Bcl-2 Expressed Using a Retroviral Vector Is Localized Primarily in the Nuclear Membrane and the Endoplasmic Reticulum of Chicken Embryo Fibroblasts

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Abstract

A complementary DNA for human bcl-2 was cloned into the replication competent avian retrovirus vector RCASBP, and the resulting virus was used to express human Bcl-2 protein at high levels in chicken embryo fibroblasts. The expression of Bcl-2 did not transform or significantly alter the longevity of the chicken embryo fibroblasts in the presence of normal amounts of serum. However, the expression of Bcl-2 blocked c-Myc-induced apoptosis in these cells. Fractionation of the infected chicken embryo fibroblasts indicated that the protein was distributed equally between nuclear and high density cytoplasmic membranes.

Immunofluorescence analysis by confocal microscopy and immunoelectron microscopy showed that the Bcl-2 protein was primarily associated with the nuclear membrane and with the endoplasmic reticulum. Reduced amounts of the protein were associated with other membranes in the cytoplasm. These data show that, in this system, the Bcl-2 protein associates with the nuclear membrane and intracytoplasmic membranes but is not preferentially associated with mitochondria.

Introduction

The bcl-2 gene was first identified by a t(14;18) chromosomal translocation in human follicular B-cell lymphomas (1–3). The translocation of the bcl-2 gene to the immunoglobulin heavy chain locus resulted in elevated levels of the Bcl-2 RNA and protein which is thought to contribute to the pathogenesis of these B-cell neoplasias (4, 5). Bcl-2 protein has been shown to be involved in the regulation of apoptosis, a process of programmed cell death (6–11). High levels of Bcl-2 protein can protect human B-lymphocytes under stress (12, 13) and prolong cell survival when over-expressed in transgenic mice (14–19). Constitutive expression of Bcl-2 blocks cell death in hematopoietic cell lines that are deprived of growth factors (20–23). Immunohistochemical analysis showed that the Bcl-2 protein is present in tissues that contain long-lived or highly proliferative cells (8). The expression of Bcl-2 is more widespread in fetal tissues than it is in adult tissues, and the distribution of the protein suggests that Bcl-2 plays a role in morphogenesis (24). Taken together, these studies suggest that Bcl-2 plays an important role in homeostasis and that inappropriate Bcl-2 expression can disrupt homeostasis and contribute to neoplasia.

The mechanism by which Bcl-2 affects the process of cell death in mammals is not known. Although the expression of Bcl-2 has been shown to inhibit the generation of reactive oxygen species in neurons, B-cells, and yeast (25, 26), it is not clear whether this is a direct or an indirect effect. The expression of human bcl-2 in Caenorhabditis elegans prevents cell death and suggests that bcl-2 may have a role that is similar to the nematode gene ced-9, which is involved in the regulation of programmed cell death (11). Although the genes involved in this pathway are not well defined in mammals, bcl-2 clearly plays a significant role, and the subcellular localization of the Bcl-2 protein could provide clues to its mechanism of action. In vitro studies have shown that the Bcl-2 protein associates with membrane bilayers via its carboxyl-terminus (27). However, there has been controversy in deciding which intracellular membranes Bcl-2 is associated with. The protein was reported to migrate with the membrane fractions that contain the enzyme succinate dehydrogenase, suggesting that it was a component of the inner mitochondrial membrane (7). Immunocytochemical analysis of cryostat sections of human tonsils suggested that Bcl-2 is present in mitochondria and weakly associated with the nucleus and nuclear clefts of germinal center B-cells (8, 28). Other reports have indicated that Bcl-2 is associated predominantly with the perinuclear membrane, with membrane components from the cytoplasm (29), as well as with the outer membrane of mitochondria (30, 31). Most of these studies analyzed neoplastically transformed cell lines, which does not necessarily indicate the localization of Bcl-2 in normal cells. There are also reports on the localization of Bcl-2 in insect cells using a baculovirus vector system (32) and on the effects of Bcl-2 expression in human fibroblasts that lack mitochondrial DNA (33). These reports bring into question not only the claim that Bcl-2 is localized primarily in the mitochondria but also whether localization in the mitochondria is relevant to the physiological effects of Bcl-2 on the cell.

