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The effects of α B-crystallin on mitochondrial death pathway during hydrogen peroxide induced apoptosis

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ABSTRACT

α B-crystallin, a major small heat shock protein, has recently been shown to exert inhibitory effects on apoptosis, while the responsible mechanisms remain largely unknown. In the present study, we discovered that α B-crystallin protected mouse myoblast C₂C₁₂ cells against oxidative stress-induced apoptosis. During hydrogen peroxide-induced apoptosis, α B-crystallin showed that it decreased the redistribution level of phosphatidylserine (PS), reduced the release of cytochrome C and Smac/Diablo from mitochondria into cytoplasm, and decreased the cleavage of Bid. Interestingly, immunoprecipitation experiments with anti- α B-crystallin and anti-myc-tag antibodies demonstrated respectively an interaction between α B-crystallin and p53 during hydrogen peroxide induced apoptosis. Both the NH₂-terminal and COOH-terminal regions of α B-crystallin could interact with p53, suggesting two domains of α B-crystallin are necessary for the interaction. Electrophoresis mobility shift assay (EMSA) and luciferase assay further demonstrated that α B-crystallin inhibited the upregulation of the DNA-binding, as well as the transactivation activity of p53 induced by hydrogen peroxide. Our results show that α B-crystallin has a protective role in oxidative stress induced apoptosis by interference with the mitochondrial death pathway.

Keywords: α B-crystallin, p53, Cytochrome C, Smac/Diablo, Bid, mitochondria, hydrogen peroxide, apoptosis

INTRODUCTION

Apoptosis is an important cellular process in many physiological and pathologic conditions. This endogenous death event processes via two principal signaling pathways (Zhao et al. 2002; Gill, et al. 2002). One is the death receptor signaling pathway and the other is the mitochondrial death signalling pathway, in which many of the stress stimulus triggers the permeabilization of mitochondrial outer membrane and the release of proapoptotic proteins, such as

cytochrome c and Smac/Diablo into the cytoplasm, resulting in the activation of Caspases cascade and the induction of apoptosis (Adrain et al. 2001; Kuwana et al, 2003). In this signalling pathway, the permeabilization of mitochondrial outer membrane (MOMP) is considered to be the key step in the early apoptotic events (Green et al. 2004), and is likely to be controlled and influenced by many factors, including the Bcl-2 family members (Kuwana et al. 2003; Harada et al. 2003), transcription factor p53 and nuclear factor- κ B (NF- κ B) (Haupt

et al. 2003; Chipuk, et al. 2004; Baptiste et al. 2004; Karin et al. 2002, Zamora et al. 2004), and some heat shock proteins (HSPs) (Gill et al. 2002, Parcellier et al. 2003). So far, two groups of Bcl-2 family proteins (pro-apoptotic proteins, Bax, Bak, Bad, Bid, Bcl-Xs and anti-apoptotic proteins, Bcl-2, Bcl-X_L) are the most well characterized regulators. The balance between these two functional groups dominates the status of MOMP and the determination of apoptosis (Kuana et al. 2003; Green et al. 2004; Harada et al. 2003). p53, another regulator of apoptosis, works through either the transcription regulation of some Bcl-2 family members, or the direct interaction with these proteins after the translocation to mitochondrial from cytoplasm (Zhao et al. 2002; Haupt et al. 2003; Chipuk et al. 2004, Baptiste et al. 2004).

Recently, several heat shock proteins (HSPs) have been shown to inhibit apoptosis by selectively interfering with mitochondrial death signalling pathway in various cell types (Parcellier et al. 2003). For instance, HSP90 plays an important anti-apoptotic role via binding with Apaf-1 to prevent apoptosome formation (Pandey et al. 2000), interacting with Bid to inhibit its cleavage (Zhao C et al. 2004), and regulating the Bcl-2 expression (Dias et al. 2002). HSP70 inhibits apoptosis through its interaction with apoptosis-inducing factor (AIF) (Gurbuxani et al. 2003) and Apaf-1 (Saleh et al. 2000). HSP70 also reduces the release of cytochrome c from the mitochondria (Tsuchiya et al. 2003). HSP60 suppresses apoptosis by modulating and interacting with Bcl-2 family members (Shan et al. 2003; Gupta et al. 2002). HSP27 negatively regulates apoptosis by sequestering both procaspase 3 and cytochrome C (Bruey et al. 2000; Concannon et al. 2001). HSP27 delays the release of cytochrome c from mitochondria (Paul et al. 2002).

α B-crystallin, a major small heat shock protein, is abundantly expressed in eye lens and striated muscle tissues (heart and skeleton muscle) (Clark et al. 2000). It also

shows the resistance to apoptosis induced by different stresses in cells and animals (Morrison et al. 2003, 2004; Mao et al. 2001; Andley et al. 2000, Kamradt et al. 2002), however. But the mechanisms are poorly understood. Recent studies indicate that α B-crystallin negatively regulates TNF- α and DNA damage-induced apoptosis by directly binding to p24, a partially processed caspase-3 intermediate, therefore inhibits the autoproteolytic maturation of caspase-3 (Kamradt et al. 2001). This inhibition of α B-crystallin on caspase-3 activation has been proven in several kinds of cells (Morrison et al. 2003; Kamradt et al. 2002; Alge et al. 2002; Liu et al. 2004; Kamradt et al. 2005). More recently, studies reveal that α B-crystallins protect human lens epithelial cells from Staurosporine-induced apoptosis through binding Bax and Bcl-Xs, which are two pro-apoptotic Bcl-2 family proteins which function in the upstream of mitochondrial death pathway, and by sequestering their translocation into mitochondria (Mao et al. 2004).

In the present study, we discovered that α B-crystallin protected mouse myoblast C₂C₁₂ cells against oxidative stress-induced apoptosis. During hydrogen peroxide-induced apoptosis, α B-crystallin in C₂C₁₂ cells reduced the redistribution level of phosphatidylserine (PS) and the release of cytochrome C and Smac/Diablo from mitochondria, and decreased the Bid cleavage. Immunoprecipitation experiments with anti- α B-crystallin and anti-myc-tag antibodies demonstrated that an interaction of α B-crystallin with p53 existed during apoptosis. Both the NH₂-terminal (Met1-Ser59) and COOH-terminal (Trp60-Lys175, " α -crystallin domain") regions of α B-crystallin, (Narberhaus et al. 2002) could interact with p53. By electrophoresis mobility shift assay (EMSA) and luciferase assay, we further demonstrated that α B-crystallin inhibited hydrogen peroxide induced the upregulation of DNA-binding and transactivation activities of p53. Our results indicated that an interaction of α B-crystallin and p53 existed, and α B-crystallin

also decreased the p53 transcriptional activity during oxidative stress induced apoptosis.

MATERIALS AND METHODS

1. Plasmid construction

Wild type α B-crystallin and its NH₂-terminal region (Met1-Ser59) and COOH-terminal region (Trp60-Lys175, containing “ α -crystallin domain”) were constructed into pcDNA3.1-myc-his(-)B(Invitrogen) and pEGFP-C₂(Clontech), respectively. The primers were used as following: for wild type α B-crystallin(1-175): CG GAA TTC GAC ATA GCC ATC CAC CAC CCC (forward) and GG GGT ACC CTT CTT AGG GGC TGC AGT GAC (reverse); for NH₂-terminal region(Met1-Ser59) of α B-crystallin: □GG GAA TTC GAC ATA GCC ATC CAC CAC CCC (forward) and GG GGT ACC CCA GCT AGG TGC CCG CAG GAA(reverse); for COOH-terminal region (Trp60-Lys175) of α B-crystallin: □GG GAA TTC ATT GAC ACT GGG CTC TCA GAG (forward) and GG GGT ACC CTT CTT AGG GGC TGC AGT GAC (reverse). *EcoR* I and *Kpn* I sites were created at each end of the peptide by polymerase chain reaction according to the structure of plasmids. The constructs were amplified in DH5 α and purified by plasmid purification kit (Qiagen).

2. Culture of mouse myoblast C₂C₁₂ cells

Mouse myoblast C₂C₁₂ cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% newborn calf serum (Gibco). The medium was prepared in ion-exchanged double-distilled water supplemented with 26mmol/L NaHCO₃ and 100U/ml penicillin and streptomycin. The medium were sterilized by filtration through 0.22- μ m filters with pH adjusted to 7.2. Cells were kept in 5% CO₂ and 95% air and at 37°C.

