virus, AcNPV) DNA (20, 21).

**Western Blot Analysis.** Sf9 cells (2 × $10^7$) were harvested at 20–96 hr postinfection. Cells were washed with Grace's medium and samples of 2 × $10^5$ cells were solubilized in SDS sample buffer, boiled for 5 min, and then electrophoresed in SDS/12% polyacrylamide gels. Proteins were then transferred electrophoretically to nitrocellulose filter paper, incubated with the S006 polyclonal antibody (25), and detected

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with the Amersham ECL (enhanced chemiluminescence) detection system (Amersham).

**Immunofluorescence.** Immunofluorescence detection and localization of the recombinant BCL2 expressed in Sf9 cells, using the anti-BCL-2-100 monoclonal antibody (26), was essentially as described (21).

**Phosphorylation of BCL2 in Sf9 cells.** At 28 hr postinfection; Sf9 cells ($4 \times 10^7$) were incubated for 4 hr with 40 $\mu$Ci of $^{32}P_i$ (1000 mCi/mmol, ICN; 1 Ci = 37 GBq) in 12 ml of phosphate-free Grace's insect medium (21). After labeling, cells were washed twice with Grace's medium, lysed on ice (20 min) in 50 mM Hepes, pH 7.2/1% (vol/vol) Nonidet P-40/1 mM phenylmethylsulfonyl fluoride, and centrifuged at $16,000 \times g$ for 15 min at 4°C. The cell lysates were immunoprecipitated with the anti-BCL-2-100 monoclonal antibody and analyzed by SDS/PAGE and autoradiography exactly as described (21, 27).

**Subcellular Fractionation.** Insect cells grown in 175-cm$^2$ tissue culture flasks and infected with the full-length or truncated *BCL2* baculoviruses were harvested at 44–48 hr postinfection. Cells from one flask ($4 \times 10^7$ cells) were lysed as described (8) in 1 ml of hypotonic Hepes buffer (10 mM Hepes, pH 7.4/42 mM KCl/5 mM $MgCl_2$/1 mM phenylmethylsulfonyl fluoride) by homogenization (30 strokes) in a 1-ml glass homogenizer. The lysed cells were then centrifuged at $200 \times g$ to obtain the crude nuclei (N1) and the supernatant containing the heavy membranes (HM). The crude nuclei were washed twice in Hepes buffer and resuspended in 1 ml of Hepes buffer. Samples (0.1 ml) of the HM and N1 fractions were centrifuged at $10,000 \times g$ and $200 \times g$, respectively. The pellet from each fraction was suspended in 0.2 ml of SDS sample buffer. The supernatant of the HM fraction was mixed with 0.1 ml of SDS sample buffer. The N1 crude nuclei were further purified by ultracentrifugation at $150,000 \times g$ over a double cushion of 1.6 M and 2 M sucrose. Pure nuclei trapped at the interface between the 1.6 M and 2 M sucrose cushions (N2) and at the bottom of the 2 M sucrose cushion (N3) were collected, washed twice in Hepes buffer, and resuspended to their original density in Hepes buffer. A sample (0.1 ml) of the N2 and the N3 nuclei was pelleted at $5000 \times g$ and suspended in SDS sample buffer.