We developed a retroviral vector that can be used to induce high level expression of human Bcl-2 protein in...
Fig. 1. Immunoprecipitation of human Bcl-2 from lysates of CEF infected with RCASBPbcl-2 virus. Cells were labeled with [35S]methionine, and lysates were prepared and reacted with control serum or anti-Bcl-2 serum. Details are given in “Materials and Methods.” Immune serum (i) but not preimmune serum (p) precipitated a protein migrating at an apparent molecular weight of 26,000 from lysates of RCASBPbcl-2 infected cells (Lanes 5 and 6). This protein was not precipitated from lysates of control CEF (Lanes 1 and 2). Bcl-2 protein made by in vitro translation migrates to the same position in the gel as the immunoprecipitated Bcl-2 (Lane 4).

Fig. 2. Western blot analysis of human Bcl-2 protein in various fractions prepared from lysates of CEF infected with RCASBPbcl-2 and from uninfected controls. Equal amounts of protein (20 μg) were loaded in each lane. Both the nuclear (N) and heavy membrane (HM) fractions contain similar amounts of Bcl-2 and are enriched compared with the unfractonated cell lysate (T). Uninfected CEF do not contain human Bcl-2 protein. T, total; HM, heavy membrane; LM, light membrane; C, cytosol.

Results

Expression of the Bcl-2 Protein in Chicken Embryo Fibroblasts Using an Avian Retroviral Vector Containing the Human bcl-2 cDNA. A human bcl-2 cDNA (34) was inserted into the RCASBP(A) and RCASBP(B) retroviral vectors (35,36; “Materials and Methods”). Clones that contained the insert in the correct (sense) orientation were identified by restriction mapping. CEF were transducted with RCASBPbcl-2 DNA to produce viral stocks. Expression of the Bcl-2 protein and of the viral vector was confirmed after three cell passages by immunofluorescence analysis of fibroblasts grown on coverslips (see “Materials and Methods”). The culture supernatant contained approximately 10^5 infectious virions per ml.

After approximately four passages, infected and uninfected fibroblasts were incubated with [35S]methionine, and the cell lysates were analyzed for expression of the Bcl-2 protein by immunoprecipitation with a rabbit polyclonal antibody. As shown in Fig. 1, immune serum, but not preimmune serum, precipitated a protein corresponding in size to the M₅ 26,000 human Bcl-2 protein from lysates of RCASBPbcl-2 infected fibroblasts but not from normal fibroblasts.

No evidence of transformation was noted in the infected fibroblast cultures when these cultures were compared with fibroblast cultures infected in parallel with the parental RCASBP vector. The morphology of the cells, their inability to grow in soft agar, and the longevity of the cells cultured in the presence of normal levels of serum (23–25 passages) were essentially the same for cultures infected either with RCASBPbcl-2 or with the RCASBP vector.

Bcl-2 Protein Localizes to Nuclear and Cytoplasmic Membranes. We analyzed the distribution of Bcl-2 protein in RCASBPbcl-2 infected fibroblasts that had been passaged five times after transfection and that expressed high levels of Bcl-2 protein. The cells were lysed, and membrane fractions were isolated by differential centrifugation. Equal amounts of protein from fractions representing the total, nuclear, and cytoplasmic heavy and light membrane fractions were fractionated by electrophoresis on a sodium dodecyl sulfate-polyacrylamide gel and transferred to a membrane for Western blotting. The mouse monoclonal antibody that was used to detect the human Bcl-2 protein (37) was made against a peptide whose sequence is not shared with the mouse or the chicken Bcl-2 (38) and, therefore, does not cross-react with the endogenous chicken Bcl-2 protein (data not shown). As shown in Fig. 2, both the nuclear and high density cytoplasmic membrane fractions contained similar amounts of human Bcl-2 and were enriched compared with the unfractonated cell lysate. However, no human Bcl-2 protein was detected in the cytosol or in the low density membrane fractions.