3. Stable transfection and analysis

pcDNA3.1-myc-his plasmids and pEGFP-C₂ plasmids expressing wild type α B-crystallin(1-175), NH₂-terminal region(Met1-Ser59) and COOH-terminal region (Trp60-Lys175) of α B-crystallin, were each stably

transfected into C₂C₁₂ cells using Lipofectamine2000(Invitrogen). The transfected cells were then subjected to 1,000 μ g/ml of G418 selection for 4-6 weeks before individual clones were obtained and identified by Western blot analysis. All these stable clones were maintained with 100 μ g/ml of G418.

4. Treatment of hydrogen peroxide (H₂O₂)

Cells were grown in DMEM containing 10% newborn calf serum until confluence. After 2 \times 10⁶ cells were plated into a 60cm² culture bottles for 12 h, the medium were replaced with 10ml of DMEM containing 0.5mmol/L of H₂O₂ for the induction of apoptosis. In control groups, cells were treated with DMEM without H₂O₂. Finally, the cells were collected and subjected to the indicated analysis procedures.

5. Protein extraction

For total protein: Whole-cell extracts were prepared essentially as described (Li *et al.* 1998). Cells were incubated with lysis buffer (10mmol/L Tris-HCl, pH 8.0, 1mmol/L EDTA, 2% SDS, 5mmol/L DTT and 10mmol/L PMSF, supplemented with 1 tablet/10ml of protein inhibitor cocktail) for 30min on ice. After sonication, the lysates were centrifuged at 13,000rpm for 10min at 4°C; the supernatants were collected as total soluble protein for further analysis.

For mitochondria protein: Cells were harvested by centrifugation at 850 g for 2 min. Intact mitochondrial fractions were isolated using OptiprepTM mitochondria isolation kit (Pierce) according to the instruction, except that the final centrifugation was taken at 3,000g instead of 12,000g to get purer mitochondrial fraction.

6. Western Blot analysis

After protein quantification by the BCA assay Reagent (Pierce), 100 μ g aliquots of protein lysate were mixed with sample buffer and boiled for 5 min. The samples were transferred to a Polyvinylidene Difluoride (PVDF) membrane (Millipore) after the separation by electrophoresis on a 10% SDS-

PAGE. The membrane was incubated in blocking buffer (PBS containing 5% skim milk and 0.1% Tween20) for 2 h, followed by incubation with the primary antibody diluted in the same buffer. After washing in PBS for 5 times, the membrane was then incubated with the proper secondary antibody for 1h. All the incubations were performed at room temperature. At last, the membrane were detected by supersignal chemiluminescence system (Pierce) followed by exposure to an auto-radiographic film.

7. Immunoprecipitation

After apoptosis induction, cells were lysed in ELB buffer (140 mmol/L NaCl, 0.5% NP40, 50 mmol/L Tris-HCl, pH8.0, plus protease and phosphatase inhibitors) at a final concentration of 1×10^6 cells/ml, and incubated on ice for 30min. Lysates were then centrifuged at 12,000rpm at 4°C for 15min. The supernatant was taken and quantified with BCA assay. Protein G-Sepharose beads (Amersham Biosciences) together with extracts were precleared in 1 ml of lysis buffer at 4°C for 30 min. After precleared, the supernatant was incubated with 5 μ l of rabbit anti- α B-crystallin antibody or rabbit IgG for 1 h at 4°C. After incubation, 50 μ l of protein G-sepharose was added into each incubated sample. These samples were then incubated at 4°C overnight while gently shaking. On the following day, beads were washed 5 times with lysis buffer plus inhibitors and boiled in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer for loading onto a gel of 12% SDS-PAGE for Western Blotting assay.

8. Apoptosis Assay and Annexin V-EGFP assays by flow cytometry

For analysis of apoptosis attached and floating cells were harvested, rinsed with PBS by centrifugation, resuspended in 10 mmol/L HEPES pH 7.4, 140 mmol/L NaCl, 5 mmol/L CaCl_2 , and fixed in 70% ethanol. Cells were stained with propidium iodide (100 μ g/ml; Molecular Probes) to label dead cells and annexinV-EGFP (BD) to label

apoptotic cells before sorting on a FACS flow cytometer (Becton-Dickinson). Apoptosis was determined as the number of cell staining positive for annexinV.

9. Electrophoresis mobility shift assay (EMSA)

For EMSA (LightShift™ Chemiluminescent EMSA Kit, Pierce), 5 μ g of nuclear extract was incubated with 2mmol/L of the end-labeled with biotin double-stranded oligonucleotide probes in reaction buffer for 20min at room temperature. For supershift reactions, 1 μ l of p53 antibody was titration into the EMSA reaction mixture to the final concentration of 1mM. Samples were resolved on a nondenaturing 4% polyacrylamide-2% glycerol gel, transferred to Biodyne® B Nylon membrane, avidin-HRP to probes, and visualized and quantitated with a phosphorImager. The double-stranded probes were synthesized as follows: 5'biotin-TCC TAC AGA ACA TGT CTA AGC ATG CTG GGG ACT G-3'.

10. Luciferase assay

Cell were co-transfected with p53 luciferase reporter DNA and β -galactosidase reporter plasmids by the Lipofectamine 2000 (Invitrogen). The p53-pGL3 luciferase reporter plasmid, which contained the firefly luciferase gene under the control of p53-responsive element, was a generous gift of Prof. Yuan ZM (Department of Cancer Cell Biology, Harvard University School of Public Health, USA). After harvest, cells were lysed in Reporter Lysis Buffer (Promega, Madison, WI) after the transfection for 24 h, and assayed immediately, 20 μ l of cell extracts plus 100 μ l luciferase assay mix were mixed and assayed for 10s in Lumi-Scint. All luciferase activities were normalized for protein concentration and transfection efficiency using BCA and RSV- β -galactosidase assay respectively. All experiments were performed in triplicate and repeated at least three times.

RESULTS

1. Overexpression of α B-crystallin in C₂C₁₂ cells.

To study the mechanisms by which α B-crystallin protects cells from hydrogen peroxide-induced apoptosis, we constructed a fusion protein of α B-crystallin with an epitope tag of Myc-His₆ using the pcDNA3.1-myc-his(-)B vector. The expression construct (pcDNA 3.1- α B) and the vector construct (pcDNA 3.1) were both stably transfected into mouse myoblast C₂C₁₂ cells by Lipofectamine 2000. The cells stably expressing high levels of α B-crystallin (pcDNA 3.1- α B- C₂C₁₂) were obtained after the 4-6 weeks selection period by G418 (1,000 μ g/ml). As shown in Figure 1, the fusion protein was highly expressed in C₂C₁₂ cells (Clone1 and 2) determined by Western blot analysis using anti- α B-crystallin and anti-His₆ tag antibodies respectively (Figure 1).

2. α B-crystallin prevented hydrogen peroxide-induced phosphatidylserin externalization

α B-crystallin has been shown to prevent apoptosis induced by various agents including staurosporine, etoposide, hydrogen peroxide, TNF, UVA and calcimycin (Kamradt et al. 2005). Recent studies demonstrate that α B-crystallin could bind to caspase-3 intermediate and suppress its activation (Kamradt et al. 2001). However, the roles of α B-crystallin in the early apoptosis stage remains to be unknown. Redistribution of phosphatidylserine(PS) from the inner leaflet to the outer leaflet of the plasma membrane occurs early and is a hallmark of the apoptotic process. Annexin V, a high affinity to PS, has been used to detect early apoptotic cells (van Engeland et al. 1998; Green et al. 2002). Here we used an EGFP labeled Annexin V and flow cytometry to detect the degree of PS Redistribution. As shown in Figure 2, after H₂O₂ (0.5 mmol/L) treatment for 3 h, the ratio of PS positive cells in vector group (pcDNA 3.1) significantly increased (from

0.33% to 6.78%), while in α B-crystallin cells (pcDNA 3.1- α B), the ratio just slightly increased (from 0.23% to 0.96%), suggesting α B-crystallin could obviously prevent hydrogen peroxide induced apoptosis in the early stage.

