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Synergistic efficacy of LBH and αB-crystallin through inhibiting transcriptional activities of p53 and p21

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LBH is a transcription factor as a candidate gene for CHD associated with partial trisomy 2p syndrome. To identify potential LBH-interacting partners, a yeast two-hybrid screen using LBH as a bait was performed with a human heart cDNA library. One of the clones identified encodes αB-crystallin. Co-immunoprecipitation and GST pull-down assays showed that LBH interacts with αB-crystallin, which is further confirmed by mammalian two-hybrid assays. Co-localization analysis showed that in COS-7 cells, αB-crystallin that is cytoplasmic alone, accumulates partialy in the nucleus when co-transfected with LBH. Transient transfection assays indicated that overexpression of LBH or αB-crystallin reduced the transcriptional activities of p53 and p21, respectively, Overexpression of both αB-crystallin and LBH together resulted in a stronger repression of the transcriptional activities of p21 and p53. These results showed that the interaction of LBH and αB-crystallin may inhibit synergistically the transcriptional regulation of p53 and p21. [BMB reports 2010; 43(6): 432-437]

INTRODUCTION

LBH (limb-bud and heart) is a highly conserved putative transcriptional regulatory protein in vertebrates that do not exhibit any known structural motifs. It displays a unique spatiotemporal gene expression pattern during early mouse heart development (1, 2). When fused to the Gal4 DNA-binding domain, LBH can activate gene expression in a transcriptional reporter assay (3). Further functional studies using Carp-LBH transgenic mice implicate LBH as a candidate gene for congenital heart disease (CHD) associated with partial trisomy 2p syndrome that is characterized by complex malformations of the outflow and inflow tracts, defects in cardiac septation, heart position, as well as abnormal ventricular development (4). Overexpression of LBH in cultured mammalian cells represses the synergistic activity of key cardiac transcription factors, Nkx2.5 and Tbx5, leading to reduced activation of the common target gene, Anf (Nppa), suggesting an important role of LBH in transcriptional control during normal cardiogenesis (5). Based on the molecular characteristics and the domain-specific expression pattern, it is possible that LBH functions in synergy with its protein partners and the signals known to be required for heart development.

αB-crystalli (CryAB) is the most abundant small heat shock protein in the heart and possesses molecular chaperone activity (6, 7). Significant upregulation of CryAB in the heart has been associated with familial hypertrophic cardiomyopathy and desmin-related cardiomyopathy (DRM), and its downregulation has been reported in end-stage congestive heart failure (8, 9). Also previous reports revealed that overexpression of αB-crystallin inhibited apoptosis through, at least in part, repression of the p53-dependent apoptotic pathway (10). Though αB-crystalli localization was predominantly cytoplasmic (11) and was not involved in transcription directly, it may induce transcriptional repression by interacting with other transcriptional repressor (12).

Recently, we have cloned and characterized the human LBH gene that is most abundantly expressed in embryonic and adult heart (13). To identify potential LBH-interacting partners, a yeast two-hybrid screen using LBH as a bait was performed with a human heart cDNA library. One of the clones identified encodes αB-crystallin. Co-immunoprecipitation and GST pull-down assays showed that LBH interacts with αB-crystallin, which is further confirmed by mammalian two-hybrid assays. Co-localization analysis showed that in COS-7 cells, αB-crystallin that is cytoplasmic alone, accumulates partialy in the nucleus when co-transfected with LBH. Overexpression of both LBH and αB-crystallin together downregulate the transcriptional activities of p21 and p53. These results showed that the interaction of LBH and αB-crystallin may inhibit synergistically the p53 and p21 transcriptional activities.

