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Biochemical and Biophysical Research Communications 354 (2007) 109–114
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Small heat shock protein αB-crystallin binds to p53 to sequester its translocation to mitochondria during hydrogen peroxide-induced apoptosis

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Received 18 December 2006
Available online 28 December 2006

Abstract

Apoptosis is a highly conserved procedure of cell death and occurs under various stimuli, including oxidative stress. A small heat shock protein, αB-crystallin, is found to process resistance to apoptosis in some cells and tissues. But the mechanisms under this protective role are not fully understood. In the present study, we reported the early protective role of αB-crystallin against hydrogen peroxide-induced apoptosis in mice myogenic C_2C_{12} cells. αB-Crystallin interacted with p53, a proapoptotic protein, during cell apoptosis and such protein interaction mainly occurred in the cytoplasm of the cells, suggesting that the interaction of αB-crystallin with p53 might prevent the translocation of p53 from cytoplasm to mitochondria. Hence, this study provides a hint that αB-crystallin affects on p53 mitochondrial translocation during oxidative stress-induced apoptosis.

Keywords: αB-Crystallin; Hydrogen peroxide; p53; Interaction; Translocation; Mitochondria; Apoptosis

Apoptosis is a genetically controlled and evolutionarily conserved form of cell death in physiological conditions and diseases. Apoptosis occurs under various stress conditions once the stimuli is strong enough. Accumulating evidence indicates that oxidative stress plays a major role in the initiation and progress of apoptosis. Oxidative stress is a state in which excess reactive oxygen species (ROS) overwhelm endogenous antioxidant systems. Hydrogen peroxide (H_2O_2) is a form of ROS generated as a result of oxidative stress. H_2O_2 and other ROS can react with various cellular targets including DNA and proteins and cause cell damage. It is now considered that H_2O_2 can act as a signaling molecule that triggers and regulates apoptosis [1–3].

The p53 tumor suppressor, a transcription factor which could be induced by DNA-damaging and oxidative stress-generating agents, has been found to mediate apoptosis by mechanisms that are both dependent and independent of its transcriptional activity [4]. Recent studies indicate that p53 directly translocates to mitochondria and binds to multiple targets in the mitochondria, such as p53AIPI, Bak, and mitochondrial Mn-SOD, to exert its activity as an apoptosis inducer [5–7]. The translocation of p53 to mitochondria occurs rapidly after the onset of oxidative stress and precedes its nuclear translocation [7].

The small heat shock protein, αB-crystallin, which is abundantly expressed in lens and striated muscle tissues, is found to process resistance to apoptosis in some kinds of cells [8–12], but the mechanisms are not fully understood. Recent studies indicate that αB-crystallin negatively regulates apoptosis by directly binding to partially processed caspase-3 (p24 intermediate) and Bax and Bcl-x_s (two proapoptotic Bcl-2 family members), thus inhibits the proapoptotic function of these proteins [8,13]. Since most heat shock proteins (such as HSP70, HSP90,

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doi:10.1016/j.bbrc.2006.12.152

HSP27) exert anti-apoptotic functions through multiple mechanisms [14–16], whether αB-crystallin plays other mechanisms in the protection of cells against apoptosis is not known.

In the present study, we found that αB-crystallin could protect mouse myogenic C_2C_{12} cells from oxidative stress (H_2O_2)-induced apoptosis at early stage. αB-crystallin bound to p53 in the cytoplasm and decreased its translocation to mitochondria after oxidative stress.

Materials and methods

Plasmid construction and stable transfection. The 522 bp mouse αB-crystallin cDNA was constructed into a mammalian vector pcDNA3.1-myc-his (−) B (Invitrogen) to create an αB-crystallin expressing plasmid called pcDNA3.1-αBC as we described [17]. The plasmid and the control plasmid pcDNA3.1 were stably transfected into a mouse myogenic cell line C_2C_{12} using Lipofectamine2000 (Invitrogen). The transfected cells were then subject to 1000 μg/ml of G418 selection for 4–6 weeks. Individual clones stably expressing high amounts of αB-crystallin were selected by Western blot identification using αB-crystallin antibody. The stable cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibcol) supplemented with 100 μg/ml of G418.

