Human bcl-2 Gene Attenuates the Ability of Rabbit Lens Epithelial Cells against H₂O₂-induced Apoptosis through Down-regulation of the αB-crystallin Gene*

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It is well established that the proto-oncogene, bcl-2, can prevent apoptosis induced by a variety of factors. Regarding the mechanism by which BCL-2 prevents cell death, one theory suggests that it acts by protecting cells from oxidative stress. In the lens system, oxidative stress-induced apoptosis is implicated in cataractogenesis. To explore the possibility of anti-oxidative gene therapy development for cataract prevention and also to further test the anti-oxidative stress theory of BCL-2 action, we have introduced the human bcl-2 gene into an immortalized rabbit lens epithelial cell line, N/N1003A. The stable expression clones of both vector- and bcl-2-transfected cells have been established. Treatment of the two cell lines with H₂O₂ revealed that bcl-2-transfected cells were less capable of detoxifying H₂O₂ than the control cells. Moreover, bcl-2-transfected cells are more susceptible to H₂O₂-induced apoptosis. To explore why bcl-2-transfected cells have reduced resistance to H₂O₂-induced apoptosis, we examined the expression patterns of several relevant genes and found that expression of the αB-crystallin gene was distinctly down-regulated in bcl-2-transfected cells compared with that in vector-transfected cells. This down-regulation was specific because a substantial inhibition of BCL-2 expression through antisense bcl-2 RNA significantly restored the level of αB-crystallin and, moreover, enhanced the ability of the bcl-2-transfected cells against H₂O₂-induced apoptosis. Introduction of a mouse αB-crystallin gene into bcl-2-transfected cells also counteracted the BCL-2 effects. Down-regulation of αB-crystallin gene was largely derived from changed lens epithelial cell-derived growth factor activity. Besides, αB-crystallin prevents apoptosis through interaction with procaspase-3 and partially processed procaspase-3 to prevent caspase-3 activation. Together, our results reveal that BCL-2 can regulate gene expression in rabbit lens epithelial cells. Through down-regulation of the αB-crystallin gene, BCL-2 attenuates the ability of rabbit lens epithelial cells against H₂O₂-induced apoptosis.

The proto-oncogene, bcl-2, was identified by its translocation (t(14;18)) and elevated expression in the follicular B-cell lymphomas (1). Subsequent studies revealed that BCL-2 is also expressed in developing B- and C-cells (2–5) and non-lymphatic tissues including the ocular lens (6). BCL-2 was initially found to prevent interleukin-3-dependent cells from apoptotic death upon withdrawal of the cytokine (7). Since then, BCL-2 has been shown to prevent cell death induced by a large number of factors such as calcium ionophore, serum and growth factor depletion, and γ-irradiation (reviewed in Refs. 8–10).

Regarding the mechanism by which BCL-2 prevents cell death, one theory suggests that it acts by protecting cells from oxidative stress (11, 12). BCL-2 could either reduce cellular generation of reactive oxygen compounds or block the activity of these compounds after they are formed (11, 12). Supportive evidence for this theory comes from the finding that in certain cell lines, bcl-2-transfected cells show greater resistance to various pro-oxidant treatment than mock-transfected cells (13–15), and anti-oxidants protect some cells from apoptosis induced by non-oxidative agents (11, 16–18).

However, in other cell lines, expression of BCL-2 does not protect the transfected cells against oxidative stress and oxidative stress-induced apoptosis (19, 20). Why BCL-2 does not protect against oxidative stress-induced apoptosis in these cells remains largely unknown. Furthermore, it is also reported that oxygen depletion has no effect on the induction of apoptosis and that BCL-2 protects against apoptosis without inhibiting the production or activity of reactive oxygen compounds (21, 22). Thus, depending on the types of cells and also the intracellular metabolic status, BCL-2 may provide protection through different mechanisms.

To study the mechanism by which BCL-2 prevents stress-induced apoptosis in the lens system, we have introduced the human bcl-2 gene into rabbit lens epithelial cells, N/N1003A (23). By using the established stable expression lines, pSFFV-N/N1003A (vector-transfected) and pSFFV-BCL-2-N/N1003A (bcl-2-transfected), we found that the bcl-2-transfected cells had an attenuated ability to metabolize H₂O₂ and to resist H₂O₂-induced apoptosis compared with the vector-transfected cells. To understand this attenuation, we have examined expression of several relevant genes in these two types of cells. Whereas expression of the anti-oxidative stress genes was hardly changed, expression of the endogenous αB-crystallin gene was significantly down-regulated. This down-regulation is specific as demonstrated with antisense inhibition of BCL-2 expression. When BCL-2 expression is substantially inhibited through antisense bcl-2 RNA, the endogenous αB-crystallin in