## RESULTS AND DISCUSSION

**Expression of BCL2 in Sf9 cells.** The cDNAs for the full-length BCL2 and the truncated BCL2 lacking the entire C-terminal hydrophobic domain were subcloned into the expression vector pVL1393. Several recombinant baculovirus clones encoding either the full-length or the truncated form of human BCL2 were generated and purified by strategies similar to those outlined previously (21, 27). The recombinant *BCL2* baculovirus clones were characterized by Southern blot analysis of purified viral DNA digested with several restriction enzymes and by Western blot analysis of total cellular proteins. Southern blot analysis confirmed the correct orientation and the identity of both full-length and truncated *BCL2* cDNAs (data not shown). Similarly, all recombinant clones that were positive by Southern blot analysis directed the synthesis of the correct form of BCL2 as confirmed by Western blot analysis using BCL2-specific antibodies (data not shown). To examine in more detail the expression of BCL2 in insect cells infected with baculoviruses encoding full-length or truncated BCL2, we performed a time-course study using Western blot analysis. Multiple immunoreactive species or isoforms of full-length and truncated BCL-2 proteins were expressed maximally at 48–72 hr postinfection. The major full-length BCL2 migrated in SDS/PAGE as a 26-kDa protein that was detectable at 20 hr postinfection by Western blotting (Fig. 1*B*) and at 48–96 hr by Coomassie staining (Fig. 1*C*). This size is consistent with that calculated from the deduced amino acid sequence of BCL2 and also was comparable to the size of the authentic mammalian BCL2 protein immunodetected in the human pre-B-cell line 380 (Fig. 1*D*). A minor 23-kDa BCL2 isoform was detectable at 48–96 hr postinfection by both methods (Fig. 1 *B* and *C*). The 23-kDa isoform could be a translation product from the internal ATG start site at position +46 or a proteolytic product of the larger major form. Tsujimoto and Croce (23) detected two BCL2 isoforms in a reticulocyte lysate that expressed BCL2 from the same *BCL2* cDNA as employed in this study to generate recombinant baculoviruses. Larger isoforms of BCL2 migrating as 29–31 kDa were also detected by Western blotting but not by Coomassie staining. Analysis of the expression of the truncated BCL2 revealed a similar pattern of immunoreactive protein species. The calculated molecular mass of the truncated BCL2 is 21.7 kDa, which is consistent with the observed migration of the major truncated isoform in SDS/PAGE (Fig. 1 *A* and *C*). Two truncated isoforms migrating at 21 and 22 kDa were seen clearly by Coomassie staining at 48–96 hr postinfection. The intensity of the 22-kDa isoform is the highest, suggesting that it is the major translational product. The smaller isoform is probably the internal translation product. In the case of both the full-length and the truncated BCL2, the large immunoreactive isoforms could be posttranslational modifications of the smaller major isoforms (Fig. 1*A* and *B*). Whatever posttranslational modifications are responsible for this pattern must occur in the N-terminal domain, since both full-length and truncated BCL2 share that domain. To our knowledge, the expression of multiple species of BCL2 in mammalian lymphocytes has not been described before. This could be due to the low abundance of BCL2 in lymphocytes, which makes the detection of these species very difficult. When we used 20 times less BCL-2 than we normally use in our immunoblots, we detected only the major (26-kDa) BCL2 isoform (data not shown). This was also true for the truncated BCL2.

Several posttranslational modifications may give rise to large protein isoforms, particularly glycosylation or fatty acid acylation. We used tunicamycin to inhibit N-glycosylation. However, we were unable to reduce the molecular size of the large BCL2 isoforms as detected by one-dimensional SDS/PAGE, thus ruling out the possibility that these isoforms represent N-glycosylated products of BCL2 (data not shown). We have also obtained a negative result using [$^3$H]palmitate as a precursor (data not shown).

An important posttranslational modification that is known to regulate the function of several proteins is phosphorylation. Phosphorylation of BCL2 was not detected previously in mammalian lymphocytes, due to the low abundance of BCL2. Fig. 2 shows immunoprecipitates from $^{32}P_i$-labeled Sf9 cells infected either with wild-type virus or with recombinant virus encoding full-length BCL2. A major radioactive 27-kDa BCL2 band was present in the immunoprecipitates from the recombinant virus-infected cells but not in the wild-type immunoprecipitates. The phosphorylated BCL2 has a slightly higher molecular weight than the major BCL2 band detected by immunoblotting (Fig. 1 *B* and *C*). It is possible that the phosphorylated BCL2 is the intermediate band between the 26- and 29-kDa immunoreactive bands shown in Fig. 1*B*. Several large radioactive proteins were also seen to coimmunoprecipitate with BCL2 but not with the immunoprecipitates from the wild-type virus (Fig. 2). This suggests that BCL2 is a "sticky" protein that may interact specifically or nonspecifically with other phosphoproteins. Similar results were obtained with the truncated BCL2 (data not shown). To learn whether BCL2 was phosphorylated in mammalian cells, we performed a preliminary experiment with the 380 pre-B-cell line, in which BCL2 is overexpressed as a result of a chromosomal translocation. Antibody-precipitated BCL2 was also phosphorylated in this line but