Hydrogen peroxide (H_2O_2) treatment. Cells were grown in DMEM containing 10% newborn calf serum with or without G418 (100 μg/ml) until confluence. Then 2×10^6 cells were plated into a 60 cm^2 culture bottles. After 12 h of growth, the medium were replaced with 10 ml DMEM containing 0.5 mmol/L H_2O_2. In control groups, cells were treated with DMEM not containing H_2O_2. The treatment was continued for indicated time, and then cells were collected and subjected to the indicated analysis procedures.

Hoechst 33258 staining. After H_2O_2 (0.5 mmol/L) treatment for indicated time, all the cells (including attached and floating cell) were harvested and fixed with 4% formaldehyde/PBS for 10 min, then stained with Hoechst 33258 (1:100 dilution). Cells were observed under a fluorescence microscopy. Apoptotic cells showed blue, peripherally clumped or fragmented chromatin.

Annexin V-EGFP assays. After H_2O_2 (0.5 mmol/L) treatment for indicated times, all the cells (including attached and floating cells) were gently harvested. For analysis of early apoptosis, cells were labeled with annexin V-EGFP (ApoAlert™ Annexin V Apoptosis Kits, BD) for indicating phosphatidylserine redistribution. Positive cells presented bright and green fluorescent staining of cell membrane under a fluorescence microscope.

Protein extraction

For total protein. Cells were washed with ice-cold PBS and incubated in lysis buffer (10 mmol/L Tris–HCl, pH 8.0, 1 mmol/L EDTA, 2% SDS, 5 mmol/L DTT, and 10 mmol/L PMSF, supplemented with protease inhibitors cocktail) for 30 min on ice. The lysates were centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was collected as total soluble protein for further analysis.

For mitochondrial protein. Cells were harvested by centrifugation at 850*g*. Mitochondrial fractions were isolated using Optiprep™ mitochondria isolation kit (Pierce). Simply, add 800 μl of Mitochondria Isolation Reagent A and then transfer cell suspension to in a Wheaton homogenizer tube and carefully homogenized for 30 strokes on ice. Return lysed cells to original tube and adds 800 μl of Mitochondria Isolation Reagent C. Centrifuge tube at 700*g* for 10 min at 4 °C. Transfer the supernatant and centrifuge at 3000*g* for 15 min at 4 °C to obtain a more purified fraction of mitochondria. Transfer the supernatant (cytosol fraction), which is centrifuged at 12,000*g* for more purified faction, to a new tube. The pellet contains the isolated mitochondria. Add 500 μl Mitochondria Isolation Reagent C to the pellet, and centrifuge at 12,000*g* for 5 min. The mitochondrial pellet was resuspended in 300 μL of mitochondria isolation buffer containing 0.1% Triton X-100 and protease inhibitors. This fraction was labeled as the mitochondria fraction and kept at −80 °C.

Western blot analysis. Protein samples were mixed with sample buffer and boiled for 5 min. The samples were then separated by electrophoresis on a 10% SDS–PAGE, and transferred to a nitrocellulose (NC) membrane. The membrane was incubated in blocking buffer (PBS containing 5% skim milk and 0.1% Tween 20) for 2 h, followed by incubation with the primary antibody diluted in blocking buffer. After washing, the membrane was then incubated with the proper secondary antibody for 1 h. All the incubations were performed at room temperature. At last, the membrane was detected by super signal chemiluminescence's system (Pierce) followed by exposure to an auto-radiographic film.