To analyze the distribution of Bcl-2 on the membranes more precisely, cells were fixed with methanol and stained for indirect immunofluorescence using anti-Bcl-2 mouse monoclonal antibody and fluorescein isothiocyanate-labeled donkey anti-mouse antibody. The Bcl-2 staining is specific for human Bcl-2; uninfected CEF and CEF infected with the vector do not react with the antibody. The staining of infected CEF was blocked with the peptide used for immunization (Fig. 3). The stained cells were examined by confocal microscopy. The level of Bcl-2 expression varies from cell to cell (Fig. 4). However, when we used different optimal contrast and adjusted the brightness for the different levels of expression, the overall pattern of subcellular localization of Bcl-2 was quite consistent in all of the cells and did not
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In order to evaluate the cytoplasmic staining in more detail, a series of images were made in adjacent focal planes, and an overlay of the maxima was used to compose the final image (Fig. 6). This image shows that the majority of the staining, and by implication, the Bcl-2 protein, was localized in the nuclear membrane and endoplasmic reticulum.

We also analyzed sections of CEF that overexpress Bcl-2 by immunoelectron microscopy. This analysis also showed that the majority of the Bcl-2 protein is in the nuclear membrane and the rough endoplasmic reticulum (Figs. 7 and 8). In sections that showed clear cross-sections of the nuclear membrane, both the inner and outer aspects of the nuclear membrane were labeled (Fig. 7b). By counting grains, we estimate that of the Bcl-2 associated with the nuclear membrane, the majority (more than 60%) is on the inner aspect of the nuclear membrane. In these sections, many of the membrane structures in the cytoplasm were labeled; however, the intensity of the labeling appeared to be lower than the labeling of the nuclear membrane and the endoplasmic reticulum.

Bcl-2 is Biologically Active in CEF. For the subcellular localization to be relevant, it is important to show that Bcl-2 is biologically active in these cells. Expression of high levels of c-Myc, in combination with low levels of serum, can induce apoptosis (39), an effect that can be blocked by the high level expression of Bcl-2 (40–42). We first showed that high levels of c-Myc expression caused apoptosis when the cells were placed in media that contained no serum.

CEF were transfected with a virus RCASBP/Aimyc DNA, which contains a cDNA for the chicken c-myc gene and induces high level expression of the chicken c-Myc protein. This virus morphologically transforms but does not immortalize CEF. The virus was allowed to spread throughout the culture. A portion of the plates that were infected with the c-Myc virus were serum starved as were control plates that did not contain the c-Myc virus. The c-Myc-expressing cells were much more susceptible to the effects of serum starvation than were control CEF which were infected by the viral vector that did not contain a DNA insert (see Fig. 9). A large number of the c-Myc-expressing cells rounded up and floated off the dish; control CEF cultures that were infected by the vector had considerably fewer floating cells. After 20 h of serum starvation, the plate of cells expressing c-Myc had 40-fold more floating cells relative to an unstarved plate. The vector-infected cells showed only a 4-fold increase in the number of floating cells after starvation. Both the floating cells and the attached cells were examined for apoptosis.

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4 C. J. Petropoulos and S. H. Hughes, unpublished data.
Cells were stained with DAPI, and their nuclei were visualized. Virtually all of the floating cells from the serum-starved c-Myc-expressing culture had fragmented nuclei characteristic of apoptosis; moreover, a significant number of attached cells from these cultures showed similar fragmented nuclei after 48 h of serum starvation (Fig. 10). Although there were considerably fewer floating cells in the vector-infected control CEF cultures, the floating cells in these cultures were apoptotic. The majority of the vector-infected CEF, which remain on the plate in the absence of serum, show few of the morphological signs characteristic of apoptosis. To demonstrate unequivocally that the c-Myc expressing cells were apoptotic, we examined the cells by time lapse laser cinemicroscopy (Fig. 11, a-d) and by transmission electron microscopy (Fig. 11 e). We also observed that DNA from the apoptotic cells produced a “ladder” on an agarose gel (data not shown). By all of these criteria, the cells were indeed apoptotic.