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RESULTS

LBH interacts with αB-crystallin in yeast two-hybrid assay

To identify LBH interacting proteins, we first performed a yeast two-hybrid screening using full-length LBH protein as the bait and human fetal heart cDNA library as a prey. The transactivational activity of the GAL4-LBH fusion protein in yeast was inhibited by 25 mM 3-AT. Approximately 2×10^6 transformants were screened and thirty clones were obtained on a SD/-Trp/-Leu/-His/-Ade medium supplemented with 25 mM 3-AT. Sequence analysis revealed that one of the clones was identical to human CRYAB cDNA previously cloned from a glioblastoma cell line (14).

LBH and αB-crystallin are co-immunoprecipitated in COS-7 cells

To further demonstrate the possible interaction between LBH and αB-crystallin in mammalian cells, co-immunoprecipitation analysis was performed to identify the results from yeast and Mammalian Two-Hybrid assays, in which LBH was tagged with Myc and αB-crystallin was tagged with Flag. As showed in Fig. 1A and 1B, αB-crystallin was precipitated by Myc tagged LBH (Fig. 1A, lane1) but not by control mouse IgG (Fig. 1A, lane 3). LBH was co-immunoprecipitated with Flag tagged αB-crystallin (Fig. 1B, lane1) but not by control rabbit IgG (Fig. 1B, lane 2). These results indicated that LBH and αB-crystallin can be found in the same complex in mammalian cells, and αB-crystallin may directly or indirectly interact with LBH.

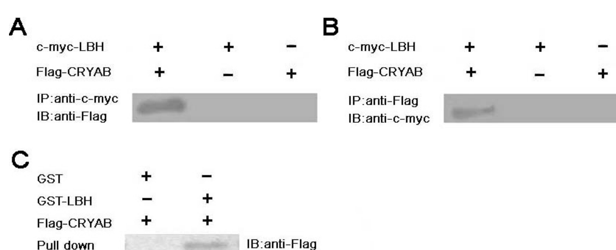

Fig. 1. Identification of the protein-protein interaction between LBH and αB-crystallin by co-immunoprecipitation and pull-down assays. (A) Western blot analysis with rabbit polyclonal anti-Flag antibody of Myc-LBH precipitated protein from COS-7 cells cotransfected with pCMV-Myc-LBH and pCMV-Tag2B-CRYAB (lane 1). Myc-LBH precipitated protein from COS-7 cells transfected with pCMV-Myc-LBH alone (lane 2) and immunoprecipitates with rabbit preimmune serum were used as negative control (lane 3). (B) Western blot analysis with mouse monoclonal anti-Myc antibody of Flag-αB-crystallin precipitated protein from COS-7 cells cotransfected with pCMV-Myc-LBH and pCMV-Tag2B-CRYAB (lane 1). Flag-αB-crystallin precipitated protein from COS-7 cells transfected with pCMV-Tag2B-CRYAB alone (lane 2) and co-immunoprecipitates with mouse preimmune serum were used as negative control (lane 3). (C) pull-down assays were performed to analyze the interaction between LBH and αB-crystallin.

LBH and αB-crystallin interact directly *in vitro*

It is possible that LBH and αB-crystallin interaction may be indirect because other protein factors in the whole cell extract may be involved in mediating the interaction, e.g. acting as 'bridging' factors. Therefore we next decided to examine a possible direct interaction between the two proteins using GST pulldown assays. Fig. 1C showed that αB-crystallin was pulled-down by GST-LBH fused proteins (Fig. 1C, lane 2), but not by GST alone (Fig. 1C, lane 1), indicating that LBH and αB-crystallin specifically interact directly *in vitro*.

Mammalian two-hybrid system detects LBH-αB-crystallin interaction

To further analysis the interaction between LBH and αB-crystallin, LBH were fused in frame to the carboxyl (C) terminus of the GAL4-DNA binding domain (pCMV-BD-LBH), and CRYAB were fused to the C-terminus of NF-kB p65 activation domain (pCMV-AD-CRYAB). These pairs of plasmids were cotransfected with the reporter plasmid pFR-Luc into COS-7 cells, and their luciferase activities were monitored. As shown in Fig. 2,