Immunoprecipitation. After H_2O_2 (0.5 mmol/L) treatment for 1 h, cells were washed with ice-cold PBS and lysed in 1 ml ELB buffer (140 mmol/L NaCl, 0.5% NP-40, 50 mmol/L Tris–HCl, pH 8.0, supplemented with protease inhibitor cocktail) to a final concentration of 2×10^6 cells/ml on ice for 30 min. The lysates were then centrifuged for 15 min at 12,000 rpm at 4 °C. For pre-clear treatment, 50 μl Protein G–Sepharose beads (Amersham Biosciences) were added into cell lysate containing 1 mg of total protein and gently rotated for 30 min at 4 °C. Then the lysates were transferred to another tube and incubated with 5 μg of antibody or control IgG for 1 h at 4 °C. Then the samples were incubated with another 50 μl protein G–sepharose by gently shaking at 4 °C overnight. The beads were washed for five times with ice-cold ELB buffer and dissolved in loading buffer. Then Western blot analyzed the samples for indicated proteins.

Purification of the His_6-tagged αB-crystallin under native condition. After H_2O_2 (0.5 mmol/L) treatment for 1 h, cells were washed with ice-cold PBS and lysed in 1 ml ELB buffer (as previous described) on ice for 30 min. The lysates were then centrifuged for 15 min at 12,000 rpm at 4 °C. The supernatant (500 μg) was applied to a pre-equilibrated Ni^+–nitrilotriacetic acid (NTA)-spin column (Qiagen). The column was then washed twice with washing buffer containing 20 mM imidazole. The protein bound to the resin was eluted with 200 μl of elution buffer containing 250 mM imidazole. The elution fraction was analyzed by Western blot for indicated proteins.

Results

αB-Crystallin prevented H_2O_2-induced cell death in C_2C_{12} cells

To delineate the antiapoptotic mechanisms of αB-crystallin in C_2C_{12} cells, we used our established stable cell lines pcDNA3.1/αBC cells (C_2C_{12} cells overexpressing αB-crystallin) and pcDNA3.1 cells (control cells). After grown to 100% confluence, these cells were subjected to treatment with 0.5 mmol/L H_2O_2 for 6, 12, and 24 h respectively. The percentage of apoptotic cells was analyzed with a viability assay by Hoechst 33258 staining. As shown in Fig. 1a, after 6, 12, and 24 h treatment of the vector-transfected cells, the percent of apoptotic cells, which contained peripherally clumped or fragmented chromatins (arrows indicated), increased gradually from $29.41 \pm 2.03\%$, $66.66 \pm 9.65\%$, and $92.0 \pm 8.21\%$ ($n = 3$). In contrast, the percent of apoptotic cells in the pcDNA3.1/αBC group increased only from $13.89 \pm 0.89\%$, $33.33 \pm 3.95\%$, $60.98 \pm 8.17\%$ ($n = 3$), respectively.

To further determine the role of αB-crystallin in the early phase of apoptosis, the externalization of phosphatidylserine (PS), which is an early mark of cell viability, was verified by Annexin V-EGFP binding assay as shown in Fig. 1b. After the treatment of H_2O_2 (0.5 mmol/L) for