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these double-transfected cells is significantly restored. Restoration of a-B-crystallin expression enhanced the ability of the cells to resist H2O2-induced apoptosis. Moreover, an exogenous mouse aB-crystallin gene introduced into bcl-2-transfected cells also counteracted the BCL-2 effects. To understand how BCL-2 may down-regulate expression of aB-crystallin gene, we have examined the DNA binding activity of LEDGF, a positive regulator of aB-crystallin (24, 25), in vector- and bcl-2-transfected cells. Our results revealed that the DNA binding activity of LEDGF was also substantially down-regulated in BCL-2 expression cells. Moreover, overexpression of LEDGF in BCL-2 infected cells. Our results revealed that the DNA binding activity of LEDGF, a positive regulator of aB-crystallin, which prevents apoptosis, we have conducted immunoprecipitation-linked Western blot analysis. Our results revealed that aB-crystallin prevents induced apoptosis through interaction with both procaspase-3 and partially processed procaspase-3. Taken together, our results demonstrate that BCL-2 can down-regulate expression of the aB-crystallin gene in rabbit lens epithelial cells through modulating transactivity of LEDGF and possibly other transcription factors. Through down-regulated expression of aB-crystallin, which prevents apoptosis by preventing caspase-3 activation, BCL-2 attenuates the ability of N/N1003A cells to resist oxidative stress-induced apoptosis. Thus, our results reveal a unique mechanism explaining why BCL-2 is unable to prevent oxidative stress-induced apoptosis in these cells.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Various molecular biology reagents were purchased from Life Technologies, Inc.; Stratagene, La Jolla, CA; New England Biolabs, Beverly, MA; and Promega Biotech, Madison, WI. DNA and protein size markers were purchased from Life Technologies, Inc. Various antibodies were obtained from Roche Molecular Biochemicals and Transduction Laboratories, San Diego, CA. Radioactive compounds were obtained from Amersham Pharmacia Biotech. The culture medium and most other chemicals and antibiotics were purchased from Sigma and Life Technologies, Inc.

**Cell Culture**—The rabbit lens epithelial cells, N/N1003A, were grown in Eagle’s minimum essential medium (Sigma catalog number M0643) containing 10% rabbit serum (Sigma catalog number R4505) as described previously (23). The medium was prepared in ion-exchanged double-distilled water to give an osmolarity of 300 mosmol to 5°C and 7.2. All cells were kept at 37

**Establishment of Stable Expression Cell Lines**—The mammalian expression vector, pSFFV-neo, and the BCL-2 expression construct, pSFFV-Bcl-2, were cloned by restriction digestion and amplified in bacterial strain SBT. The plasmids containing the cDNAs encoding BCL-2 (27), catalase (33), glutathione peroxidase (34), 2-actin (35), GAPDH (36), and aB-crystallin (28) were amplified in bacterial strain DH5a and purified by two rounds of CsCl ultracentrifugation according to Ausbel et al. (29). The cDNAs were inserted into a retroviral vector by double gel purification (29), and labeled with [32P]dATP (Amersham Pharmacia Biotech, PR10204) according to Feinberg and Vogelstein (37).

**RNA Preparation and Northern Blot**—Total RNAs were extracted from N/N1003A and various stable expression cell lines as described previously (32) using a RNA extraction buffer, Trizol reagent (Life Technologies, Inc., catalog number 15596-026). For Northern blot, 25 μg of total RNAs was used for each sample. Other procedures such as gel electrophoresis, pre-hybridization, hybridization, washing, and exposure were conducted as described previously (30–32).

**Protein Preparation and Western Blot**—The total proteins were prepared from N/N1003A or various stable cell lines using 300 μg of extraction buffer. The extraction buffer contained 1% Nonidet P-40, 0.1% SDS, 0.1% deoxycholate, 0.1% DEPC, 0.1% H2O2, 30 μg/ml aprotinin, and 5 μg/ml pepstatin A with pH of the preparation adjusted to 7.4. After homogenization by passing through a 21-gauge needle, additional 10 μl of PMSC was added to each sample, which was incubated on ice for 30 min. After the cell lysate was centrifuged at 10,000 × g for 20 min at 4°C, the supernatant of each sample was collected and stored in aliquots at −70°C. For each sample, the protein concentration was determined according to Peterson (38). Fifty micrograms of total proteins in each sample were resolved by 6% (for LEDGF), 10% (for GFP), or 12% (for aB-crystallin) SDS-polyacrylamide gel electrophoresis and transferred into supported nitrocellulose membranes. The protein blots were blocked with 5% milk in TBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) overnight at 4°C and incubated with antihuman BCL-2 antibody or anti-GFP antibody (both from Roche Molecular Biochemicals), anti-caspase-3 antibody (Transduction Laboratories), anti-aB-crystallin antibody (a kind gift from Dr. Joseph Horwitz), or anti-LEDGF antibody (a kind gift from Dr. Toshimichi Shinobara) at a dilution of 1 to 1000 to 2000 μg/ml in 5% milk prepared in TBS. The secondary antibody was anti-mouse IgG (for anti-BCL-2, GFP, and caspase-3 antibodies) or anti-rabbit IgG (for anti-aB-crystallin and anti-LEDGF antibody)

1 The abbreviations used are: LEDGF, lens epithelial cell-derived growth factor; CAT, chloramphenicol acetyltransferase; GFP, green fluorescence protein; GFP-mcB, green fluorescence and mouse aB-crystallin fusion protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MEM, minimum essential medium; PMSC, phenylmethylsulfonyl fluoride; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; DTT, dithiothreitol.
anti-Ledgf/Pgim antibodies) at a dilution of 1:1000 (Amersham Pharmacia Biotech). Immunoreactivity was detected with an enhanced chemiluminescence detection kit according to the company’s instructions (ECL, Amersham Pharmacia Biotech).