To test the effect of Bcl-2, both the RCASBP(A)myc infected cells and uninfected CEF were infected with RCASBP(B)bcl-2. Because the c-Myc virus is subgroup (A), the Bcl-2 virus, which in this experiment was subgroup (B), was able to infect the c-Myc-expressing cells. Bcl-2 expression has a dramatic effect on the ability of c-Myc to induce apoptosis. In contrast to CEF that express c-Myc alone, most of which round up and become apoptotic when serum starved, CEF that express both c-Myc and Bcl-2 do not become apoptotic in the absence of serum (Fig. 9, j and k, and Fig. 10).

CEF infected with the vector RCASBP(B) will die if they are serum starved. Cells begin to die after 3 days of serum deprivation; after 12 days, all of the cells are dead. The presence of c-Myc increases the sensitivity of the cells to serum starvation; CEF infected with RCASBP(A)myc are all dead after 3 days of starvation. In contrast, CEF infected with both RCASBP(A)myc and RCASBP(B)bcl-2 and deprived of serum, survive for more than 1 month. These doubly infected cells cease to divide when serum starved; however, if these cells are refed serum even after a month of starvation, they will resume normal cell division. If the doubly infected cells are rescued by refeeding after 6 days of serum deprivation,
they are still capable of the same total number of passages (approximately 25) as unstarved control CEF.

The expression of Bcl-2 alone is not as effective in protecting CEF from the effects of serum deprivation as are c-Myc and Bcl-2 in combination. CEF infected with RCASBP(B)bcl-2 begin to die after 13 days of serum starvation.

Discussion

There is convincing evidence that the expression of Bcl-2 can prolong the life of cells that would otherwise die of apoptosis. Although it seems reasonable to propose that this function is important in preserving certain cells during development, the inappropriate expression of Bcl-2 appears to play a role in the immortalization of certain malignant cells. For this reason, it is important to understand the mechanism of action of Bcl-2 and, by implication, to understand its role in human carcinogenesis. To understand the role of Bcl-2, it is essential to determine its site of action. Although it has been clear for some time that Bcl-2 is associated with membranes in the cell, it has been less clear which particular membranes are involved. There have been reports that suggest that Bcl-2 is associated with both nuclear and cytoplasmic membranes (29–33); however, it has also been reported that the majority of the Bcl-2 protein is associated with the mitochondria, especially with the inner mitochondrial membrane (7). We have examined the subcellular location of Bcl-2 using a replication competent avian retroviral vector to express high levels of the human Bcl-2 protein in CEF. This system has the advantage that, despite the fact that the protein is expressed at high levels in the cells, the cells have a normal growth rate, morphology, and life span and do not grow in soft agar. This suggests that the high levels of expression of human Bcl-2 do not significantly perturb the CEF.

More importantly, Bcl-2 does have a significant and biological effect in this system; expression of high levels of Bcl-2 prolongs the survival of CEF in the absence of serum; in addition, Bcl-2 expression blocks c-Myc-induced apoptosis in the absence of serum. Since the CEF are not immortalized.

Fig. 7. Immunoelectron microscopy of human Bcl-2 in RCASBP(bcl-2) infected CEF. The colloidal gold spheres, which indicate the presence of human Bcl-2, are associated with both the inner and outer nuclear membrane. The cristae of the rough endoplasmic reticulum (E) are more sparsely labeled. A lobe of the membranes of the nuclear poles can be identified at the periphery (asterisk). Because the nuclear envelope in the region of the pole has been sectioned tangentially, the colloidal gold spheres do not show a clear association with the nuclear membrane. Labeling of the nuclear envelope can be seen in b and c of the rough endoplasmic reticulum (E) in e, a, × 32,500; b, × 30,000; c, × 52,000. Nu, nucleus; E, rough endoplasmic reticulum.
nor are these cells immortalized in the course of the experiments either by c-Myc or by Bcl-2, the normal processes that control growth and division are not precluded. These data suggest that this is a good system in which to test the biochemistry and subcellular localization of Bcl-2.