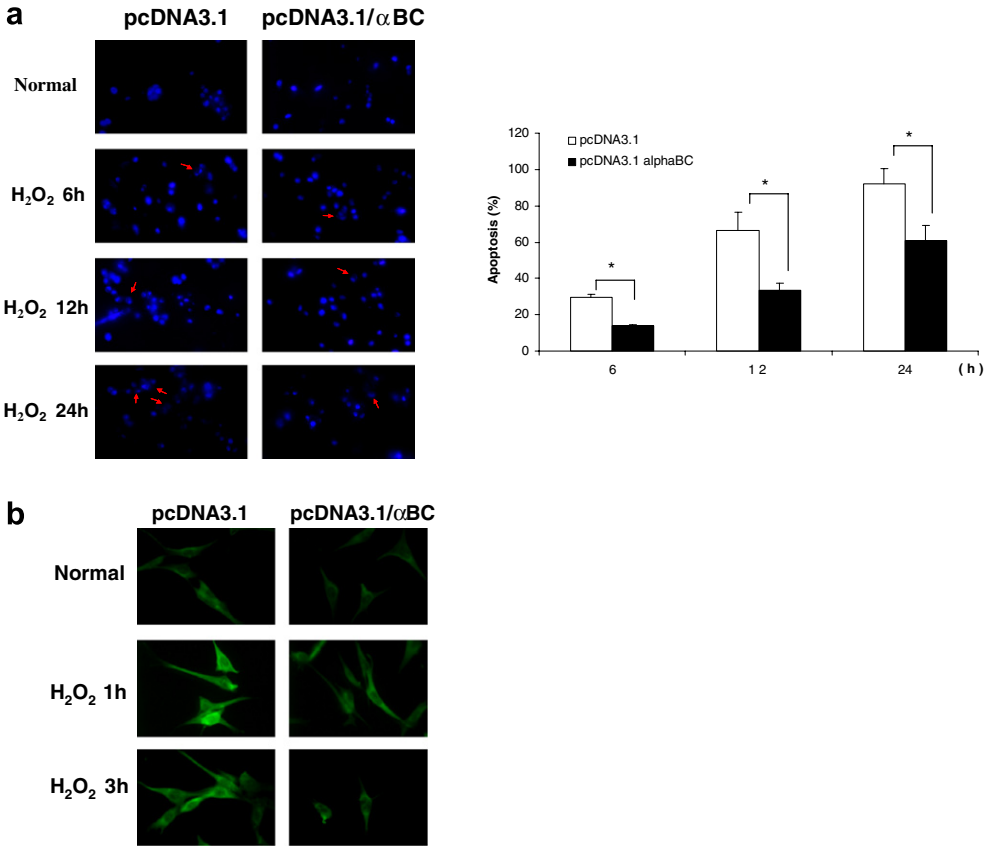

Fig. 1. αB-Crystallin overexpression decreased H_2O_2 (0.5 mmol/L)-induced cell apoptosis. (a) Hoechst 33258 nuclear staining analysis of cells. After grown to 100% confluence, cells were treated with 0.5 mmol/L H_2O_2, and then the percentage of apoptotic cells was observed and calculated with a viability assay by Hoechst 33258 staining (The arrows indicated apoptotic cells. Columns, means of three independent experiments; bars, SE. $^*P < 0.01$ when compared with control (pcDNA3.1 cells). (b) Phosphatidylserine (PS) externalization analysis of cells. After grown to 100% confluence, cells were treated with 0.5 mmol/L H_2O_2 for 1 and 3 h, respectively. Then the cells were harvested and labeled with annexin V-EGFP and positive cells were observed under a fluorescence microscope. In control groups (pcDNA3.1 cells), cells displayed a clear pattern of PS externalization after treatment of H_2O_2, while the pcDNA3.1/αBC cells displayed much weak signaling of PS externalization. Figures are representative of three independent experiments with similar results.

3 h, the vector-transfected cells displayed a clear pattern of PS externalization as bright green staining of cell membrane. However, the pcDNA3.1/αBC cells only displayed a much weaker PS externalization signaling. Thus, it suggested that αB-crystallin could provide marked protection against oxidative stress (H_2O_2)-induced apoptosis in C_2C_{12} cells.