Immunoprecipitation-linked Western Blot Analysis—Parental BCL-2 expression cells (pSFFV-Bcl-2-N/N1003A), vector-transfected BCL-2 expression cells (pEGFP/pSFFV-Bcl-2-N/N1003A), and mouse α-crystallin-transfected BCL-2 expression cells (pEGFP-mαC/pSFFV-Bcl-2-N/N1003A) were grown and treated with 10 μg/ml of anti-GFP antibody or 50 μg/ml of normal mouse serum and 50 μl of protease inhibitor mixture for 1 h on ice. After incubation, 50 μl of protein A/G plus-agarose was added into each incubated sample. These samples were then incubated overnight in a 4 °C refrigerator attached to a slow motion rotator. At the end of incubation, these samples were washed four times with RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) by spinning down 5 min at 10,000 × g. After the final wash, the pelleted samples were subjected to Western blot analysis as described above or for immunoprecipitation-linked Western blot analysis. To conduct immunoprecipitation-linked Western blot analysis, 500 μg of total proteins from vector- and mouse α-crystallin-transfected BCL-2 expression cells were incubated with 10 μg (in 50 μl) of anti-GFP antibody or 50 μl of normal mouse serum and 50 μl of protease inhibitor mixture for 1 h on ice. After incubation, 50 μl of protein A/G plus-agarose was added into each incubated sample. These samples were then incubated overnight in a 4 °C refrigerator attached to a slow motion rotator. At the end of incubation, these samples were washed four times with RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) by spinning down 5 min at 10,000 × g. After the final wash, the pelleted samples were subjected to Western blot analysis as described above using specific anti-α-crystallin or anti-caspase-3 antibodies.

Analysis of Transient Gene Expression—For reporter gene activity, the construct of chloramphenicol acetyltransferase (CAT) reporter gene driven by the mouse α-crystallin gene promoter (−427 to +44 (39)), together with the control construct expressing p-galactosidase (40, both were kind gifts from Dr. Joram Piatigorsky), was introduced into both vector- and BCL-2-transfected cells using electroporation (30). The transfected cells were grown in 100-mm culture dishes and then harvested after 48 h of growth for assays of β-galactosidase and CAT activities as described previously (29, 30).

For LEDGF-regulated α-crystallin expression, the construct, pCI-αGTP-Ledgf (the LEDGF cDNA was a kind gift from Dr. Yoshimichi Shinahara), and the vector, pCI, were introduced into BCL-2 expression cells using electroporation (30). The transfected cells were grown in 100-mm culture dishes and then harvested after 48 h of growth for Western blot analysis of LEDGF and α-crystallin expression.

Preparation of Nuclear Extracts—Both pSFFV-N/N1003A and pSFFV-Bcl-2-N/N1003A cells were cultured in 175-cm² flasks until confluence. The cells were washed twice with 5 ml of ice-cold PBS and then harvested into a 1.5-ml centrifuge tube with rubber policeman. Pelleted cells were rapidly suspended in 400 μl of hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10% glycerol, 10 mM KCl, 1 mM DTT, 0.5% Nonidet P-40, 0.5 mM PMSF, 100 μg/ml aprotinin) and incubated on ice for 15 min. After incubation the samples were centrifuged at 2,200 × g for 2 min. The pellet nuclei were resuspended in buffer D (20 mM HEPES, pH 7.9, 400 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 0.5 mM PMSF, 100 μg/ml of aprotinin, 0.5% Nonidet P-40) and incubated on ice for 20 min. The nuclei were further centrifuged at 8,900 × g for 5 min. The supernatant was collected and stored at −70 °C for gel mobility shifting assay.

Gel Mobility Shifting Assays—For gel mobility shifting assays, the following oligonucleotides were used: 5′-AAATATTGGGTTTTTTTTT-3′ for LEDGF-binding site and 5′-AAATATTTAAAAATTTTTTTTTTT-3′ for mutated LEDGF-binding site. Forty μg of nuclear extracts prepared from pSFFV-N/N1003A and pSFFV-Bcl-2-N/N1003A cells were incubated with 1 × 10⁷ cpm of 32P-labeled double-stranded synthetic oligonucleotide for 30 min at 30 °C in a binding shifting buffer (1 μl/ml poly(dI-dC), 25 μM HEPES, pH 7.9, 40 mM KCl, 3 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, and 10% glycerol). For competition experiments, 5-fold of the unlabeled double-stranded synthetic oligonucleotides were preincubated with the nuclear extracts for 10 min before the labeled probe was added into the reaction. In the pre-cleared experiments, 2 μg of antibody against LEDGF was preincubated with the nuclear extracts on ice for 10 min prior to addition of the 32P-labeled oligonucleotides. After the binding reaction, the mixtures were loaded onto 5% native polyacrylamide gel electrophoresis and detected by autoradiography.

RESULTS
Expression of Human bcl-2 in Rabbit Lens Epithelial Cells, N/N1003A—To study the mechanism by which BCL-2 protects lens epithelial cells from apoptosis, we transfected the rabbit lens epithelial cells with a human bcl-2 cDNA under control of a viral gene promoter. These cells have barely detectable endogenous BCL-2 (data not shown). Both the vector (26) and the

![Fig. 1. Analysis of human bcl-2 expression in rabbit lens epithelial cells](image1.png)

**FIG. 1.** Analysis of human bcl-2 expression in rabbit lens epithelial cells. A, Northern blot to detect the bcl-2 mRNA in pSFFV-N/N1003A and pSFFV-Bcl-2-N/N1003A. Top panel, ethidium bromide staining of the RNA gel to show the equal loading of the two samples. Bottom panel, autoradiography after hybridization with [32P]dATP-labeled bcl-2 cDNA and washed under high stringency. Note, a single bcl-2 mRNA band is detected only in the pSFFV-Bcl-2-N/N1003A cells. B, Western blot. The anti-human Bcl-2 antibody detected a single protein band of 25-kDa only in the pSFFV-Bcl-2-N/N1003A cells.