3. α B-crystallin reduced the release of cytochrome C and Smac/Diablo after the treatment of hydrogen peroxide

The mitochondrial death pathway is a principal mechanism of apoptosis, which the release of cytochrome C and Smac/Diablo from mitochondria into the cytoplasm is a crucial event of early apoptosis (Zhao et al. 2002; Adrain et al. 2001). So, we observed the effect of α B-crystallin on the release of cytochrome C and Smac/Diablo from mitochondria induced by hydrogen peroxide. As data shown in figure 3, the levels of cytochrome C and Smac/Diablo in the cytosol of α B-crystallin transfected and vector transfected cells increased after H₂O₂ (0.5mmol/L) treatment for 1 h, but the release of cytochrome C and Smac/Diablo to the cytoplasm in α B-crystallin transfected cells reduced significantly as compared with the vector transfected cells. The results indicated that α B-crystallin could decrease the release of cytochrome C and Smac/Diablo from mitochondria during hydrogen peroxide induced apoptosis.

4. α B-crystallin decreased hydrogen peroxide-induced Bid cleavage

The Bcl-2 family proteins regulate mitochondrial apoptosis and consist of both anti-apoptotic and pro-apoptotic members. Bid belongs to the BH3-only subgroup of the pro-apoptotic molecules and serves as a sentinel to diverse apoptotic signals (Harada et al. 2003; Esposti et al. 2002; Goonesinghe et al. 2004; Choi et al. 2004; Wang et al. 2004). The full length of Bid becomes the activation form after proteolytic cleavage, especially by apical caspases like caspase 8. Once activated, the truncated Bid (tBid) induces the release of apoptotic factors including cytochrome C and Smac/Diablo after the translocation from cytoplasm into the mitochondria (Esposti et al. 2002;

Goonasinghe et al. 200). Thus Bid activation (tBid formation) is an important event in the upstream of mitochondrial death pathway.

To further determine whether α B-crystallin could protect cells from oxidative stress-induced apoptosis at an earlier stage of apoptosis, we examined Bid activation in α B-crystallin transfected cells and control cells after the treatment of H₂O₂ (0.5mmol/L) for 15, 30, 60 and 180 min. Data in figure 4 demonstrated that hydrogen peroxide promoted tBid formation in both vector transfected cells and α B-crystallin transfected cells, but tBid formation was obviously attenuated in α B-crystallin transfected cells as compared with the vector cells, indicating a protection role of α B-crystallin at the upstream step of mitochondrial signalling pathway.

5. Interaction of p53 and α B-crystallin increased during hydrogen peroxide induced apoptosis

α B-crystallins bind to proapoptotic proteins including p24 intermediate of caspase-3, Bax and Bcl-X_s and prevent their autoproteolytic maturation or translocation during apoptosis in tumor, lens, myofibril cells (Kamradt et al. 2001, Mao et al. 2004). In the present study, we detected the possible interactions between α B-crystallin and some other proteins related to the mitochondrial signalling pathway by immunoprecipitation experiments. In C₂C₁₂ cells, data showed that α B-crystallin bound to p53 in the normal conditions. After the treatment of hydrogen peroxide for 1 h, the interaction of p53 and α B-crystallin increased significantly (Figure 5-1). We further confirmed the interaction in α B-crystallin transfected C₂C₁₂ cells using anti-myc-tag antibody, we found that the interaction of p53 and α B-crystallin also increased after the treatment of hydrogen peroxide in figure 5-2.

To confirm that which domain of α B-crystallin is responsible for the interaction of p53, we constructed two plasmids expressing

NH₂-terminal domain of α B-crystallin (1-59 amino acid) and COOH-terminal domain of α B-crystallin (60-175 amino acid, including “ α -crystallin” domain of all sHSP family members) respectively and stably transfected in C₂C₁₂ cells (figure 5-3). Then we detected the interaction domain of α B-crystallin with p53. Data in figure 5-4 showed that p53 could bind to both domains of α B-crystallin and the interaction of NH₂- α B-crystallin with p53 was much stronger than the interaction of COOH- α B-crystallin with p53.

We also observed the distribution of GFP fusion protein of wild type α B-crystallin and its two domains in transfected C₂C₁₂ cells. Data in figure 5-5 showed that α B-crystallin and its 60-175 amino acids domain were located in the cytoplasm of C₂C₁₂ cells, but the 1-59 amino acids domain of α B-crystallin was distributed in the cytoplasm and nucleus.

6. α B-crystallin inhibited hydrogen peroxide induced p53 DNA-binding and trans-activation activity

Since p53 exerts its proapoptotic function mainly through the regulation of many apoptosis related the transcription of genes, such as Bax, Bid, PUMA, Noxa and apaf-1 (Haupt et al. 2003), we further detected whether α B-crystallin could affect the DNA binding and transactivation activities of p53 induced by hydrogen peroxide using EMSA and luciferase reporter gene analysis respectively. Data in figure 6-1 showed that hydrogen peroxide increased the DNA binding activity of p53 while α B-crystallin decreased the DNA binding activity after the treatment of hydrogen peroxide. Also, data in figure 6-2 demonstrated that hydrogen peroxide increased the transactivation activity of p53 significantly, while α B-crystallin decreased the transactivation activity of p53. The findings indicated an inhibition role of α B-crystallin on the nuclear function of p53 including DNA binding and transactivation activities during hydrogen peroxide induced apoptosis.