In this system, the majority of the Bcl-2 protein is in the nuclear membrane and in the endoplasmic reticulum. It is possible that the association with the endoplasmic reticulum may reflect sites of synthesis and/or transport of the Bcl-2 protein. The pattern of labeling suggests that Bcl-2 is associated with both the inner and outer portions of the nuclear membrane and that the majority of the Bcl-2 associated with the nuclear membrane is on the inner aspect of the nuclear membrane. We do see lesser amounts of Bcl-2 protein associated with other membranes in the cytoplasm; however, the retroviral vector system produces high levels of protein, and the relatively minor amounts of Bcl-2 that are associated with these other membranes may reflect the presence of large amounts of Bcl-2 in the cell. Although the system we used does cause high levels of expression of Bcl-2, we used confocal microscopy to examine cells that expressed different levels of human Bcl-2 and found that the distribution of Bcl-2 was quite similar in all the cells, which suggests that the distribution we see is not an artifact of overexpression.

Materials and Methods
Construction and Growth of RCASBPbcl-2 Virus. A 719-base pair restriction fragment which includes all of the human bcl-2 coding sequences (34) was subcloned into the adaptor plasmid CLA12NCO. To expedite cloning into the adaptor plasmid, the PCR was used to create an Ncol site at the bcl-2 initiator ATG. A SalI site was introduced at the TGA termination codon of the bcl-2 cDNA. The PCR product (730 base pairs) was digested with Ncol and SalI, and the resulting fragment was cloned into the adaptor vector via the Ncol and SalI sites in the plasmid. This plasmid was digested with ClaI, and the bcl-2 containing fragment was subcloned into RCASBP(A) and RCASBP(B). The ends of the insert were confirmed by DNA sequence analysis. Ten μg of either RCASBP(A)bcl-2 or RCASBP(B)bcl-2 DNA were introduced into CEF prepared from 13-day embryos of the EV-O line. The method of transfection has been described in detail elsewhere (35, 43). Infection of the fibroblasts was confirmed by detection of both the viral core protein and human Bcl-2 protein or by standard reverse transcriptase assays (35, 36). Immunofluorescence analysis was done on fibroblasts grown on coverslips. The cells were stained with an antibody to the viral protein p27 (SPAFAS, Inc.) and to the human Bcl-2 protein (37).
Fig. 9. Effects of Bcl-2 and c-Myc expression on CEF in the presence and absence of serum. Cells were grown to ~50% of confluence, washed with PBS, and placed in serum-free media or complete media. The cells were viewed and photographed using an inverted confocal microscope (see “Materials and Methods”). a–i, cells are shown after 7 h; j and k, after 72 h. CEF infected by RCASBP(B)bcl-2 are shown (a) unstarved and (b) starved. c, uninfected, starved CEF. CEF infected by both RCASBP(A)myc and RCASBP(B)bcl-2, unstarved (d) and starved (e). CEF infected by RCASBP(B), starved (f) and unstarved (g). CEF infected by RCASBP(A)myc, unstarved (h) and starved (i). CEF infected by RCASBP(A)myc, starved (j). CEF infected by both RCASBP(A)myc and RCASBP(B)bcl-2, starved (k). × 200.

Construction of the RCASBPmyc Virus. A full-length chicken c-myc cDNA clone⁵ was subcloned into the adaptor plasmid CLA12. This plasmid was digested with ClaI and subcloned into RCASBP(A). Ten μg of RCASBP(A)myc were introduced into CEF cells as described above. After three passages, all of the cells in the culture were transformed, and the culture was infected with RCASBP(B)bcl-2 virus. The cells were passed twice to allow complete spread of the RCASBP(B)bcl-2 virus, and the serum starvation experiments were initiated (see text).