![Fig. 2. Effect of BCL-2 expression on the ability of N/N1003A cells to metabolize hydrogen peroxide](image2.png)

**FIG. 2.** Effect of BCL-2 expression on the ability of N/N1003A cells to metabolize hydrogen peroxide. N/N1003A cells (parental cell line), pSFFV-N/N1003A (vector-transfected control), and pSFFV-Bcl-2-N/N1003A (bcl-2-transfected) stable expression clones were cultured in MEM with 10% rabbit serum until confluence. Then, 3 × 10⁶ cells were plated into a 60-mm culture dish. After 12 h of culture, the media in three different cultures were replaced with serum-free MEM containing 150 μM (A) and 350 μM (B) H₂O₂ (the concentration at the starting point). The treatment was continued for 90 min. Hydrogen peroxide levels in the medium without cells or with each of three different types of cells were measured as described previously (31) at intervals of 15, 30, 45, 60, and 90 min.
BCL-2 expression construct (27) were introduced into the N/N1003A cells using electroporation (30). The stable expression clones of pSFFV-N/N1003A (vector-transfected) and pSFFV-Bcl-2-N/N1003A (bcl-2-transfected) were obtained by G418 selection (400 μg/ml). Following growth to confluence, both pSFFV-N/N1003A and pSFFV-Bcl-2-N/N1003A cells were harvested for extraction of total RNA and protein. Northern and Western blot analyses were conducted to confirm expression of the human bcl-2 gene in rabbit lens epithelial cells. As shown in Fig. 1, the mRNA and protein from the human bcl-2 gene were detectable only in the pSFFV-Bcl-2-N/N1003A cells but not in the pSFFV-N/N1003A cells. Thus, human BCL-2 was successfully expressed in rabbit lens epithelial cells.

Expression of Human BCL-2 in Rabbit Lens Epithelial Cells Attenuates the Ability of the Transfected Cells to Metabolize H$_2$O$_2$—After BCL-2 was successfully expressed in the rabbit lens epithelial cells, we next tested whether the expressed protein was functional. Previous studies (11–15) have suggested that BCL-2 can protect cells from induced apoptosis through reduction of cellular oxidative stress. Whether this is true in lens epithelial cells remains to be verified. To test this possibility, we exposed N/N1003A, pSFFV-N/N1003A, and pSFFV-Bcl-2-N/N1003A cells to 150 and 350 μM H$_2$O$_2$. Degradation of H$_2$O$_2$ in the culture dishes containing either N/N1003A, pSFFV-N/N1003A, or pSFFV-Bcl-2-N/N1003A cells or containing medium alone was monitored within 90 min using a chemical method as previously described (31). Our results revealed that the pSFFV-Bcl-2-N/N1003A cells were less capable of metabolizing H$_2$O$_2$ than the pSFFV-N/N1003A cells. As shown in Fig. 2A, when the cultures were incubated with 150 μM H$_2$O$_2$, about 100 μM H$_2$O$_2$ was left in the dish containing the pSFFV-Bcl-2-N/N1003A cells after 90 min of incubation. In contrast, only 80 μM H$_2$O$_2$ remained in the dish containing N/N1003A cells or pSFFV-N/N1003A cells after the same period of incubation. This difference was highly repeatable in four independent experiments. Moreover, when the starting concentration of H$_2$O$_2$ was increased to 350 μM in the treatment of the two cell lines, by the end of 90 min of incubation the H$_2$O$_2$ left in the pSFFV-Bcl-2-N/N1003A dish was about 80 μM higher than that either in the N/N1003A cell dish or in the pSFFV-N/N1003A cell dish (Fig. 2B). In the control dishes without any cells, the H$_2$O$_2$ concentration was only decreased slightly. Thus, to our surprise, expression of human BCL-2 in rabbit lens epithelial cells attenuated the ability of the transfected cells to metabolize H$_2$O$_2$.

pSFFV-Bcl-2-N/N1003A Cells Are Less Capable of Resisting H$_2$O$_2$-induced Apoptosis Than N/N1003A and pSFFV-N/N1003A Cells—Because pSFFV-Bcl-2-N/N1003A cells are less capable of metabolizing H$_2$O$_2$ than N/N1003A and pSFFV-N/N1003A cells, we predicted that bcl-2-transfected cells might be more susceptible to H$_2$O$_2$-induced apoptosis. To test this possibility, N/N1003A, pSFFV-N/N1003A, and pSFFV-Bcl-2-N/N1003A cells were incubated with a single dose of 150 μM H$_2$O$_2$. After 6–18 h of incubation, cell viability was examined using trypan blue exclusion and further verified with Hoechst staining (32). The four clones of pSFFV-Bcl-2-N/N1003A cells displayed 20, 22, 23.5, and 25% more apoptosis than the pS-
FFP1-N1003A or the parental line, N/N1003A, after 24 h of treatment (Fig. 3A). The apoptotic nature was verified using Hoechst staining (Fig. 3, B–D).

pSFFV-Bcl-2-N1003A Cells Are Resistant to Apoptosis Induced by Staurosporine and Camptothecin—One possible explanation for the lack of protection of BCL-2 on pSFFV-Bcl-2-N1003A cells from H2O2-induced apoptosis could be that the BCL-2 expressed in rabbit lens epithelial cells is not functional. Because BCL-2 activity is defined by its ability to inhibit apoptosis, we tested this possibility by subjecting pSFFV-Bcl-2-N1003A cells to staurosporine and camptothecin treatments that have been shown to induce apoptosis in different cells (41–44). When pSFFV-Bcl-2-N1003A, pSFFV-N1003A, and the parental N1003A cells were treated with 25 nM staurosporine or 10 μM camptothecin, the viability assay, Hoechst staining, and DNA fragmentation revealed that pSFFV-Bcl-2-N1003A cells are much more resistant to apoptosis induced by these two reagents. As shown in Fig. 4, A and B, after a 6-h treatment by staurosporine, only 4% of pSFFV-Bcl-2-N1003A cells were undergoing apoptosis, whereas 19% of N1003A or pSFFV-N1003A cells were undergoing apoptosis.