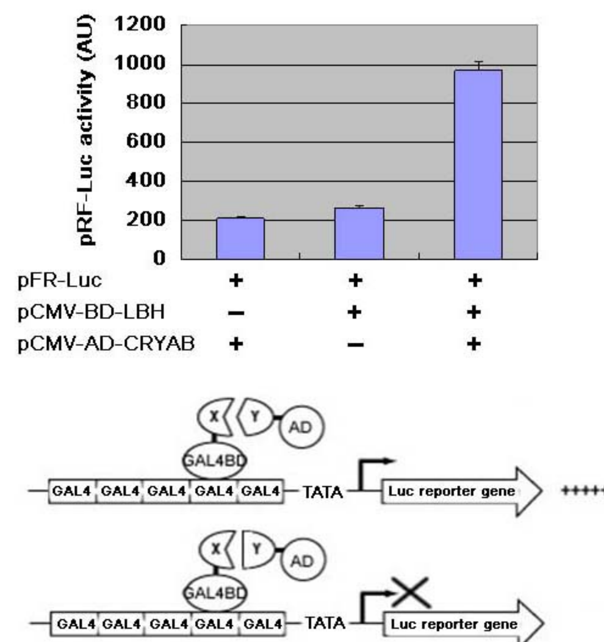

Fig. 2. Mammalian two-hybrid assay detects LBH-αB-crystallin interaction. pCMV-AD-CRYAB (NF-kB p65 activation domain-αB-crystallin fusion protein expression vector) (lane 1), pCMV-BD-LBH (GAL4DBD-LBH fusion protein expression vector) (lane 2), or both pCMV-BD-LBH and pCMV-AD-CRYAB (lane 3) were transiently transfected into COS-7 cells along with pFR-Luc report as indicated in the figure. Forty-eight hours after transient transfection, the luciferase activity assay was performed. Relative activity of luciferase was presented as the mean ± SD of three independent transfection experiments performed in triplicate each treatment.

transfections with the plasmids pCMV-BD-LBH or pCMV-AD-CRYAB alone displayed low luciferase activity, while transfection with the combination of plasmids pCMV-BD-LBH and pCMV-AD-CRYAB resulted in stronger activation of luciferase gene expression, suggesting that the expressed fusion proteins interact *in vivo*.

Subcellular localization of the LBH-αB-crystallin interacting proteins

To detect the subcullular distribution of the LBH-αB-crystallin interacting proteins, pEGFP-N1-LBH and pCMV-Tag2B-CRYAB were transiently cotransfected into COS-7 cells. As expected, the LBH protein was most commonly localized in nucleus with a diffused pattern (Fig. 3A), whereas the α B-crystallin protein distributes in cytoplasm of COS-7 cells (Fig. 3B). However, when cotransfecting both plasmids into COS-7 cells, the CRYAB signal is detected in both the nucleus and cytoplasm, and both signals of LBH and αB-crystallin were detected in the nuclei of COS-7 cells (Fig. 3C), suggesting that the LBH protein could stimulate the translocation of αB-crystallin to the nucleus. This result further indicated that αB-crystalli is one of LBH-interacting proteins.

LBH and αB-crystallin inhibit synergistically the p53 and p21 transcriptional activities

To investigate the physiological relevance of the LBH and αB-crystalli interaction, the p21-Luc or p53-Luc reporter construct was co-transfected into COS-7 cells together with the combination of plasmids pCMV-Myc-LBH and pCMV-Tag2B-CRYAB, pCMV-Myc-LBH or pCMV-Tag2B-CRYAB, respectively. As shown in Fig. 4, overexpression of αB-crystalli inhibited the transcriptional activity of p53 by approximately 45% and that of p21 by approximately 50% in COS-7 cells, while overexpression of LBH suppressed the transcriptional activity of p53 by approximately 11% and that of p21 by approximately 38% in COS-7 cells. Transfection with the combination of LBH and CRYAB significantly suppressed the luciferase activity of p53 by approximately 54% and that of p21 by approximately 74%. Taken together, these results suggested that the interaction of LBH and αB-crystallin may inhibit synergistically the p53 and p21 transcriptional activities.