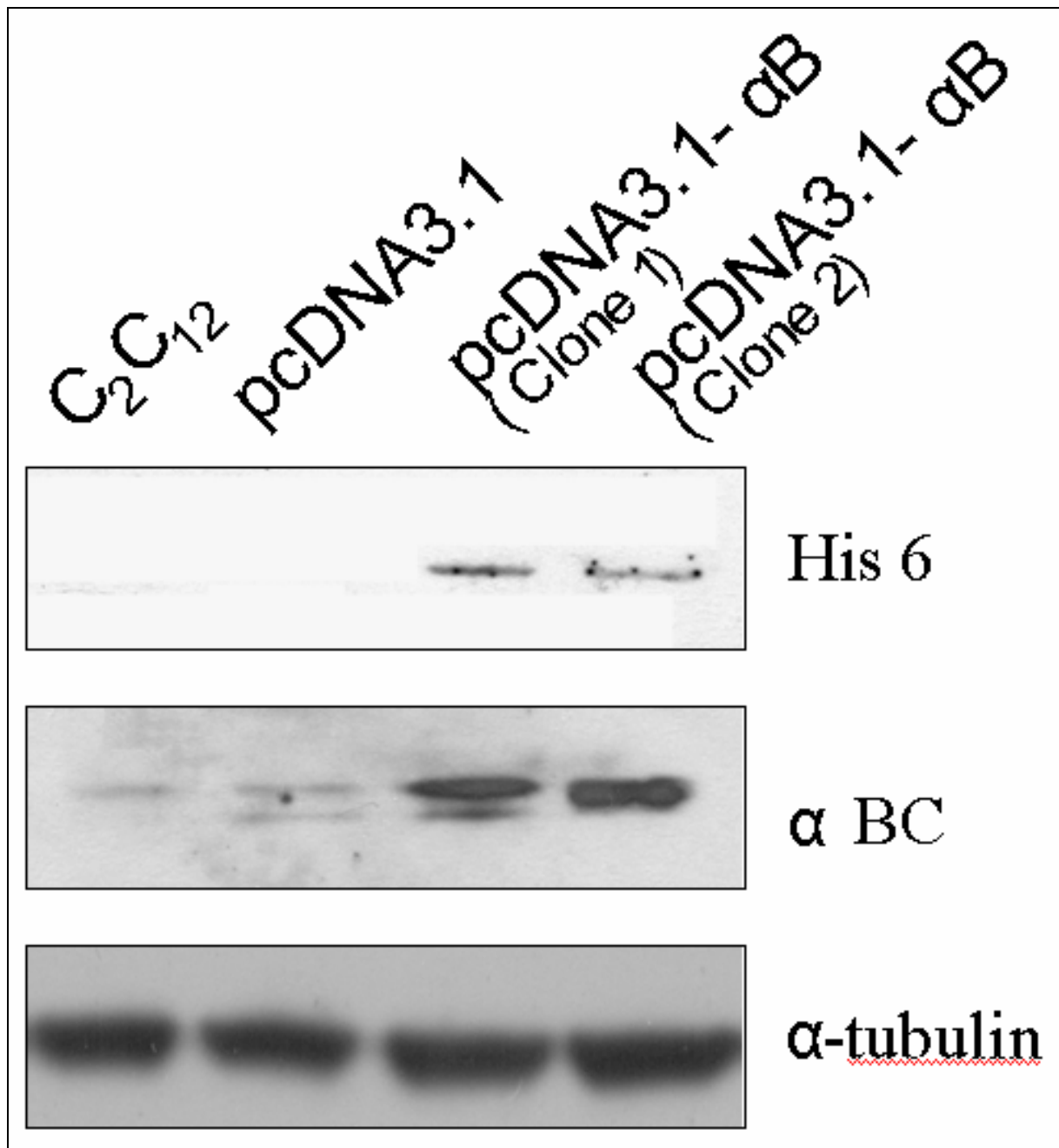


Figure 1: Overexpression of αB-crystallin in mouse myoblast C₂C₁₂ cells
 Total proteins (100μg) from parental C₂C₁₂, pcDNA3.1-αB transfected C₂C₁₂ (clone1,2) and vector transfected C₂C₁₂ cells were separated by 10% SDS- PAGE and Western blot analysis using antibodies to penta His (top panel), αB-crystallin (middle panel) and α-tubulin (bottom panel) as control. Data showed that αB-crystallin was significantly increased in αB-crystallin transfected C₂C₁₂ cells.

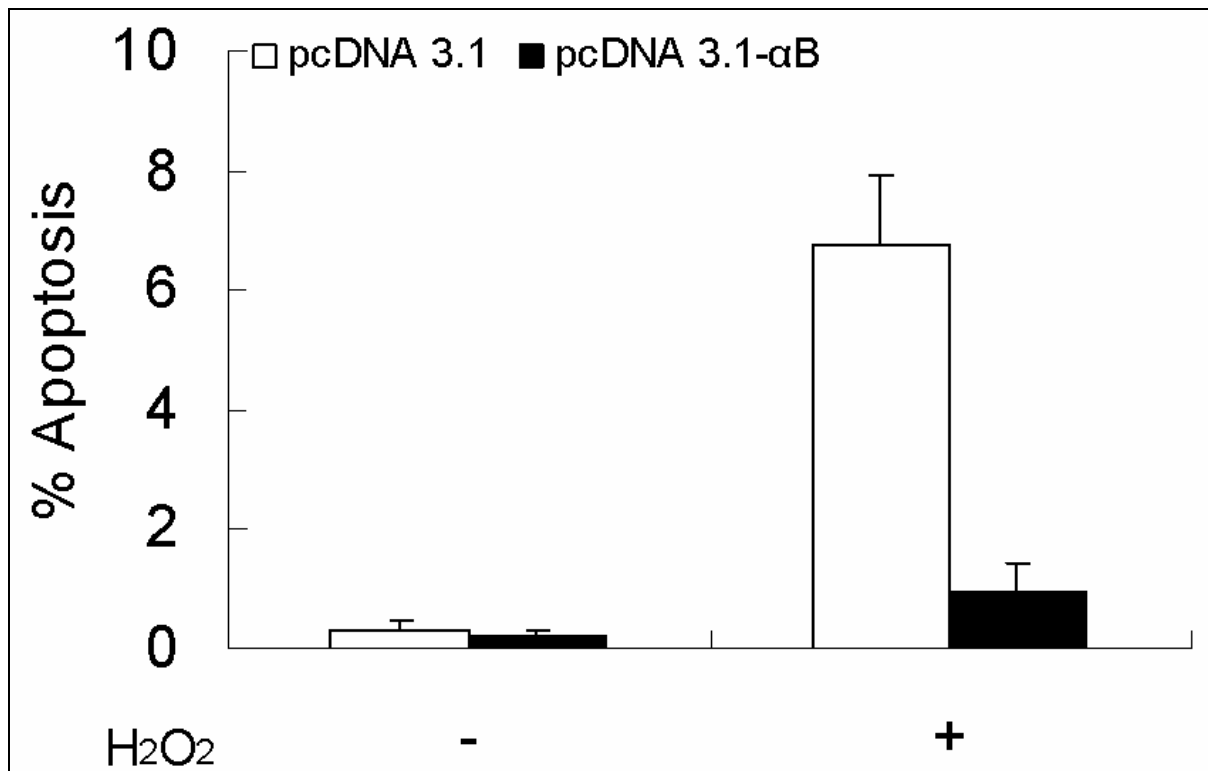


Figure 2: αB-crystallin prevented hydrogen peroxide-induced early apoptosis
 2×10^6 of αB-crystallin (pcDNA3.1-αB) or vector (pcDNA3.1) transfected C₂C₁₂ cells were treated with H₂O₂ (0.5mmol/L) for 3 h. Cells were collected and labelled with annexin V-EGFP for indicating phosphatidylserine redistribution. The percent of annexin V positive cells was measured by flow cytometry.

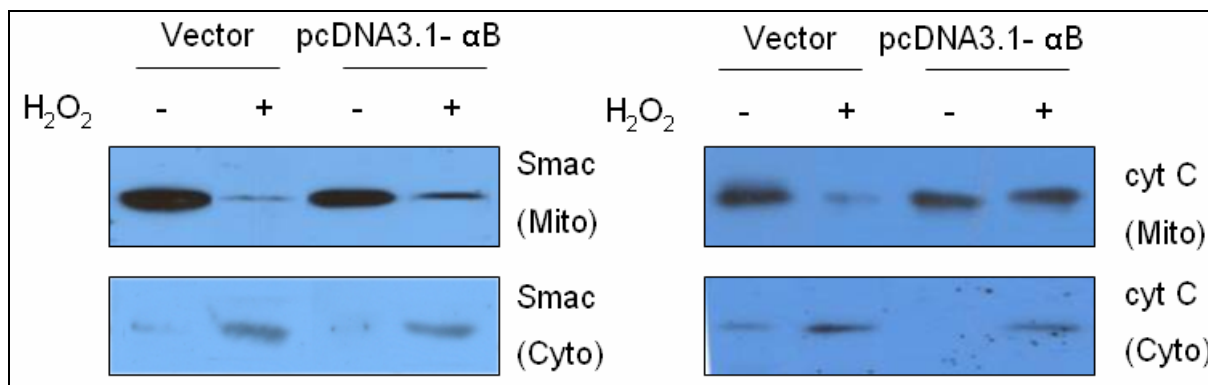


Figure 3: αB-crystallin reduced the release of cytochrome c and Smac/Diablo from mitochondria after treatment with hydrogen peroxide
 2×10^6 of αB-crystallin (pcDNA3.1-αB) or vector (pcDNA3.1) transfected C₂C₁₂ cells were treated with 10 ml DMEM containing H₂O₂ (0.5mmol/L) for 1 h. Then the mitochondrial (Mito) and cytosolic (Cyto) fractions were isolated. The distributions of cytochrome c (cyt c) and Smac/Diablo(Smac) were determined by Western blot analysis using the corresponding antibodies.