Another possible explanation for the lack of protection of pSFFV-Bcl-2-N1003A cells from H2O2-induced apoptosis could be that the expressed BCL-2 in pSFFV-N1003A, pSFFV-N1003A, and N1003A cells affects the expression of the anti-oxidative stress genes. To test this possibility, total RNAs were prepared from pSFFV-Bcl-2-N1003A, pSFFV-N1003A, and N1003A cells. An equal amount of total RNAs (25 μg per sample) from these cell lines was resolved with a 1.2% formaldehyde-agarose gel and transferred to supported nitrocellulose membranes. The RNA blot was sequentially hybridized to [α-32P]dATP-labeled catalase, glutathione peroxidase, β-actin, and GAPDH cDNA probes, washed under high stringency conditions, and then exposed to x-ray film as described previously (32). The top panel shows ethidium bromide staining of the RNA gel to display the equal loading of the three samples.

Expression of the Genes Encoding Anti-oxidative Stress Enzymes Is Not Changed in pSFFV-Bcl-2-N1003A, pSFFV-N1003A, and N1003A Cells—Another possible explanation for the lack of protection of pSFFV-Bcl-2-N1003A cells from H2O2-induced apoptosis could be that the expressed BCL-2 in rabbit lens epithelial cells affects the expression of the anti-oxidative stress genes. To test this possibility, total RNAs were prepared from pSFFV-Bcl-2-N1003A, pSFFV-N1003A, and N1003A cells. An equal amount of total RNAs (25 μg per sample) from these cell lines was resolved with a 1.2% formaldehyde-agarose gel and transferred to supported nitrocellulose filters. The RNA blot was sequentially hybridized to the following [α-32P]dATP-labeled cDNA probes: catalase, glutathione peroxidase, β-actin, and GAPDH after the previous probe was stripped with Tris-EDTA buffer (1 mM EDTA, 10 mM Tris-Cl, pH 8.0) heated to 80 °C. As shown in Fig. 5, the mRNA levels for catalase, glutathione peroxidase, β-actin, and GAPDH are very similar in the three RNA samples from pSFFV-Bcl-2-N1003A, pSFFV-N1003A, and N1003A cells, respectively. Thus, BCL-2 has almost no effect on expression of the major anti-oxidative stress genes as well as housekeeping genes in rabbit lens epithelial cells.
Expression of the αB-crystallin Gene Is Distinctly Down-regulated in pSFFV-Bcl-2-N/N1003A Cells—To further explore why BCL-2 cannot prevent pSFFV-Bcl-2-N/N1003A cells from H₂O₂-induced apoptosis, we investigated whether expression of the lens crystallin gene was affected by BCL-2. Previous studies have revealed that α-crystallins are molecular chaperones (45, 46) and can prevent apoptosis under a variety of conditions (47, 48). The N/N1003A cells express only αB-crystallin as previous studies (49) have demonstrated. Thus, we analyzed expression of αB-crystallin gene in pSFFV-Bcl-2-N/N1003A, pSFFV-N/N1003A, and N/N1003A cells. First, Northern blot analysis with total RNA from the three cell lines demonstrated that the mRNA level for αB-crystallin was barely detectable in pSFFV-Bcl-2-N/N1003A cells. In contrast, both N/N1003A and pSFFV-N/N1003A cells had similar amounts of αB-crystallin mRNA that appeared as a prominent band (Fig. 6A). To further confirm this down-regulation, we used specific anti-αB-crystallin antibody to conduct Western blot analysis. As shown in Fig. 6B, αB-crystallin protein was weakly detectable in pSFFV-Bcl-2-N/N1003A. In contrast, a strong αB-crystallin band was detected in the parental N/N1003A and pSFFV-N/N1003A cells. Thus, expression of BCL-2 in N/N1003A cells was associated with distinct down-regulation of expression of the αB-crystallin gene.

Inhibition of BCL-2 Expression Restores αB-crystallin Expression and the Ability against H₂O₂-induced Apoptosis in pSFFV-Bcl-2-N/N1003A Cells—To confirm that BCL-2 actually represses expression of αB-crystallin gene in pSFFV-Bcl-2-N/N1003A cells, we prepared an antisense bcl-2 expression construct, pZeoSV-antisense-bcl-2 using the vector, pZeoSV. Both the vector and the antisense construct were introduced into pSFFV-Bcl-2-N/N1003A cells, and the transfected cells were selected with Zeocin (300 μg/ml) and G418 (400 μg/ml). After selection, stable expression clones of pZeoSV-pSFFV-Bcl-2-N/N1003A and pZeoSV-antisense-bcl-2-pSFFV-Bcl-2-N/N1003A were obtained. Western blot analysis revealed that the antisense bcl-2 RNA was able to inhibit BCL-2 expression (top panel in Fig. 7A). When the same protein samples were analyzed for αB-crystallin expression, it was found that the level of αB-crystallin protein in pZeoSV-antisense-bcl-2-pSFFV-Bcl-2-N/N1003A cells was restored to the level close to that in pSFFV-N/N1003A cells (bottom panel in Fig. 7A). As in the pSFFV-Bcl-2-N/N1003A cells, the αB-crystallin protein in pZeoSV/pSFFV-Bcl-2-N/N1003A cells was down-regulated to a barely detectable level (bottom panel in Fig. 7A). Thus inhibition of BCL-2 expression in pSFFV-Bcl-2-N/N1003A cells restores the level of αB-crystallin expression. Most importantly, when αB-crystallin expression was increased, the ability of the transfected cells to metabolize H₂O₂ and to resist H₂O₂-induced apoptosis was also recovered (Fig. 7, C and F).