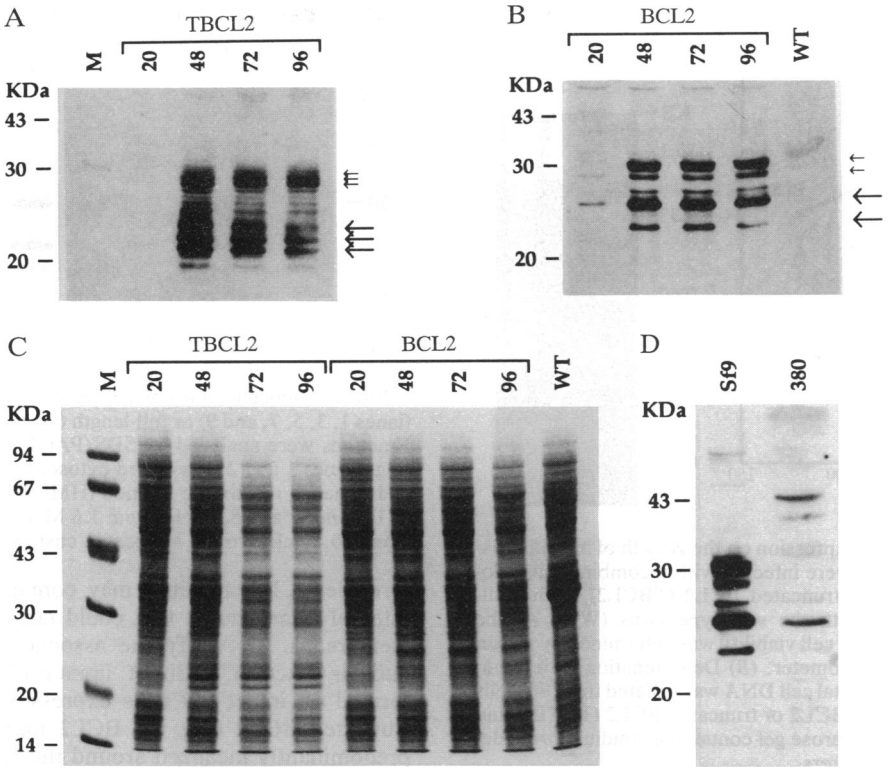


FIG. 1. Time-course analysis of the expression of full-length and truncated BCL2 in Sf9 cells. Total cell lysates from baculovirus-infected Sf9 cells ($2 \times 10^5$ cells per sample) expressing truncated (TBCL-2) or full-length BCL2 (BCL-2) were prepared at 20–96 hr postinfection and then electrophoresed in an SDS/12% polyacrylamide gel. The proteins were detected by Western blot analysis using the BCL2-specific polyclonal antibody S006 (*A* and *B*) or by Coomassie blue staining (*C*). Times after infection (hr) are shown above each lane. Lysates from wild-type virus-infected Sf9 cells isolated at 48 hr postinfection are included as controls (WT). *D* shows Western blot analysis of BCL2 from Sf9 (lane 1) and 380 lymphocyte (lane 2) lysates. Large and small arrows at right in *A* and *B* indicate the major and minor immunoreactive BCL2 protein species, respectively. Lanes M, molecular mass markers.

the major band involved a higher molecular weight species of BCL2, one that may also reflect other posttranslational alterations.

**BCL2 Prolongs the Survival of Baculovirus-Infected Sf9 Cells.** Recent observations that BCL2 inhibited apoptosis induced by a number of different agents in mammalian thymocytes (17, 18) and pre-B lymphocytes (16) led us to examine the effect of its expression on baculovirus-infected Sf9 cells. Sf9 cells were infected with the recombinant full-length or truncated BCL2 baculoviruses or with the wild-type virus and cell viability was determined at various times postinfection (Fig. 3*A*). The viability of cells infected with the wild-type virus or with the truncated BCL2 decreased sharply after 48 hr to reach a minimum at 120 hr postinfection. In contrast, cells expressing BCL2 showed very little decrease in viability during the course of the experiment. Concomitant with the decrease in cell viability, a decrease in cellular proteins was also evident in cells expressing the truncated BCL2 but not in cells expressing the full-length BCL2 (Fig. 1*C*). Because there is some evidence that baculovirus infection of insect cells causes host cell DNA degradation during viral infection (28, 29), we examined the integrity of Sf9 DNA after infection with recombinant and wild-type baculoviruses. DNA isolated from cells expressing the truncated BCL2 (Fig. 3*B*) or wild-type virus (data not shown) revealed typical internucleosomal "ladder" patterns characteristic of apoptosis. This DNA degradation was observed only late in viral infection, 48–96 hr postinfection. DNA degradation was not detectable in cells expressing full-length BCL2 even at later times after viral infection (Fig. 3*B*). Recent evidence (29) demonstrated that baculovirus encodes a specific viral gene product, p35, that blocks the apoptotic response of baculovirus-infected insect cells. Our data are consistent with those observations, although we believe that the baculovirus p35 protein delays the apoptotic response of insect cells rather than inhibiting it completely. Further protection may be conferred by expressing BCL2 in these cells. The protective effect of BCL2 in insect cells