DISCUSSION

Many proteins were identified to be involved in some important pathway mediated by protein-protein interaction (15, 16). In this paper, we used LBH as a bait to screen a hunan heart cDNA library and identified a new interacting protein αB-crystallin of LBH by yeast two-hybrid screening. As the interaction identified by yeast two-hybrid do not always occurred in mammary cells (17), it was further proved by co-immunoprecipitation, pull-down and subcellular colocalization analuses. The results indicated that LBH is interacted with αB-crystallin to form a protein complex in intro and *in vivo*.

αB-crystallin is a member of the family of small heat shock proteins and acts as molecular chaperone. Previous study suggested that αB-crystalli localization was predominantly cytoplasmic (11). αB-crystalli accumulated in myofibrillar structures, especially in the Z-disks and the intermediate structures (desmin), and is possible to stabilize and protect the myofibrillar structures during and after unaccustomed eccentric exercise (18). Overexpression of CryAB can protect cardiomyocytes from ischemia and reperfusion injury in primary cell culture and in transgenic mice (19, 20). Our experiments showed that transfection with the combination of LBH and CRYAB expression vectors, part of αB-crystallin accumulates

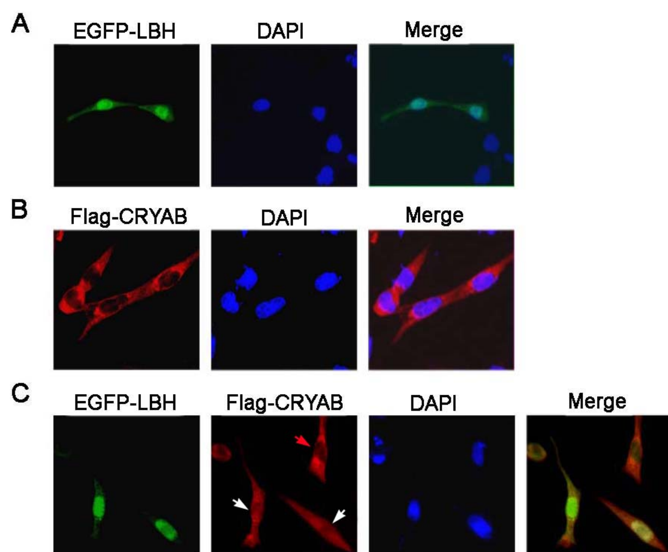

Fig. 3. Co-localization analysis of LBH and αB-crystallin proteins. (A) COS-7 cells was transfected with pEGFP-N1-LBH alone; (B) COS-7 cells was transfected with pCMV-Tag2B-CRYAB alone; (C) COS-7 cells was cotransfected with both pEGFP-N1-LBH and pCMV-Tag2B-CRYAB. The subcellular localization of LBH tagged with EGFP was observed with direct EGFP fluorescence in COS-7 cells. αB-crystallin were detected by immunofluorescence analysis with rabbit polyclonal anti-Flag antibodies and F(ab')2-PE-Cy3 goat anti-rabbit secondary antibodies. Nuclei were stained with DAPI. COS-7 cells only expressing Flag-CRYAB were marked by red arrows. Those cells expressing Flag-CRYAB and EGFP-LBH were marked by white arrows. Yellow signal indicated the overlap of the two proteins. Original magnifications: (A-C) ×400.