Expression of the Mouse αB-crystallin Counteracts the Effects of BCL-2 in Rabbit Lens Epithelial Cells—To further confirm that decreased expression of αB-crystallin accounts for the inability of the pSFFV-Bcl-2-N/N1003A cells to protect against oxidative stress-induced apoptosis, we prepared an expression construct in which the mouse αB-crystallin cDNA was inserted into a green fluorescence protein expression vector, pEGFP (48). Both the vector, pEGFP, and the expression construct, pEGFP-mαB, were introduced into the BCL-2 expression cells, pSFFV-Bcl-2-N/N1003A. Expression of the GFP-mouse αB-crystallin fusion protein was confirmed by Western blot analysis using a specific anti-αB-crystallin antibody (right lane in Fig. 8A) and also fluorescence microscopy (Fig. 8, B and C). Whereas the green fluorescence protein was distributed homogeneously in BCL-2 expression cells (Fig. 8B), the GFP-mαB fusion protein was localized only in the cytoplasm (Fig. 8C). When the parental cells, vector-, and mouse αB-transfected BCL-2 expression cells were subjected to H₂O₂ treatment, it was found that cells expressing both BCL-2 and αB-crystallin were more resistant to H₂O₂-induced apoptosis than the BCL-2 and GFP-expression cells or the BCL-2-transfected cells (Fig. 8D). Associated with the protection of cell death by exogenous mouse αB-crystallin expression, activation of caspase-3 found in pSFFV-Bcl-2-N/N1003A cells was largely repressed (Fig. 8E). These results suggest that the down-regulation of αB-crystallin in N/N1003A cells by BCL-2 is responsible for the attenuated ability of the pSFFV-Bcl-2-N/N1003A cells to protect against H₂O₂-induced apoptosis.

The Exogenous Mouse αB-crystallin Promoter Is Also Down-regulated in BCL-2 Expression Cells—To determine whether BCL-2 can regulate αB-crystallin gene from another species, we have introduced a CAT reporter gene driven by the mouse αB-crystallin gene promoter (39) into both vector- and BCL-2-transfected cells. As shown in Fig. 9, transient assays of relative CAT and β-galactosidase activities revealed that the mouse αB-crystallin gene promoter was also down-regulated by more than 2-fold. Thus, expression of human BCL-2 in rabbit
lens epithelial cells down-regulates both endogenous and exogenous αB-crystallin gene.

Down-regulation of αB-crystallin Is Largely Derived from Changed Activity of LEDGF in pSFFV-Bcl-2-N/N1003A Cells—To explore how BCL-2 may down-regulate expression of αB-crystallin gene, we have examined the DNA binding activity of LEDGF, a nuclear transcription factor, which can positively regulate expression of αB-crystallin gene (24, 25). As shown in Fig. 10A, when the nuclear extracts prepared from vector- and BCL-2-transfected cells were assayed, gel mobility shifting assays revealed that the vector-transfected cells contain a substantially higher level of binding activity to the LEDGF sites than the BCL-2-transfected cells (1st and 2nd lanes of Fig. 10A). This binding activity is contributed by LEDGF for three reasons. First, the unlabeled oligonucleotides containing LEDGF-binding site can compete off the labeled probe during the binding shifting assay (3rd and 4th lanes of Fig. 10A). Second, the pre-cleared nuclear extracts with anti-LEDGF antibody no longer gave DNA binding (5th and 6th lanes of Fig. 10A). Third, the oligonucleotides containing a mutated LEDGF site did not display any interaction with the nuclear extracts (7th and 8th lanes of Fig. 10A). Thus, down-regulation of αB-crystallin gene is parallel to decreased activity of LEDGF. To demonstrate that the decreased LEDGF activity is responsible for down-regulation of αB-crystallin expression, we have introduced the LEDGF expression construct into BCL-2 expression cells. As shown in Fig. 10B, expression of an exogenous LEDGF cDNA in BCL-2 expression cells substantially increases the expression level of αB-crystallin. Thus, down-regulation of αB-crystallin gene is largely derived from BCL-2-modified LEDGF activity.

αB-crystallin Prevents Apoptosis through Interaction with Procasps-3 and Partially Processed Procasps-3 in Rabbit Lens Epithelial Cells—Our previous study (48) revealed that αB-crystallin can prevent apoptosis by repressing caspase-3 activation. In BCL-2 expression cells, αB-crystallin also prevents caspase-3 activation by H2O2 (Fig. 8E). To explore how αB-crystallin represses caspase-3 activation, we have conducted immunoprecipitation-linked Western blot analysis. First, Western blot analysis revealed that the anti-GFP anti-
the fusion protein GFP-mN1003A cells (lane in A of either GFP alone from the vector (normalization against 
activity is expressed as counts/min per microgram of protein after 
sidase and CAT activities as described previously (29– 
H2O2 treatment. The pSFFV-Bcl-2-N/ 
imported into both vector- and BCL-2-transfected cells using electro-
together with the control construct expressing 
plasm (described under 
Expression of apoptosis and caspase-3 activity 
dishes and then harvested after 24 h of growth for assays of 
poration (30, 48). The transfected cells were grown in 100-mm culture 
introduced into pSFFV-Bcl-2-N/N1003A cells (left lane in A). Expression of either GFP alone from the vector (B) or 
the fusion protein GFP-maB (C) was also 
monitored by fluorescence microscopy. The 
vector-transfected clone (B) displays a ho-