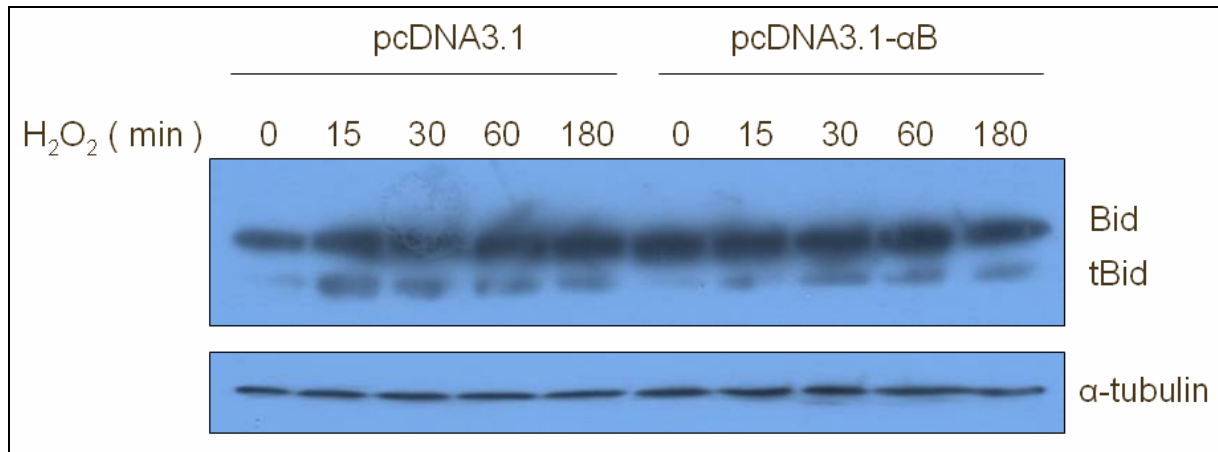


Figure 4: αB-crystallin decreased hydrogen peroxide-induced Bid cleavage
 2×10^6 of αB-crystallin (pcDNA3.1-αB) or vector (pcDNA3.1) transfected C₂C₁₂ cells were treated with H₂O₂ (0.5 mmol/L) for 0, 15, 30, 60 and 180 min, respectively. Total proteins were extracted and detected for Bid and tBid by Western blot analysis using the corresponding antibody.

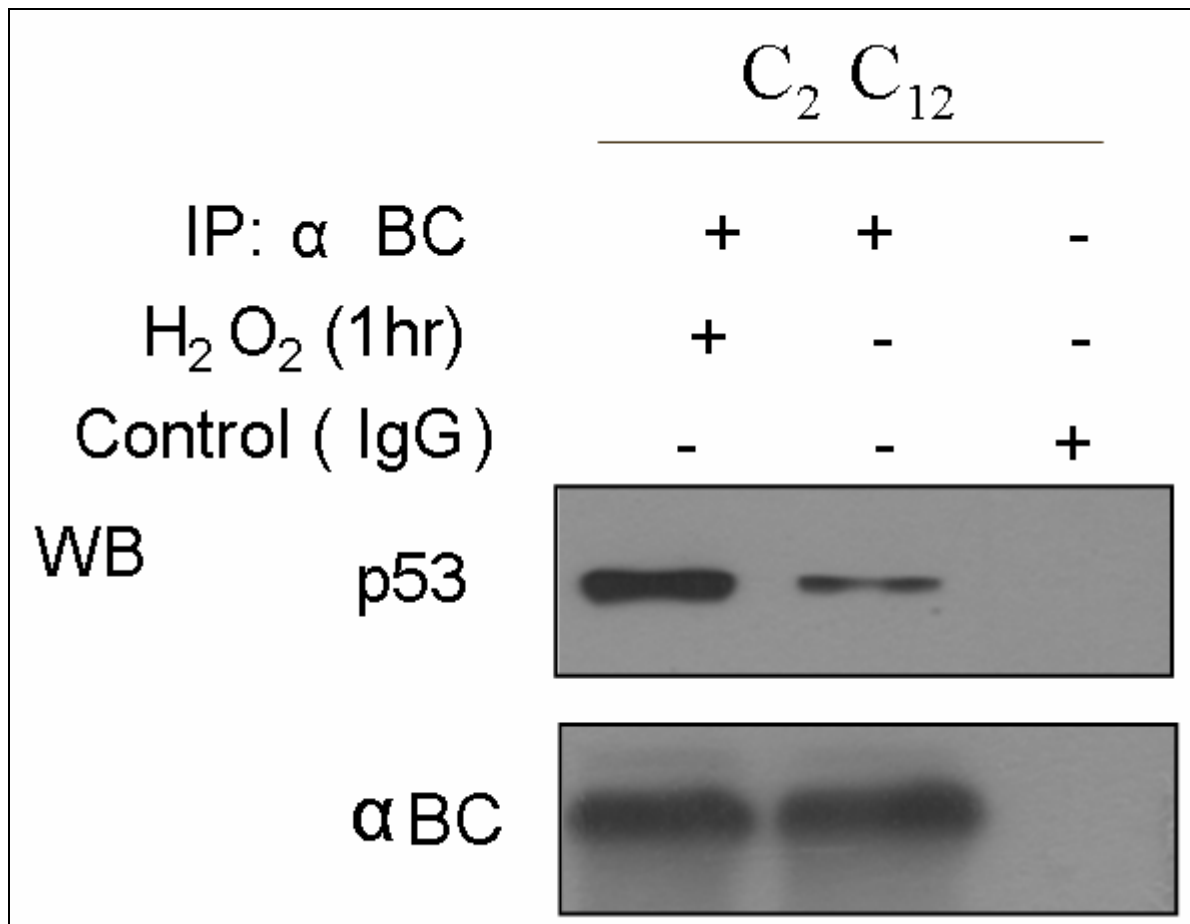


Figure 5-1: αB-crystallin bound to p53 during hydrogen peroxide induced apoptosis
 C₂C₁₂ cells were treated with H₂O₂ (0.5 mmol/L) for 1h. Total proteins were extracted under native conditions for immunoprecipitation with antibody against to αB-crystallin. The precipitated samples were then sequentially blotted with anti-p53 antibody.

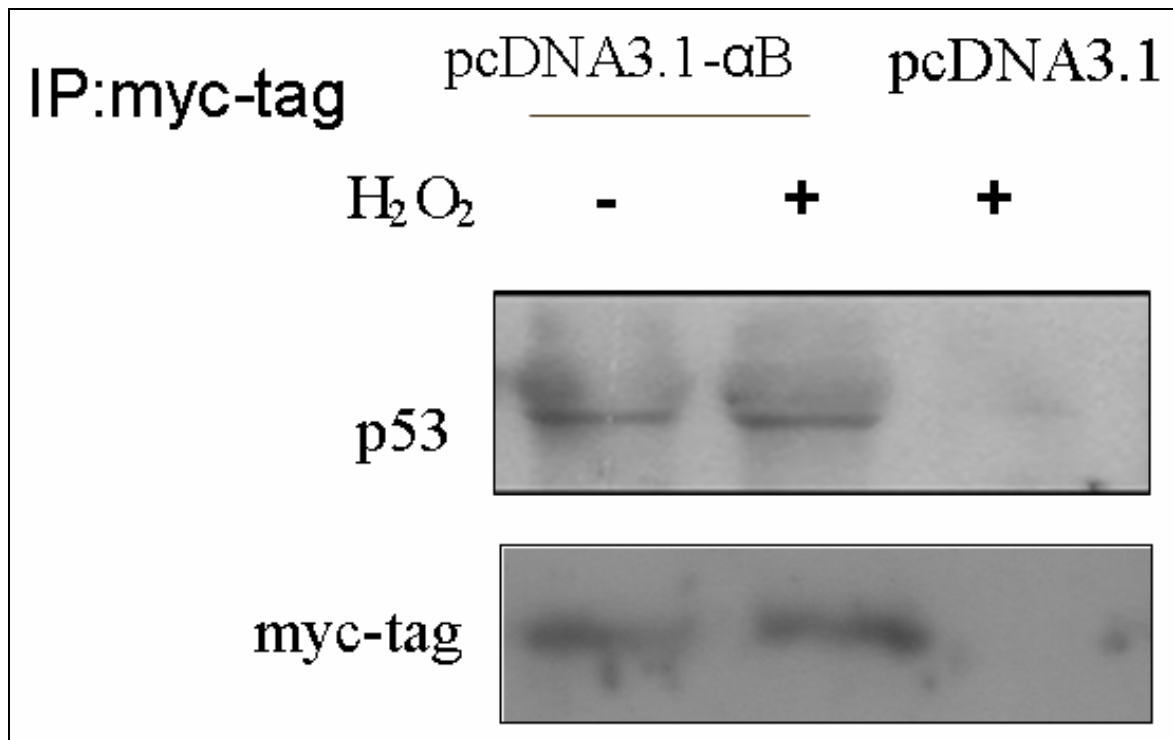


Figure 5-2: α B-crystallin (pcDNA3.1- α B) and vector (pcDNA3.1) transfected C₂C₁₂ cells were treated with H₂O₂ (0.5mmol/L) for 1h. Total proteins were extracted under nature conditions for immunoprecipitation with antibody to myc-tag. The precipitated samples were then sequentially blotted with anti-p53 antibody.