Western Blotting. Protein concentrations were determined using bicinchoninic acid reagent (Pierce, Inc., Rockford, IL). Equal amounts of protein were fractionated by electrophoresis in 12.5% sodium dodecyl sulfate-polyacrylamide gels. The proteins were transferred to an Immobilon-P membrane (Millipore Corp., Bedford, MA) by

⁵ C. J. Petropoulos and S. H. Hughes, manuscript in preparation.
electrotransfer. The filter was pretreated with 0.3% H₂O₂ in methanol for 30 min at room temperature and rinsed with PBS. The filter was then blocked with 1% milk in PBS containing 0.02% azide at room temperature for 30 min before incubating with the mouse anti-Bcl-2 monoclonal antibody [clone 124; (37)] in 3% milk in PBS-azide at 4°C overnight. The filter was then washed four times, 5 min each, with 0.3% Tween 20 in PBS-azide. The filter was then reacted with a biotinylated goat anti-mouse IgG antibody, and subsequent development of the staining reaction was carried out using a Vectastain Kit using conditions recommended by the manufacturer (Vector Laboratories, Inc., Burlingame, CA; and ECL chemical luminescence/ECL Western blotting detection reagents; Amersham, Arlington Heights, IL).

Membrane Fractionation. CEF cultures were transfected with the RCASBPbcl-2 plasmid. After the 4th passage, cells were removed from the plate using trypsin-EDTA. The cells were then washed with PBS and suspended in a hypotonic buffer [42 mM KCl, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), and 5 mM MgCl₂]
and lysed by passage through a 26-gauge needle. The lysed cells were separated into subcellular fractions as described previously (7). Nuclei were pelleted at 200 × g for 10 min. The supernatant was subjected to centrifugation at 10,000 × g for 10 min, and the heavy membrane pellet was collected. The remaining supernatant was subjected to centrifugation at 150,000 × g for 90 min to give a light membrane pellet and a cytosolic supernatant.

Immunoprecipitation of Bcl-2 Protein. Labeling and immunoprecipitation of the Bcl-2 protein was performed essentially as described previously (44). CEF were transfected with RCASBPbcl-2 DNA and passaged four times. These CEF were shown to be infected and were then incubated with [35S]methionine (NEN; 1000 Ci/m mole) containing medium. Uninfected CEF were labeled in parallel. Cells were harvested, and lysates were prepared. The [35S]-labeled cell lysates were incubated with a polyclonal rabbit serum prepared to the human Bcl-2 protein (44) and in parallel with preimmune serum. The antigen-antibody complexes were isolated by incubation with protein A-agarose (Pharmacia). The immunoprecipitated proteins were released from the sepharose beads and analyzed on a 12% sodium dodecyl sulfate-polyacrylamide gel. The human Bcl-2 protein was translated in vitro from RNA made using T7 RNA polymerase (BRL). The RNA used for the in vitro translation was transcribed from a bcl-2 cDNA cloned into a vector pT3T7-18 (BRL).

Immunofluorescence and Microscopy. The primary antibody to human Bcl-2 (clone 124) was prepared using a peptide that corresponds to sequences in the NH2 portion of the human Bcl-2 protein (37), a region that is not present in the chicken Bcl-2 protein. As a consequence, this antibody does not cross-react with the endogenous chicken Bcl-2 protein. Cells were grown on 16-chamber Labtek slides (Nunc, Roskild, Denmark) for 20 h. After two washes with PBS, cells were fixed for 30 min in cold methanol (−20°C) and washed extensively with PBS. For immunofluorescence assays, the cells were blocked for 10 min using the blocking reagents in the Biomeda immunostaining kit (Biomed, Foster City, CA). The primary antibody (124 monoclonal) was diluted 1:100 in tissue culture media and incubated overnight at room temperature at a 1:1 dilution in primary antibody dilution buffer (Biomed, Foster City, CA). In the competition assay, 10 µg of the peptide was added to the primary antibody for 30 min at 37°C before incubation with the cells. The secondary antibody, a donkey antibody to mouse immunoglobulin coupled to fluorescin isothiocyanate, was diluted 1:100 in PBS (Jackson Immuno Research Laborato-