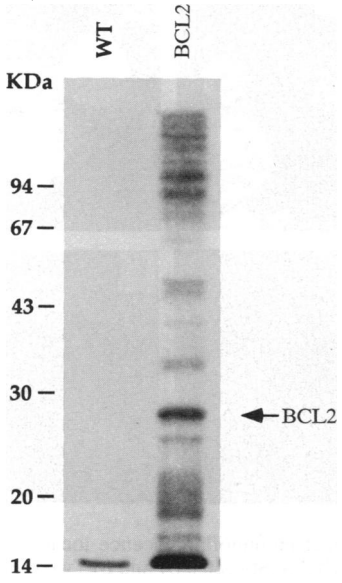


FIG. 2. Phosphorylation of BCL2 in Sf9 cells. Immunoprecipitates from $^{32}P_i$-labeled Sf9 cells infected with the wild-type (WT) virus or recombinant BCL2 baculovirus were electrophoresed in an SDS/12% polyacrylamide gel. The gel was stained, dried, and autoradiographed.

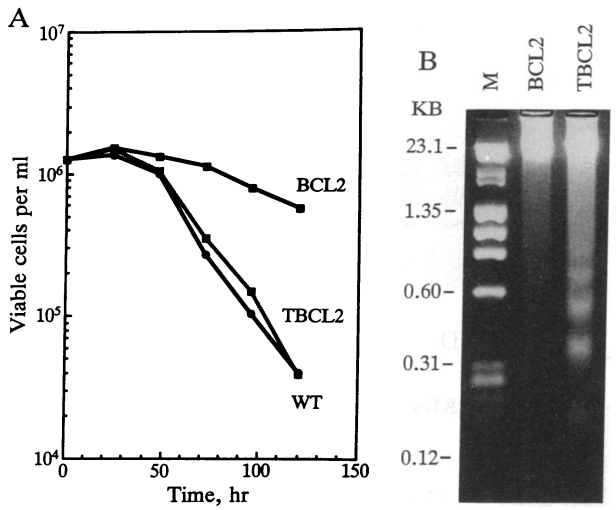


FIG. 3. Effect of BCL2 expression on the growth of baculovirus-infected Sf9 cells. Sf9 cells were infected with recombinant baculoviruses encoding either the truncated BCL2 (TBCL2) or the full-length BCL2 (BCL2) or with the wild-type virus (WT). At the indicated times postinfection, cell viability was determined by trypan blue exclusion in a hemocytometer. (*B*) Determination of internucleosomal DNA cleavage. Total cell DNA was isolated from Sf9 cells expressing either full-length BCL2 or truncated BCL2 (TBCL2) and electrophoresed in a 1.5% agarose gel containing ethidium bromide. Lane M, molecular size markers.

supports the possibility that apoptosis is regulated by a common mechanism in both mammalian and insect cells that has been conserved in evolution. The absence of a protective effect of the truncated BCL2 implies that the C-terminal hydrophobic domain is the functional domain or that its presence is important for BCL2 function.