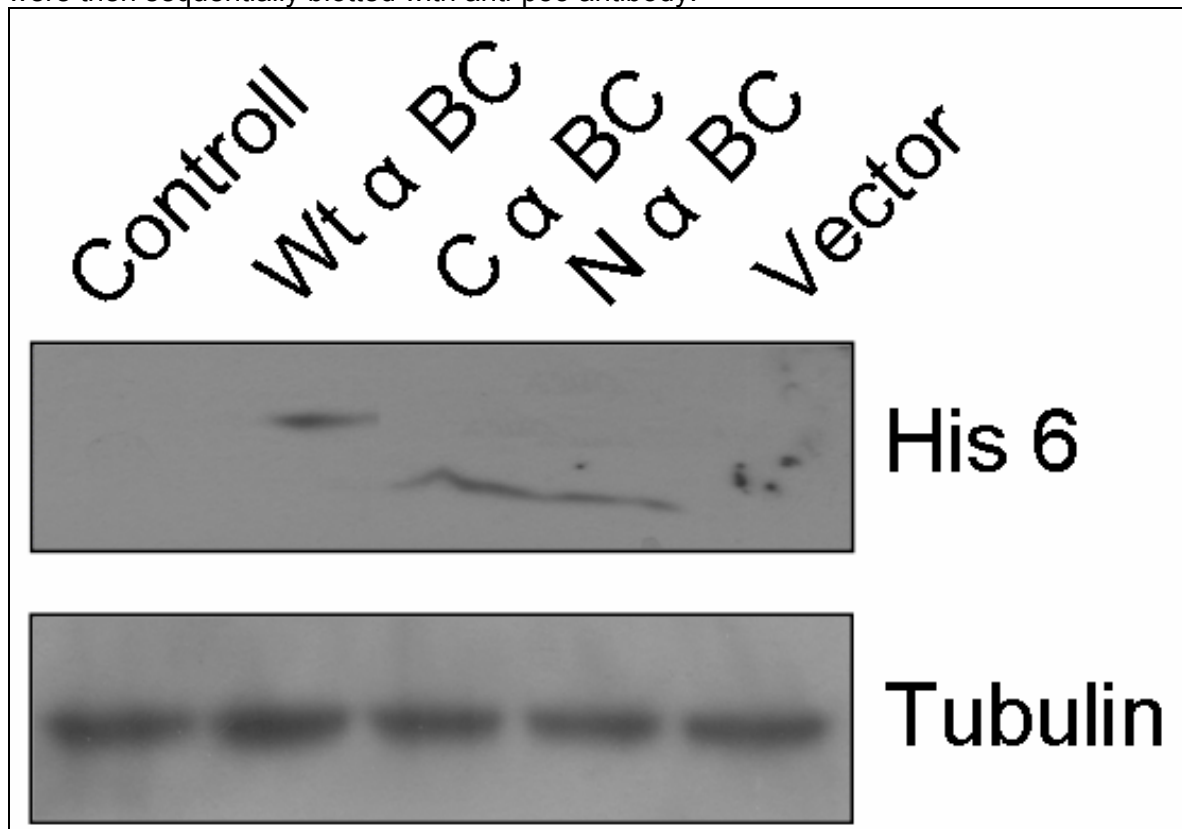


Figure 5-3: α B-crystallin mutants were stably transfected into C₂C₁₂ cells. Total proteins 100 μ g were separated by 12% SDS- PAGE and Western blot analysis was performed using an antibody against penta His. Data showed that the 1-59 amino acid domain (NH₂) and the 60-175 amino acid domain (COOH) of α B-crystallin have been successfully transfected into C₂C₁₂ cells.

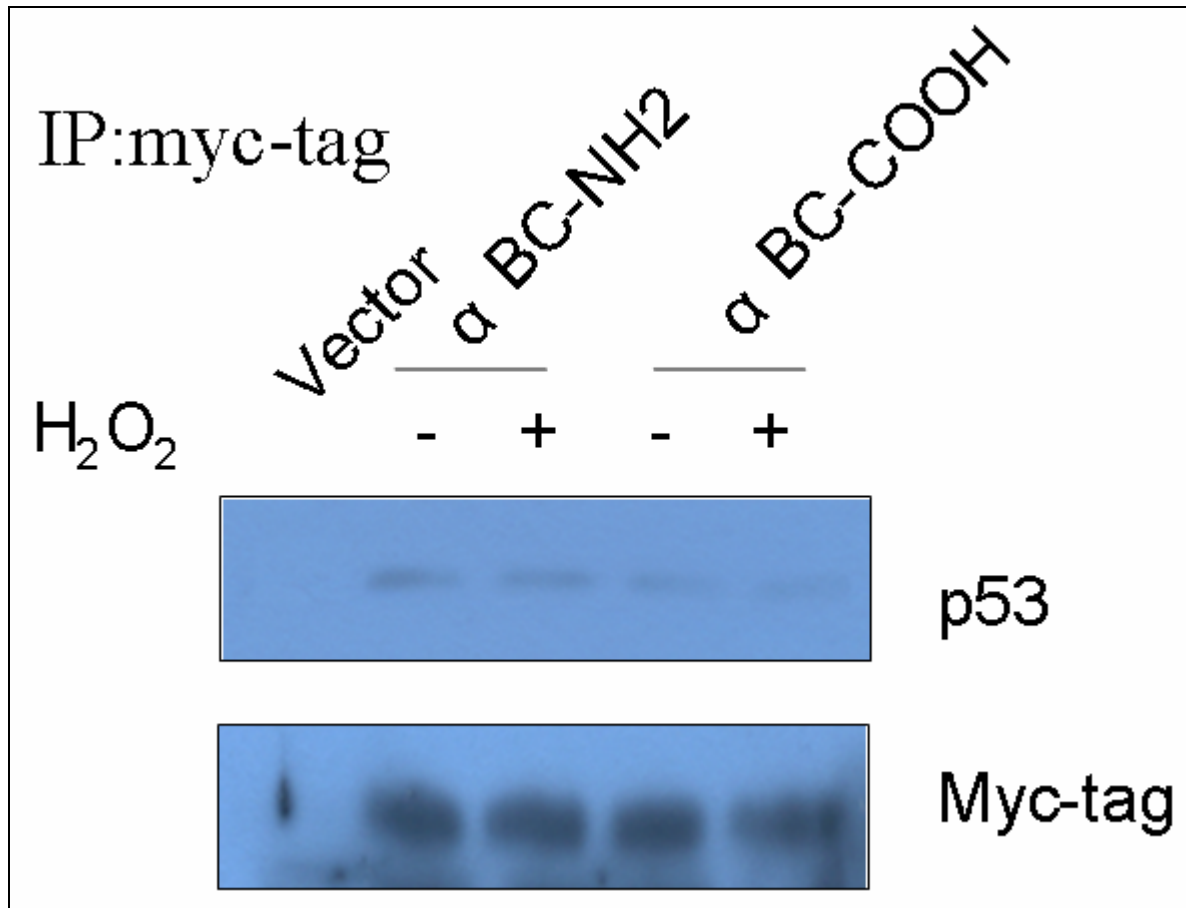


Figure 5-4: Mutants of αB-crystallin transfected C₂C₁₂ cells were treated with H₂O₂ (0.5 mmol/L) for 1h. Total proteins were extracted under nature conditions for immunoprecipitation with antibody against to myc-tag. The precipitated samples were then sequentially blotted with anti-p53 antibody.