Western blot analysis with anti-aB-crystal-
lin antibody revealed that GFP-maB fusion 
proteins were detected only in the pEGFP-maB or pSFFV-Bcl-2-N/N1003A cells (right lane in A) but not in pEGFP/pSFFV-Bcl-2-
N1003A cells (left lane in A). Expression of either GFP alone from the vector (B) or 
the fusion protein GFP-maB (C) was also 
monitored by fluorescence microscopy. The 
vector-transfected clone (B) displays a ho-

body recognized both GFP and the fusion protein of GFP and 
mouse aB-crystallin (Fig. 11A). This anti-GFP antibody was 
used to conduct immunoprecipitation of the total proteins ex-
tracted from parental BCL-2 expression cells, pEGFP vector, 
and mouse aB-transfected BCL-2 expression cells. The immu-
noprecipitated samples were then used for Western blot anal-
ysis. As shown in Fig. 11B, the specific anti-aB antibody iden-
tified the same 48-kDa band of GFP and mouse aB-crystallin 
fusion protein as the anti-GFP antibody did in pEGFP-maB/ 
pSFFV-Bcl-2-N/N1003A cells (top panel of Fig. 11B and also 
Fig. 11A). As expected, the specific anti-aB antibody did not 
recognize any specific protein in the precipitated samples from 
pSFFV-Bcl-2-N/N1003A cells and pEGFP/pSFFV-Bcl-2-N/ 
N1003A cells. When the immunoprecipitated samples were 
analyzed with anti-caspase-3 antibody, it was found that in 
pEGFP-maB/pSFFV-Bcl-2-N1003A cells, two specific bands 
were recognized, one migrated slightly above 29 kDa and an-
other below 29 kDa (bottom panel of Fig. 11B). The sizes of the 
two bands were equal to the 31-kDa procaspase-3 and the 
partially processed procaspase-3 (24 kDa), respectively. These 
two bands were disappeared when the anti-caspase-3 antibody 
was preincubated with purified recombinant caspase-3 protein 
(data not shown). Immunoprecipitation with normal mouse 
signal did not pull down either the 48 kDa GFP-maB-crystallin 
fusion protein or the procaspase-3 and partially processed pro-
caspase-3 bands (data not shown). Together, our results sug-
gest that mouse aB-crystallin can form a complex with both 
procaspase-3 and partially processed procaspase-3, which can 
be immunoprecipitated by anti-GFP antibody.

**DISCUSSION**

In the present communication, we have demonstrated the following. 1) BCL-2, when expressed in rabbit lens epithelial cells, can protect the transfected cells from apoptosis induced by a general protein kinase inhibitor, staurosporine, and also by a topoisomerase I inhibitor, camptothecin, suggesting that the expressed BCL-2 is functional in the lens epithelial cells. 2) BCL-2 expression cells are less capable of metabolizing H2O2 and of resisting H2O2-induced apoptosis. Thus, in lens cells,
Bcl-2 prevents apoptosis in certain non-anti-oxidative stress pathway. 3) BCL-2 can specifically down-regulate expression of the αB-crystallin gene. 4) The down-regulation of αB-crystallin gene is largely derived from changed activity of LEDGF. 5) The down-regulation of αB-crystallin gene leads to attenuation of the BCL-2-transfected cells against H$_2$O$_2$-induced apoptosis. 6) αB-crystallin prevents apoptosis by interacting with procaspase-3 and partially processed procaspase-3 to repress caspase-3 activation.

The Protective Role of BCL-2—The protective role of BCL-2 has been documented extensively (8–10) in many different cell and tissue types. Regarding the protection mechanism, one of the theories suggests that BCL-2 prevents cells from apoptosis by protecting them from oxidative stress. The supportive evidence is 2-fold. First, a number of laboratories have reported (11–15) that bcl-2-transfected cells show a greater resistance to various pro-oxidant treatments than the mock-transfected cells. Second, antioxidants protect some cells from apoptosis induced by non-oxidative reagents (16–18).

In the lens, oxidative stress and oxidative stress-induced apoptosis are implicated in cataractogenesis (31, 50–52). Prevention of stress-induced apoptosis may lead to potential gene therapy strategy against cataractogenesis. Thus, we have studied the mechanism of BCL-2 action for two reasons. First, if BCL-2 could prevent oxidative stress-induced apoptosis in the lens, it would be a candidate for gene therapy in preventing cataractogenesis. Second, exploration of the BCL-2 action in lens cells may contribute to clarification of whether BCL-2 prevents apoptosis through anti-oxidative stress pathway. For these purposes, we have introduced the human bcl-2 gene into rabbit lens epithelial cells and established the stable line expressing hBcl-2. A mock control cell line transfected with the same vector was also established. When the two cell lines were subjected to treatment of H$_2$O$_2$ at pathological concentrations in the eye as previously suggested (53), we found that the bcl-2-transfected cells are less capable of detoxifying H$_2$O$_2$ than the control cells. Moreover, we found that bcl-2-transfected cells were more susceptible to H$_2$O$_2$-induced apoptosis when they were treated with a concentration previously shown to induce apoptosis of lens epithelial cells (31). Our results are consistent with cell lines such as EW-36 (19, 20) but different from the results in other cell lines such as mouse neural cell line (11) or Pro-B-cell line (12). Such differences among different cell lines may reflect the intracellular property of these cell lines or the functional status of the expressed BCL-2 protein. To confirm that human BCL-2 is functional in rabbit lens epithelial cells, we subjected the bcl-2- and vector-transfected cells to staurosporine and camptothecin treatment, both previously shown to induce typical apoptosis (41–44). As expected, the bcl-2-transfected cells were more resistant to staurosporine- and camptothecin-induced apoptosis than the vector-transfected cells. Thus, our results suggest that BCL-2 prevents apoptosis in a non-anti-oxidative stress pathway in lens cells.