**Subcellular Localization of BCL2.** The exact cellular localization of BCL2 is still controversial. BCL2 has been localized to different sites including the inner surface of the plasma membrane (6), the perinuclear endoplasmic reticulum (7), and the inner mitochondrial membrane (8). Although these studies do not agree on the exact localization of BCL2, all conclude that BCL2 is a membrane-associated protein. Due to the lack of an obvious N-terminal or internal membrane domain in the BCL2 sequence, it has been suggested that BCL2 associates with the subcellular membranes through its 19-amino acid C-terminal hydrophobic domain (7, 23, 30, 31). To test this possibility, we compared the subcellular localization of full-length and truncated BCL2 lacking the last 50 C-terminal amino acids, including the entire C-terminal hydrophobic domain, by using biochemical fractionation and indirect immunofluorescence analysis. Subcellular fractionation showed that neither truncated nor full-length BCL2 was present in the low-speed Sf9 cytosol (Fig. 4, lanes 1 and 2, respectively). It was surprising that both BCL2 forms were associated with the heavy membrane fraction, which includes the mitochondria (lanes 3 and 4), and predominantly with the crude nuclear fraction (lanes 5 and 6) or the nuclear fractions purified by ultracentrifugation through 1.6 and 2M sucrose cushions (lanes 7 and 8 and lanes 9 and 10, respectively). Both full-length and truncated BCL2 were extracted from the nuclear fraction with 1% Nonidet P-40, thus confirming their association with the perinuclear membranes (data not shown). Subsequent fractionation of the heavy membranes through a 30% Percoll density gradient revealed the association of BCL2 with the mitochondrial fraction (data not shown). Because of the lack of any effect of C-terminal truncation of BCL2 on its association with the subcellular membranes, we conclude that the C-terminal hydrophobic domain is not necessary for its membrane association. Therefore the BCL2 sequence may contain other N-terminal or internal signal motifs that could target it to the subcellular membranes. To verify the association of BCL2 with the nuclear fraction, indirect immunofluorescence was performed on intact Sf9 cells expressing either full-length or truncated BCL2 (Fig. 5). BCL2 immunofluorescence was predominantly localized around the nucleus, particularly in the perinuclear endoplasmic reticulum region, in both cells expressing the full-length BCL2 (Fig. 5 *A* and *B*) and cells

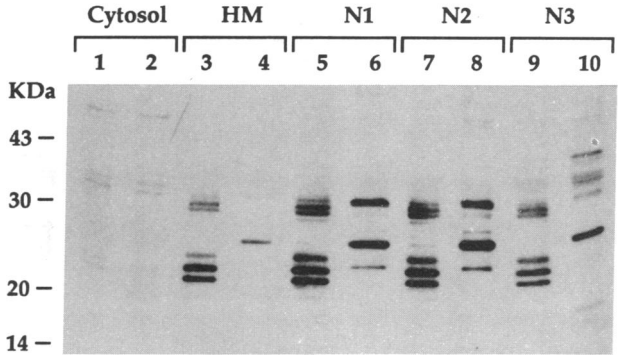


FIG. 4. Subcellular fractionation of Sf9 cells expressing truncated (lanes 1, 3, 5, 7, and 9) or full-length (lanes 2, 4, 6, 8, and 10) BCL2. Fractions were analyzed by SDS/PAGE followed by immunoblotting. Lanes 1 and 2, low-speed cytosolic fraction (cytosol); lanes 3 and 4, heavy membrane fraction (HM); lanes 5 and 6, crude nuclei (N1); lanes 7 and 8, nuclei from 1.6 M sucrose cushion (N2); lanes 9 and 10, nuclei from 2 M sucrose cushion (N3).