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Fig. 11. Time lapse laser confocal microscopy of CEF-infected with RCASBP-Amyc virus and ultrastructure of floating cells. Cells were serum starved at 50% of confluence. After 5.5 h of serum starvation, the medium was changed to remove floating cells, and the cells were photographed every 10 min using an inverted confocal microscope. Time points: a, 10 min; b, 70 min; c, 100 min; d, 140 min. Membrane blebbing characteristic of apoptosis can be seen. e, floating cells from CEF infected by RCASBP-Amyc; and by both RCASBP-Amyc and RCASBP (RbcI-2) were analyzed by electron microscopy. A representative apoptotic cell is shown. a-d, ×1000; e, ×7200.
ries, Westgrove, PA) and incubated for 1 h at room temperature. After extensive washing, cells were mounted on slides with gel mount (Biomeda). The cells were examined by CLSM as described previously (45).

**Serum Starvation Experiments.** Cells infected with RCASBP(B) vector, RCASBP(A/myc, RCASBP(B)bcl-2, and both RCASBP(A/myc and RCASBP(B)bcl-2, were grown to ~50% of confluence. Cultures were washed with PBS and placed in complete media (10% serum) or serum-free media. After 7 h, the cells were imaged using a Zeiss (Oberkochen, Germany) laser scanning confocal microscope (CLSM 410). This is an inverted microscope equipped with transmitted light, argon, and HeNe lasers. The cells were imaged using phase optics, and the images were printed using a Codonics NPK600 (Ohio) electronic printer.

For the time lapse cinemicroscopy, CEF infected with RCASBP(A/myc were grown to 50% of confluence, washed with PBS, and placed in serum-free media. After 5.5 h of serum starvation, the medium was changed to remove floating cells, and the cells were imaged at 10-min intervals using the CLSM 410 as described above.

**DAPI Staining.** CEF infected with the RCASBP(B) vector, with RCASBP(A/myc, with RCASBP(B)bcl-2, and with both RCASBP(A/myc and RCASBP(B)bcl-2 were grown to 50% of confluence. The cells were then washed with PBS and placed either in serum-free media or complete media. At various times, both the floating cells and the attached cells were washed with PBS and fixed with 3.7% formaldehyde for 10 min at room temperature. The cells were then washed with PBS and stained with DAPI (Boeringher Mannheim) at a concentration of 0.1 μg/ml in PBS for 10 min at room temperature. The cells were washed extensively, mounted on slides with gel mount (Biomeda), and examined using a Nikon Microphot-FXA Epifluorescence microscope with a Fluor 40X 0.05 n.a. objective. The cells were photographed with Kodak Ektachrome 400 film.

**Immunoelectron Microscopy.** The techniques used for postembodied immunoelectron microscopy are described in detail elsewhere (46, 47). Briefly, cells were washed two times with PBS, fixed in 4% paraformaldehyde and 0.5% glutaraldehyde for 20 min at 4°C, then dehydrated with a series of methanol washes and embedded in L.R. Gold (Electron Microscopy Sciences, Fort Washington, PA) at −20°C. Thin sections were cut at LKB Nova Ultramicrotome and picked up with formvar-carbon coated, glow-discharged 200 mesh gold grids. For postembodied immunolabelling of human Bcl-2 protein, thin sections were washed with PBS three times, incubated in 1% bovine serum albumin for 30 min, incubated with anti-Bcl-2 monoclonal antibody (clone 124) for 2 h at room temperature, thoroughly washed, and incubated with goat anti-mouse IgG-Gold 10 nm (Sigma, St Louis, MO) for 1 h at room temperature. The sections were then washed and stained with uranyl acetate and lead citrate. Sections were prepared from CEF infected with the RCASBP(bcl-2) virus and in parallel with CEF infected with the RCASBP vector. All preparations were observed at 80 kV with a Philips EM 410 transmission electron microscope.

**CEF infected with the RCASBP(A/myc virus and with both RCASBP(A/myc and RCASBP(B)bcl-2 viruses were serum starved for 24 h, and both the floating cells and the attached cells were fixed in 2.5% glutaraldehyde for 2 h and processed for normal transmission electron microscopy observation.**

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