H_2O_2 increased the interaction of αB-crystallin and p53 in vivo

Recently, it has been found that αB-crystallin binds to several apoptotic-related proteins such as p24 (intermediate of caspase-3), Bax and Bcl-x_s, and disturbs the function of these proteins in some kinds of cells. Since αB-crystallin is a chaperon, whether it binds to other proteins during apoptosis is unknown. We conducted immunoprecipitation assay to identify the possible interactions between αB-crystallin with other proteins. The myc-tag antibody (specifically recognizing the peptide label on the exogenous protein) was used to precipitate the αB-crystallin and associated proteins from C_2C_{12} cells. The proteins precipitated down were further analyzed with antibodies against some proteins and we found a positive signal using antibody of p53. As shown in Fig. 2a and b, p53 was bound to myc-tag of αB-crystallin. To confirm that the interaction actually occurs *in vivo*, coimmunoprecipitates using anti-p53 antibody was further probed with anti-αB-crystallin antibodies. As expected, αB-crystallin was coimmunoprecipitated from C_2C_{12} cells using anti-p53 antibody. Also it seemed that αB-crystallin and p53 had slightly better affinity to each other after H_2O_2 (0.5 mmol/L) treatment.

Also, we performed the affinity chromatography experiment to further confirm the interaction of αB-crystallin and p53 *in vivo*. Since purification of His_6-tagged protein under native conditions allows copurification of its associated proteins, and nonspecific binding can be reduced by including a certain concentration of imidazole in the lysis and wash buffers, results in Fig. 2c showed that p53 was co-purified with His_6-tagged αB-crystallin from cell lysate, and H_2O_2 (0.5 mmol/L) treatment significantly increased the amount of p53 co-purified with αB-crystallin. Thus these results indicated that αB-crystallin interacted with p53 *in vivo* and oxidative stress (H_2O_2) could increase such interaction.

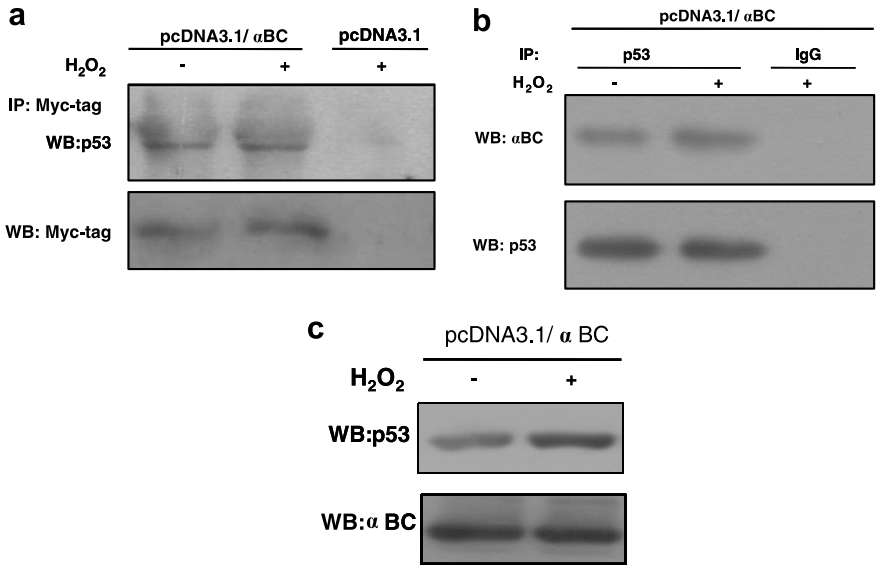

Fig. 2. H_2O_2 (0.5 mmol/L) increased the interaction of αB-crystallin and p53. (a) Immunoprecipitation of p53 by anti-myc-tag antibody. Cell extracts (1 mg) obtained from pcDNA3.1/αBC cells treated or not with H_2O_2 for 1 h and pcDNA3.1 cells treated with H_2O_2 for 1 h were incubated with 5 μg of mouse anti-myc-tag antibody coupled to Protein G–Sepharose beads. Immunoprecipitates were analyzed by Western blot using p53 and myc-tag antibodies. Blots are representative of three independent experiments with similar results. (b) Immunoprecipitation of αB-crystallin by anti-p53 antibody. Cell extracts (1 mg) obtained from pcDNA3.1/αBC cells treated or not with H_2O_2 for 1 h were incubated with 5 μg of mouse anti-p53 antibody or nonspecific antibody (IgG) of the same class coupled to Protein G–Sepharose beads. Western blot using αB-crystallin and p53 antibodies analyzed the immunoprecipitates. Blots are representative of four independent experiments with similar results. (c) Ni^{+}–NTA affinity chromatography under native conditions to analyze the interaction of αB-crystallin and p53 in vivo. Cell extracts (1 mg) obtained from pcDNA3.1/αBC cells treated or not with H_2O_2 for 1 h were applied to a pre-equilibrated Ni^{+}–NTA spin column. The elution fractions were analyzed by Western blot using p53 and αB-crystallin antibodies. Blots are representative of four independent experiments with similar results.