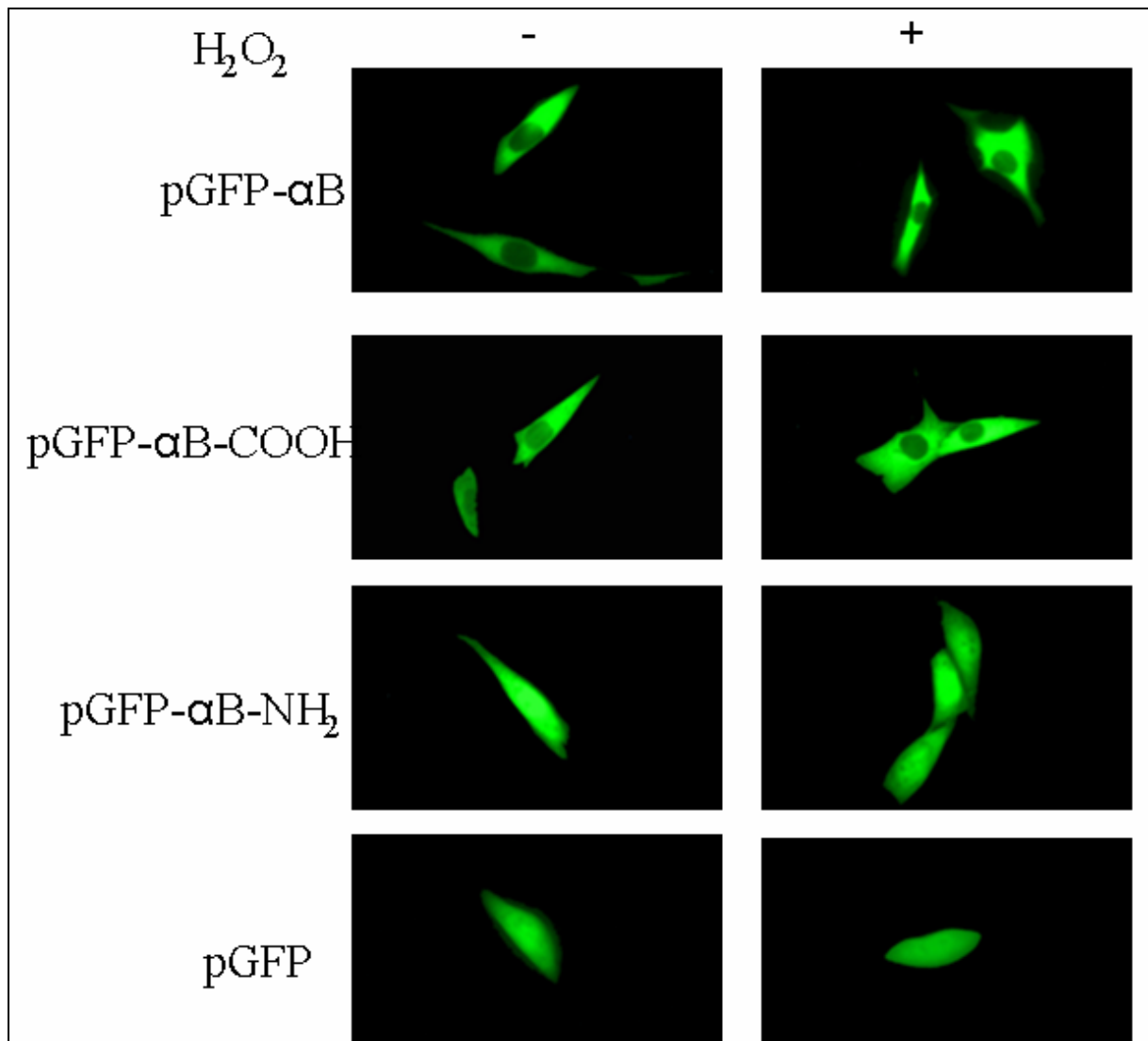


Figure 5-5: Mutants of αB-crystallin fusion to GFP protein transfected C₂C₁₂ cells were treated with H₂O₂ (0.5 mmol/L) for 1h. The cells were directly visualized with a fluorescent microscope.

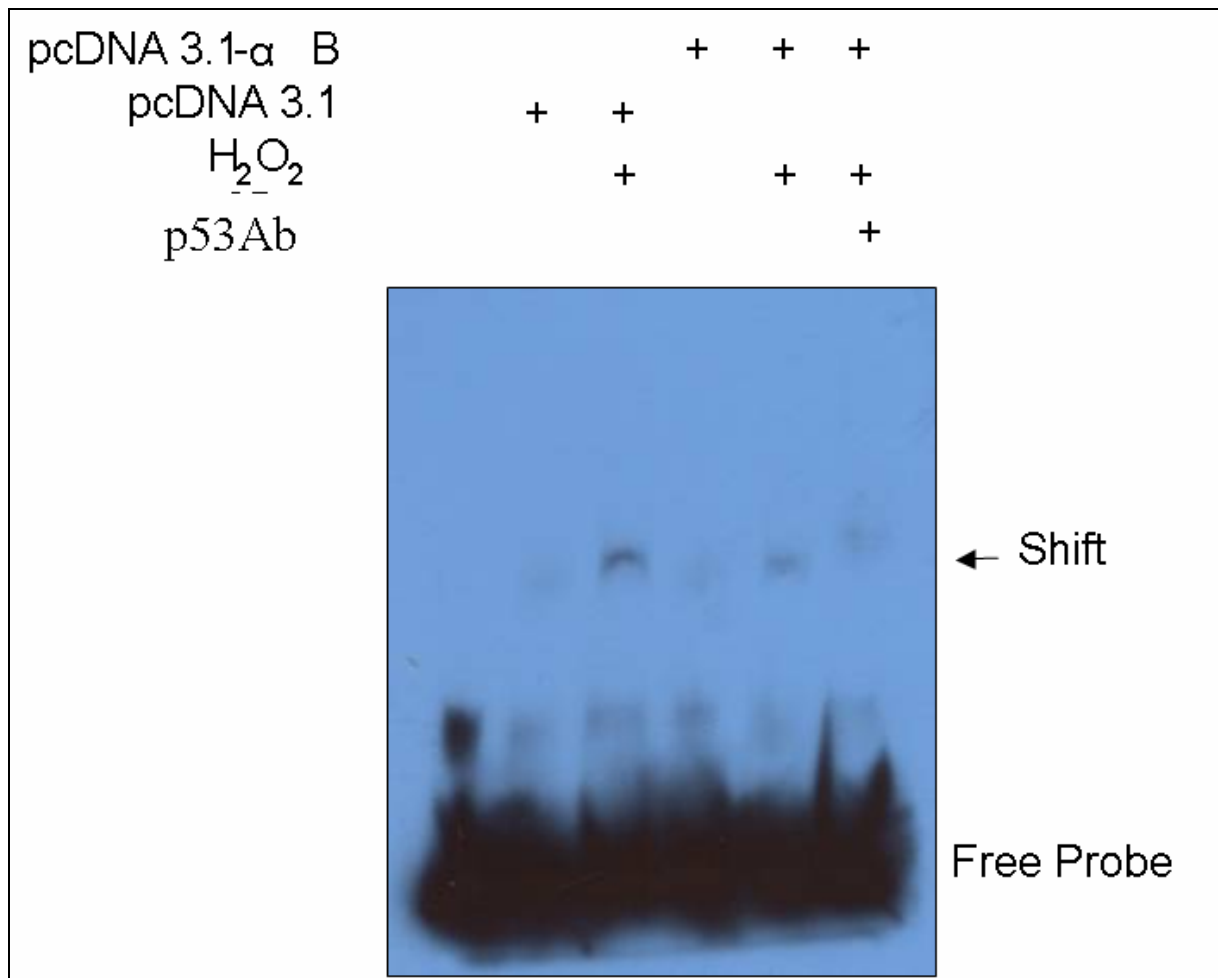


Figure 6-1: α B-crystallin inhibited hydrogen peroxide induced p53 DNA-binding and transactivation activity
 α B-crystallin (pcDNA3.1- α B) and vector (pcDNA3.1) transfected C₂C₁₂ cells were treated with H₂O₂ (0.5 mmol/L) for 1 h. The binding to biotin-labelled of p53 from the nuclear extracts from the cells was examined by EMSA and supershift in the absence or presence of p53 antibody (DO-1).