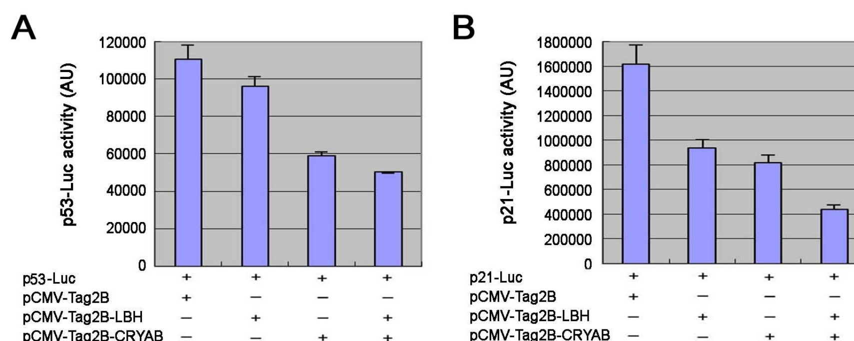

Fig. 4. Luciferase reporter assay of the synergistic efficacy of LBH and αB-crystallin through inhibiting transcriptional activities of p53 and p21. pCMV-Tag2B-LBH, pCMV-Tag2B-CRYAB, or both pCMV-Tag2B-LBH and pCMV-Tag2B-CRYAB were transiently transfected into COS-7 cells along with p53-Luc (A) or p21-Luc (B) reporter respectively, as indicated in the figure. Fortyeight hours later, the luciferase activity assay was performed. The data are means of three repeats in a single experiment after normalization for β-galactosidase activity. Each experiment was repeated at least three times.

in the nucleus and co-localized with LBH in the nuclei of COS-7 cells, suggesting that the interaction of LBH with αB-crystallin could stimulate the translocation of αB-crystallin to the nucleus, and the LBH-αB-crystallin protein complex could be involved in the transcription regulation of potential substrate proteins occuring in the process of the protective role. However, the interacting mechanism of the LBH-αB-crystallin protein complex remains to be investigated.

It has been reported that overexpression of αB-crystallin inhibit the transcriptional activity of p53 (21). As discussed above, we have shown that αB-crystallin is a LBH-interacting protein. Overexpression of LBH and αB-crystallin could give a synergistic effect on the transcriptional activity of p53. Indeed, overexpression of CRYAB and LBH together enhance the repression effect on the activity of p53 when compared to transfection of LBH or CRYAB alone (Fig. 4A). It is known that p21 is one of the downstream genes of p53 and p53 strongly induces p21 expression (13). As expected, the stronger repression effect on the activity of p53 is obseved when overexpression of CRYAB and LBH together compared to transfection of LBH or CRYAB alone. Therefore, the synergistic efficacy of LBH and αB-crystallin through inhibiting transcriptional activities of p53 and p21 provides a further evidence that CRYAB and LBH interact to form a protein complex *in vivo*, which is involved in the process of cell and orgain development.

MATERIALS AND METHODS

Plasmid construction

The pRF-Luc, p53-Luc and p21-Luc constructs used were generated previously in the lab (22). For yeast two-hybrid screening, full-length cDNA of LBH was ligated in frame with the GAL4 DNA-binding domain of the pGBKT7 vector resulting in pGBKT7-LBH. To generate a fusion protein of LBH with enhanced green fluorescent protein (EGFP) or GAL4, the LBH was amplified by PCR (primers pLBH-F2/pLBH-R3 or pLBH-F1/pLBH-R2) (Supplement Table 1) and then subcloned into the *Xho* I and *Hind* III sites of the pEGFP-N1 vector or *Eco*RI and *Sal* I site of the pCMV-BD. To generate a fusion protein of CRYAB with NF-kB or FLAG tag, the CRYAB ORF was amplified by PCR with primers pCRYAB- F1/pCRYAB-R1 (Supplement Table 1) and then subcloned in-frame into the *Eco*RI and *Sal* I site of the pCMV-AD or pCMV-Tag2B, respectively.

Yeast two-hybrid screen

Yeast two-hybrid screening was performed according to the instructions (Clontech, San Jose, USA) using pGBKT7-LBH as bait and human fetal heart cDNA library (Clontech, San Jose, USA) as prey. Yeast strain AH109 was sequentially transformed with pGBKT7-LBH and human fetal heart cDNA library, and plated on quadruple dropout medium (SD/-Trp/-Leu/-His/-Ade) containing X-alpha-gal. Approximately 2×10^6 yeast transformants were screened and these positive colonies were retested using two independent yeast strains AH109 contained pGBKT7-LBH or pGBKT7, respectively. The plasmids isolated from these true positive colonies were sequenced in both directions and analyzed by bioinformatics.