αB-Crystallin interacted with p53 in the cytoplasm

Given that αB-crystallin and p53 were coimmunoprecipitated from cell lysate, we wondered in which part of the cells the interaction occurred. We prepared mitochondrial and cytoplasmic extract from pcDNA3.1/αBC cells and repeated the immunoprecipitation assay using αB-crystallin antibody. Immunoblot assay of precipitate showed that p53 was mainly detected in the cytoplasmic fraction of cell but nearly undetectable in the mitochondrial fraction (Fig. 3a). According with this, immunoblot analysis of cell extracts showed that αB-crystallin distributed mainly in the cytoplasm of cells (Fig. 3b). These results demonstrated that the interaction between αB-crystallin and p53 mainly occurred in the cytoplasm of C_2C_{12} cells.

αB-Crystallin reduced the translocation of p53 from cytoplasm to mitochondria

It was reported that oxidative stress is a potential source of p53 induction. In our results, we also observed the increases of p53 after 1 h of H_2O_2 treatment in both pcDNA3.1/αBC cells and pcDNA3.1 control cells. Since it demonstrated that a fraction of stress-induced wild-type p53 protein translocates rapidly into mitochondria during p53-dependent cell death. We tested whether αB-crystallin could affect p53 distribution to mitochondria during oxidative stress-induced apoptosis. The pcDNA3.1/αBC and pcDNA3.1 cells were either treated with 0.5 mmol/L H_2O_2 and DMEM for 1 h; the mitochondrial-associated and soluble proteins were isolated from cells. Then, the same amounts of mitochondrial and cytosolic proteins were immunoblotted using antibody against p53. As shown in Fig. 4a, in pcDNA3.1 control cells, p53 was equally distributed in the cytoplasmic fraction and the mitochondrial-associated fraction before H_2O_2 treatment. After treatment with 0.5 mmol/L H_2O_2, most of p53 translocated into mitochondria, leading to its dominant distribution in mitochondria and much less amount in the cytosolic fraction. In contrast, in pcDNA3.1/αBC cells, the H_2O_2-induced translocation of p53 was largely blocked as reflected by the equally distribution of p53 in cytoplasmic and mitochondria fraction after or before 0.5 mmol/L H_2O_2 treatment, although αB-crystallin overexpression did not prevent the increase of p53 levels induced by H_2O_2 as indicated in Fig. 4b. These results demonstrated that αB-crystallin could prevent the translocation of p53 to mitochondria after oxidative stress (H_2O_2) stimulation.

Discussion

Being a major small heat shock protein, αB-crystallin has recently been found to exert multiple functions. In which, one of the most important functions is the ability to protect cells from apoptosis induced by various stress factors, including oxidative stress [8–12]. It is now consid-