Next we explored why BCL-2 expression cells were less capable of degrading H$_2$O$_2$ and more susceptible to H$_2$O$_2$-induced apoptosis. One of the possibilities tested is whether overexpression of BCL-2 in rabbit lens epithelial cells could down-regulate expression of the anti-oxidative genes as indirectly shown in the BCL-2 knockout mice (54). Hochman et al. (54) demonstrated that in the liver of the knockout mice, the activities for both catalase and glutathione peroxidase, both of which are the major enzymes responsible for detoxifying H$_2$O$_2$ (55). Our results revealed hardly any difference in the mRNA levels for the two enzymes between bcl-2-transfected and vector-transfected cells, thus excluding the possibility of differential expression of the anti-oxidative genes.

In the lens, another set of important proteins involved in cellular protection is the lens crystallin, especially the α-crystallin. In the rabbit lens epithelial cells, previous studies (49) have revealed that β- and γ-crystallins are not expressed in...
were subjected to Western blot analysis as described above using specific analysis. Five hundred down-regulation of also an anti-apoptotic protein (47, 48), it is possible that the B-cl crystallin is a molecular chaperone (45, 46) and B-crystallin gene, human BCL-2 attenuates the B-crystallin gene promoter (Fig. 9). Thus, depending upon the cell type or the specific target gene, BCL-2 can exert either positive or negative regulation of expression of other genes. A recent study from Vairo et al. (58) further supports this point. In the mouse fibroblasts, Vairo et al. (58) demonstrate that BCL-2 up-regulates accumulation of p27 and p130 proteins but down-regulates the level of p107 protein from G0 to S transition during the cell cycle of the fibroblasts. This differential regulation allows BCL-2 to retard fibroblast cells entering into the cell cycle (58).

How could BCL-2 regulate gene expression? It is well established that BCL-2 can modulate transactivities of different transcription factors. For example, NF-xB, an important transcription factor mediating multiple signaling pathways (59), is positively regulated by BCL-2 (60–62). By changing the affinity of IxB to NF-xB, BCL-2 can up-regulate the transactivity of NF-xB and expression of NF-xB-responsive genes such as that encoding the matrix metalloproteinase-9 (61). The tumor suppressor, p53, is another target negatively regulated by BCL-2. Zhan et al. (62) found that in the human Burkitt’s lymphoma WMN cell line, BCL-2 specifically suppresses the p53-mediated transactivation of p21WAF1 and GADD45 after treatment with methylmethane sulfonate or UV irradiation. In human kidney 293 cells and MCF7 cells, Froesch et al. (63) also observed that overexpression of BCL-2 down-regulates p53 transactivity without affecting nuclear accumulation of p53 protein. In BCL-2 expression rabbit lens epithelial cells, we have examined the DNA binding activity of LEDGF. This lens epithelial cell-derived growth factor is a transcription factor that positively regulates expression of B-crystallin gene (24–25). As expected, in BCL-2 expression cells where B-crystallin is distinctly down-regulated, the DNA binding activity of LEDGF is substantially decreased (Fig. 10A). This decreased LEDGF activity contributes substantially to the down-regulation of B-crystallin gene because expression of the exogenous LEDGF in BCL-2 expression cells significantly up-regulates the expression level of B-crystallin (Fig. 10B). Of course, BCL-2-modulated changes in other transcription factors may also contribute to down-regulation of B-crystallin.

Mechanism by Which B-crystallin Prevents Apoptosis—Since the first demonstration that B-crystallin is able to prevent apoptosis induced by staurosporine (47), several laboratories have demonstrated that this molecular chaperone can provide cellular protection from a variety of stress conditions.
For example, αB-crystallin has been shown to prevent apoptosis induced by UVA irradiation (64). In the transgenic mice overexpressing αB-crystallin, the expressed protein confers simultaneous protection against cardiomyocyte apoptosis during myocardial ischemia and reperfusion (65). In our recent study (48), we have demonstrated that αB-crystallin is able to prevent apoptosis induced by okadaic acid, and moreover, it does so by repressing caspase-3 activation. In the present communication, we have demonstrated that αB-crystallin also prevents cells from oxidative stress-induced apoptosis through repression of caspase-3 activation (Fig. 8E). How can αB-crystallin repress caspase-3 activation? The chaperone property of αB-crystallin suggests that it could bind to procaspase-3 to prevent caspase-3 activation by other proteases. A recent study (66) demonstrates that αB-crystallin indeed binds to the partially processed procaspase-3 intermediate. Here we present evidence to show that αB-crystallin can bind to both procaspase-3 and partially processed procaspase-3. Thus, one of the mechanisms for αB-crystallin to prevent apoptosis is to interact with procaspase-3 and partially processed procaspase-3 to repress caspase-3 activation.

It is also possible that αB-crystallin may prevent apoptosis through other mechanisms. For example, αB-crystallin is an autokine (67, 68), and it might modulate phosphorylation status of apoptosis regulators in BCL-2 family or other upstream death regulators. Recent studies (69–72) have shown that the anti-apoptotic function of BCL-2 and BCL-XL and the anti-apoptotic ability of BAD and BIK can be dramatically changed by phosphorylation. It remains to be determined whether αB-crystallin can actually phosphorylate any of these cell death regulators.

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