Immunoprecipitation and western blot analysis

COS-7 cells were co-transfected with pCMV-Myc-LBH and pCMV-Tag2B-CRYAB or transfected with pCMV-Myc-LBH or pCMV-Tag2B-CRYAB alone, respectively. Twenty four hours after transfection, the transfected COS-7 cells were washed twice with phosphate buffered saline (PBS) and lysed in RIPA buffer [50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS] with protease inhibitors. Immunoprecipitation using mouse monoclonal anti-Myc antibody (Santa Cruz Biotech) or rabbit polyclonal anti-Flag antibody (Santa Cruz Biotech) were performed as described previously (23). Co-precipitated proteins were subjected to electrophoresis on 10% SDS-polyacrylamide gel, and then western blot analysis was performed using rabbit polyclonal anti-Flag antibody or mouse monoclonal anti-Myc as the first antibody.

GST pull-down assay

GST protein, GST-LBH and Flag-αB-crystallin fusion proteins were expressed and purified according to manufacturer's instructions (Amersham). For the pull-down assay, 1-5 mg of the GST or GST fusion proteins were mixed with 40 ml of 50% suspension of glutathione-Sepharose 4B beads for 2 h in binding buffer [25 mM HEPES-NaOH (pH 7.5), 12.5 mM MgCl2,

10% Glycerol, 5 mM DTT, 0.1% NP-40, 150 mM KCl and 20 mM ZnCl2]. Then 1-5 mg of Flag-αB-crystallin fusion proteins was added followed by incubation for another 2 h. The pellets were washed extensively and boiled. The bound proteins were resolved by 13% SDS-polyacrylamide gel and analyzed by western blot analysis with rabbit polyclonal anti-Flag antibody (Santa Cruz Biotech).

Subcellular colocalization of LBH and αB-crystallin

Two methods, direct EGFP fluorescence and indirect immunofluorescence with anti-Flag antibody were used together to investigate the subcellular colocalization of LBH and αB-crystallin. COS-7 cells cultured on glass coverslips were co-transfected with pEGFP-N1-LBH and pCMV-Tag2B-CRYAB. Immunostaining was performed as previously described (24). Briefly, cells cultured 48 hours were fixed with cold methanol and were then blocked for 20 min in PBS, 1% bovine serum albumin, 10% goat serum, and 0.05% Triton X-100. Next, the cells were incubated in primary antibody diluted in PBS, 5% goat serum, and 0.2% Triton X-100 for 1.5 h followed by secondary antibodies for 1 h. The primary antibodies used were rabbit polyclonal anti-Fag. F(ab')2-PE-Cy3, goat anti-rabbit IgG (Santa Cruz Biotechnology Inc., USA) was used as secondary antibody. DAPI (4', 6'-diamidino-2-phenylindole hydrochloride, Roche, Basel, Switzerland) was used to stain the nuclei. The green and red fluorescence signals were observed with a fluorescence microscope. The COS-7 cells transfected with pEGFP-N1-LBH or pCMV-Tag2B-CRYAB vector were used as the control.

Luciferase assays

COS-7 cells used in all studies were maintained and passaged according to standard methods described previously (25). Transient transfections of cells with the reporter plasmid (pFR-Luc, p21-Luc, p53-Luc), pCMV-LacZ and the indicated expression vectors were carried out with Lipofectamine 2000 (Invitrogen). Twenty four hours after transfection, the cells were lysed and luciferase assay was performed using the luciferase assay system (Promega). pCMV-LacZ was cotransfected in all experiments, and b-galactosidase activity was used to normalize for different transfection efficiencies.

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