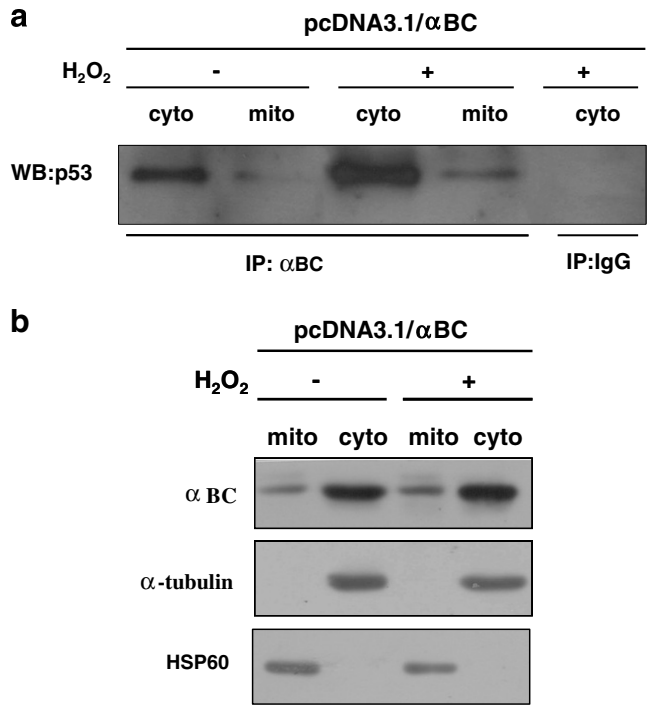

Fig. 3. αB-Crystallin interacted with p53 in the cytoplasm. (a) Immunoprecipitation of p53 by anti-αB-crystallin antibody. Cytoplasmic and mitochondrial protein were isolated from pcDNA3.1/αBC cells treated or not with H_2O_2 for 1 h, then the protein extracts (1 mg) were incubated with 5 μg of mouse anti-αB-crystallin antibody or nonspecific antibody (IgG) of the same class coupled to Protein G–Sepharose beads. Immunoprecipitates were analyzed by Western blot using p53 antibody. Blots are representative of four independent experiments with similar results. (b) Western blot analysis. Cytosol and mitochondrial protein were isolated from pcDNA3.1/αBC cells treated or not with H_2O_2 for 1 h. Top, The proteins were analyzed by Western blot for indicating the distribution of αB-crystallin. Middle and Bottom, α-tubulin and HSP60 were probed as an indicator of cytoplasmic and mitochondrial protein, respectively. Blots are representative of three independent experiments with similar results.

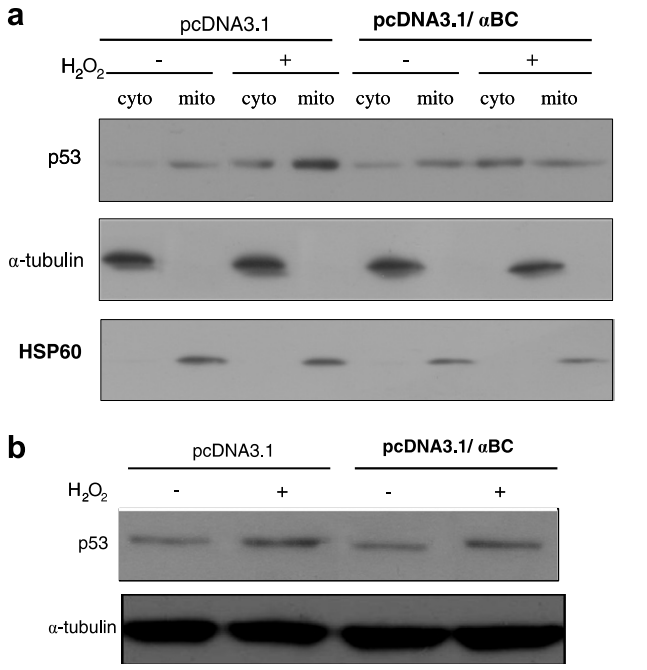

Fig. 4. αB-Crystallin reduced the translocation of p53 from cytoplasm to mitochondria. (a) Western blot analysis. Cytoplasmic and mitochondrial proteins were isolated from pcDNA3.1/αBC cells and pcDNA3.1 cells treated or not with H_2O_2 for 1 h. Top, The proteins were analyzed by Western blot using p53 antibody for indicating p53 distribution. Middle and Bottom, α-tubulin and HSP60 were probed as an indicator of cytoplasmic and mitochondrial protein, respectively. Blots are representative of four independent experiments with similar results. (b) Western blot analysis. Top, total cell protein were extracted from pcDNA3.1/αBC cells and pcDNA3.1 control cells treated or not treated with H_2O_2 for 1 h and analyzed for p53 content. Bottom, α-tubulin was probed as a loading control. Blots are representative of five independent experiments with similar results.