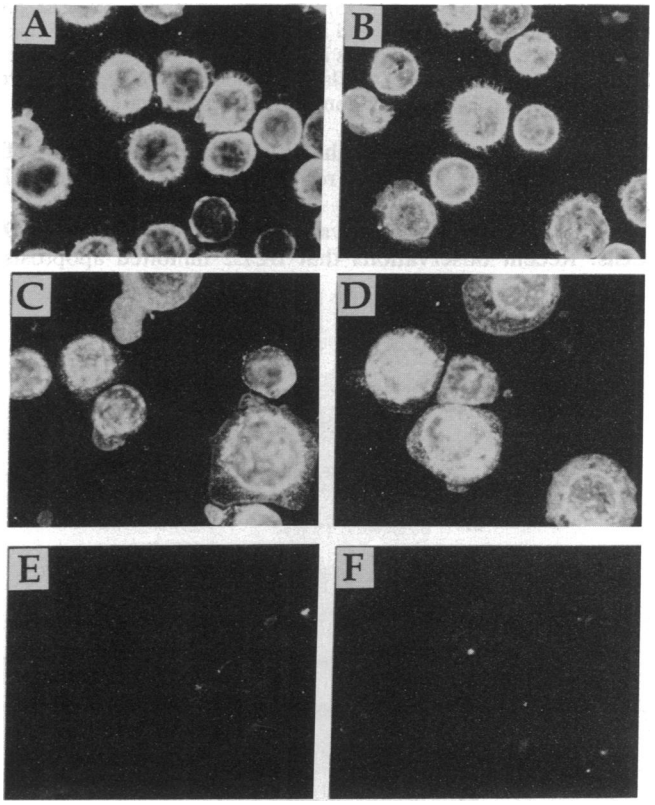


FIG. 5. Indirect immunofluorescence localization of full-length and truncated BCL2 in Sf9 cells. (*A* and *B*) Two representative fields illustrate perinuclear endoplasmic reticulum BCL2 immunofluorescence in Sf9 cells expressing full-length BCL2. (*C* and *D*) Same as *A* and *B* except that cells expressed truncated BCL2. (*E*) Sf9 cells infected with the wild-type virus. (*F*) Sf9 cells expressing full length BCL2 detected with a nonspecific antibody. All cells were harvested at 48 hr postinfection. (× 250).

expressing the truncated BCL2 (Fig. 5 *C* and *D*). No positive immunofluorescence was detected in cells infected with the wild-type virus (Fig. 5*E*) or when cells expressing BCL2 were probed with nonspecific antibody (Fig. 5*F*). Within the cytoplasm, some immunofluorescence was seen associated with the endoplasmic reticulum as a slightly fluorescent network. Punctate immunofluorescence also was seen in these cells, although it was more obvious in the case of the truncated BCL2 (Fig. 5 *A–D*). The punctate immunofluorescence suggests association with subcellular organelles, such as the mitochondria. Similar distribution was observed by Cleary and coworkers (7) in human pre-B lymphocytes. Because apoptosis is mainly a nuclear phenomenon, with internucleosomal DNA cleavage as its hallmark (32, 33), we believe that a perinuclear localization of BCL2 is more in concert with protection against apoptosis. Some evidence suggests that an early event in cells undergoing apoptosis is an increase in nuclear membrane fragility (34), which precedes nuclear membrane blebbing and internucleosomal DNA fragmentation. It is possible that the association of BCL2 with the perinuclear membrane could inhibit the increase in nuclear membrane fragility and prevent nuclear membrane blebbing, thus protecting the cell against apoptosis. Although our data support the localization of some BCL2 to the mitochondria, we speculate that the failure of Korsmeyer and coworkers (8) to detect perinuclear localization of BCL2 could be due to several causes. In this study we have used a different BCL2-specific monoclonal antibody than the one used in the previous study (8). Because different antibodies may recognize different epitopes, these epitopes may not be exposed to interact with the antibody if BCL2 has a dual membrane localization and it assumes different conformations depending on the membrane with which it associates. Interestingly, when we carried out immunofluorescence analysis with the BCL2-specific S006 polyclonal antibody directed against a BCL2 peptide, we detected only punctate cytoplasmic fluorescence (data not shown). Another reason could be that overexpression of BCL2 in insect cells may overwhelm the limited number of mitochondria in the cell, which could lead to association of BCL2 with other membranes. However, the absence of BCL2 association with the light membranes as determined by the subcellular fractionation and the plasma membrane as seen by indirect immunofluorescence suggests that the perinuclear localization of BCL2 is specific. Deletion of a hydrophobic domain in the truncated BCL2 did not appreciably change the subcellular localization of the protein but did obliterate the protective effect on cellular survival. We had considered that this moiety might represent the membrane inclusion domain, but it is clear that it serves a function in the activity of the protein, and some other domain may be responsible for the membrane localization. Although we considered that the overexpressed protein might swamp out the cellular machinery for delivering BCL2 to membranes and depositing it there, it must be noted that overexpression of epidermal growth factor receptor (35) and of influenza virus hemagglutinin (36), as two examples, resulted in membrane localization that was completely reflective of the endogenous mammalian cellular process. In addition, insect cells have been shown to process many other mammalian proteins in terms of posttranslational modifications and subcellular localization exactly as their mammalian counterparts (21, 27, 37, 38).

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