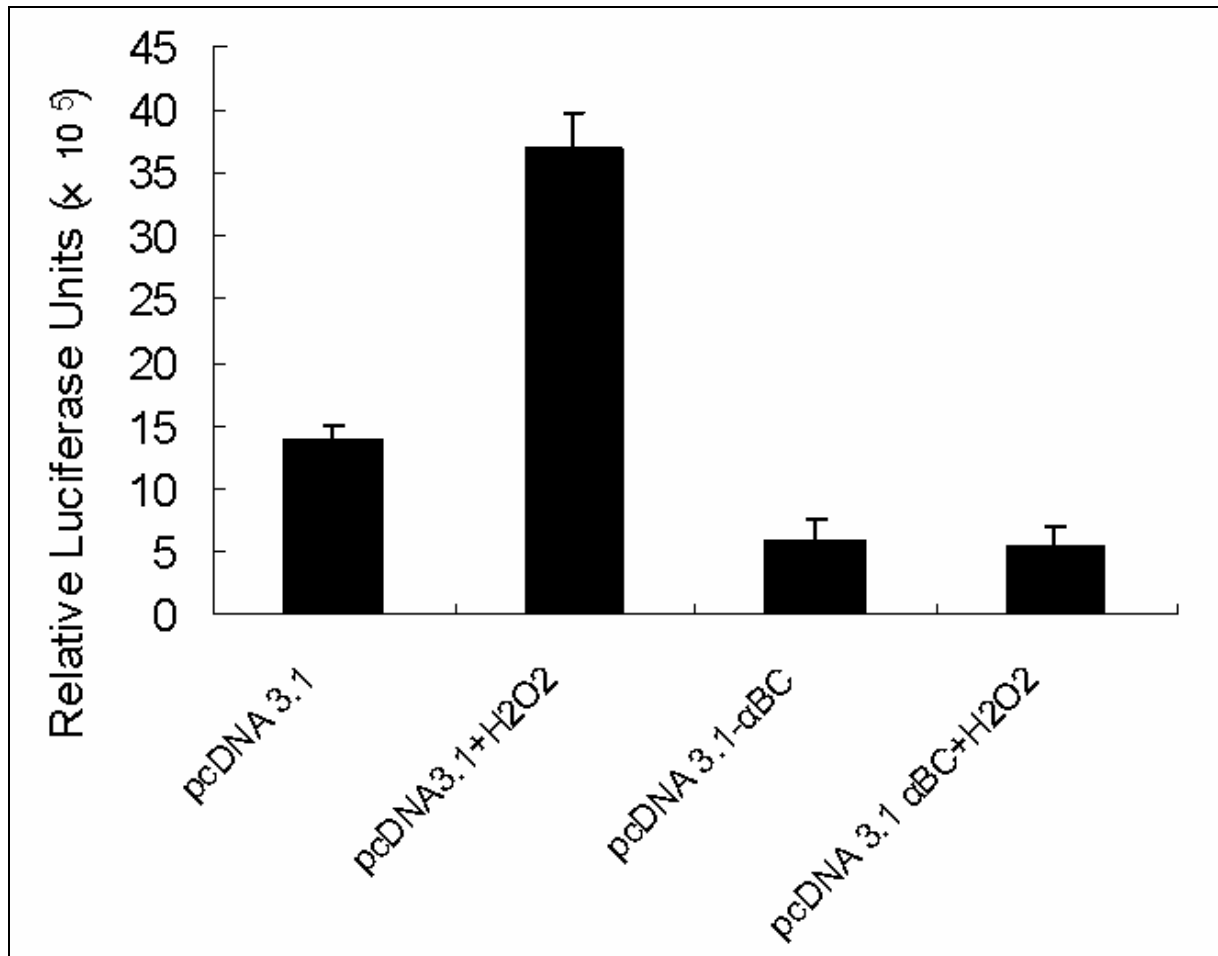


Figure 6-2: α B-crystallin (pcDNA3.1- α B) and vector (pcDNA3.1) transfected C₂C₁₂ cells were transiently transfected with constructs driving a p53-responsive element reporter gene for 24 h, and then the cells were treated with H₂O₂ (0.5mmol/L) for 1 h. Luciferase normalized to β -galactosidase activity is presented.

DISCUSSION

α B-crystallin, a major small heat shock protein, has recently been found to exert multiple functions (Koh et al. 2004; Kumarapeli et al. 2004; van Rijk et al. 2003; den Engelsman et al. 2003; Gangalum et al. 2004). One of the most important functions is to protect cells from apoptosis induced by a large numbers of stress factors, including oxidative stress. The mitochondrial death pathway of apoptosis is a crucial mechanism in many types of cells including myocardial and myofibril cells (Zhao et al. 2002, Gill et al 2002), while lately quite a number of heat shock proteins protects cells from apoptosis through disruption of this intrinsic apoptotic pathway. α B-crystallin could negatively regulate apoptosis by a distinct mechanism in several kinds of cells, in which it interacts

with partially processed procaspase-3 to prevent its activation (Kamradt et al 2001). α B-crystallin also prevents staurosporine-induced apoptosis through interactions with pro-apoptotic Bcl-2 family member protein Bax and Bcl-Xs to sequester their translocation to mitochondria (Mao et al. 2004). Since most heat shock proteins exert apoptosis-regulatory functions in multiple aspects and levels (Parcellier A et al. 2003; Pandey P et al. 2000; Zhao C, et al. 2004; Dias S, et al. 2002; Gurbuxani et al. 2003 Saleh A et al. 2000; Tsuchiya et al. 2003 ; Shan et al. 2003;Gupta et al. 2002; Bruey et al. 2000; Concannon et al. 2001; Paul et al. 2002), it is not yet clarified whether α B-crystallin could protect myoblast C₂C₁₂ cells against oxidative-induced apoptosis is not yet clarified.

In the present study, we explored the effects of α B-crystallin on several early stages of mitochondrial death pathways during hydrogen peroxide-induced apoptosis in C₂C₁₂ cells. Our findings demonstrated that α B-crystallin effectively inhibited hydrogen peroxide induced apoptosis at early stage indicated by phosphatidylserine(PS) externalization analysis. Also, α B-crystallin reduced the degree of cytochrome C and Smac/Diablo release from mitochondria and attenuated Bid cleavage. These results suggested that α B-crystallin regulated apoptosis through interference in the upstream events of mitochondrial signaling pathway.

Oxidative stress takes place under various conditions in striated muscle tissues (myocardium and skeleton muscles) (Zhao et al. 2002; Kumar et al. 2003), in which α B-crystallin is abundantly expressed. It is obvious that reactive oxygen species (ROS) could activate mitochondrial death signalling pathway and mediate cell apoptosis (Kumar et al. 2003; Zhao et al. 2004). ROS also increases the accumulation of p53 and promotes its transcriptional (Zhao et al. 2002; Haupt et al. 2003). In the present study, we found that α B-crystallin bound to p53 under oxidative stress and inhibited hydrogen peroxide induced DNA-binding and transactivation activities of p53. Both the NH₂-terminal (1-59) and COOH-terminal (60-175) domain of α B-crystallin could bind to p53, indicating that there might be at least two binding sites in α B-crystallin responsible for the interaction. The wild type α B-crystallin and its COOH-terminal domain (60-175) distributed mainly in the cytoplasm of C₂C₁₂ cells under oxidative stress, suggesting the interaction of α B-crystallin with p53 occurred in cytoplasm. The interaction of α B-crystallin with p53 might arrest the import of p53 into the nucleus and thus decreased its function.

In myocardial and skeletal muscle tissues, α B-crystallin immediately translocates to the

skeleton fractions and structural proteins such as actin, titin and desmin upon oxidative stress (Golenhofen et al. 1999a, 1999b; Clark et al. 2000). The co-localization of α B-crystallin with skeleton proteins might offer a kind of support to the skeleton structure of cells, thus helps them to resist disconstruction during apoptosis. Also, cell skeletons were considered as the channels of signalling molecules transmission and the depository site of them, thus the immediately translocation of α B-crystallin might also afford contact to some functional signalling molecules related to apoptosis. As several structural proteins have been proven to be targets of α B-crystallin *in vitro* and *in vivo* (Golenhofen et al. 2002; Wang et al. 2003; Arai et al. 1997; Wieske et al. 2001), some signalling molecules including Bax also could also be targeted by α B-crystallin during apoptosis (Kamradt et al. 2001, Mao et al. 2003).

In conclusion, we studied the effects of α B-crystallin on several early stages of apoptosis induced by oxidative stress in C₂C₁₂ cells. We revealed the binding of α B-crystallin to p53 and its inhibition of the nuclear function of p53 during apoptosis. The findings indicate that α B-crystallin protects against apoptosis induced by oxidative stress.

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