ered that the mitochondrial death pathway is a crucial mechanism mediating apoptosis. In carcinoma cells, eye epithelial cells and myocardium cells, αB-crystallin has been proved to negatively regulate mitochondrial death pathway by distinct mechanisms [18,13]. Being an important molecular chaperone in the cell, more and more proteins have been reported to be targets of and affected by αB-crystallin under normal or stressful conditions, such as Bcl-x_L [13], caspase-3 [18], tubulin [19], titin [20], FBX4 [21]. In the present study, we explored the effects of αB-crystallin overexpression on early stage of apoptosis induced by oxidative stress in mice myogenic C_2C_{12} cells and found that overexpression of αB-crystallin effectively inhibited hydrogen peroxide-induced apoptosis at early stage, indicated by Hoechst 33258 nuclear staining (Fig. 1a) and cell membrane phosphatidylserine (PS) externalization analysis (Fig. 1b).

To further explore the underlying mechanisms, we started to seek possible targets that might be affected by αB-crystallin during this procedure using immunoprecipitation and Ni^+–NTA chromatography assay. We found a protein that interacted with αB-crystallin-p53 (Fig. 2), and such interaction mainly occurred in the cytoplasm of C_2C_{12} cells (Fig. 3). It is now well accepted that the transcription factor p53 mediates apoptosis in response to diverse stimuli that cause apoptosis, including hypoxia, oxidative stress, and DNA damage [22]. In turn, p53 induces apoptosis by transactivating the expression of multiple proapoptotic genes (including *bax*, *noxa*, *puma*, *bid*, *asc*, *apaf-1*, *caspase-6*, and so on) [23]. Also, p53 participates in apoptosis induction by acting directly at mitochondria. Recently, it was reported that the translocation of p53 to mitochondria occurs rapidly in response to multiple death stimuli, including oxidative stress. In mitochondria, p53 physically interacts with antiapoptotic Bcl-2 family proteins (Bcl-x_L and Bcl-2), induces Bak oligomerization, permeabilizes mitochondrial membranes, and rapidly induces cytochrome c release and early wave of cell apoptosis. Such events precede the nuclear translocation of p53 and its target genes activation [7,24,25]. Thus, the interaction of αB-crystallin with p53 in the cytoplasmic portion of C_2C_{12} cells might prevent the translocation of p53 from cytoplasm to other places. This was confirmed by the fact that the translocation of p53 from cytoplasm to mitochondria was greatly

reduced in αB-crystallin overexpressing cells (Fig. 4). Hence, our study provided the first evidence of the effects of αB-crystallin on p53 mitochondrial translocation during oxidative stress-induced apoptosis. Since p53 mitochondrial translocation occurs at the very early stages of apoptosis, our study also suggested an early antiapoptotic role of αB-crystallin on the upstream of mitochondrial-death pathway.

The present investigation shows the relationship between p53 translocation to mitochondria under the stimuli of hydrogen peroxide and αB-crystallin prevents the translocation through interaction with p53. Further studies will include which domains involve in the interaction. Overall, this is the first study to show that αB-crystallin binds to p53 may play a direct role in the regulation of oxidative stress and inhibiting the role of p53 on the mitochondria. The results also suggest that the role of p53 in the mitochondria lead to the activation of p53 transcriptional function and subsequent induction of its proapoptotic target genes and αB-crystallin will interfere in these functions of p53.

Acknowledgments

The project was supported by Major National Basic Research Program of China (G2000056908), National Nature Science Foundation of China (30270533, 30330280) and Nature Science Foundation of Hunan province (05JJ30048).

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