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hhLIM protein is involved in cardiac hypertrophy

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Abstract

Proteins of the LIM family are critical regulators of development and differentiation in various cell types. Here we examined the roles of one new member of LIM family, hhLIM, in cardiac hypertrophic growth and cardiac muscle-specific gene expression. To model the increase in endogenous hhLIM transcriptional activity that occurs in response to hypertrophic stimulation, hhLIM was overexpressed using a recombinant plasmid for hhLIM. The results showed that overexpression of hhLIM resulted in increased cell volume in both C2C12 muscle cells (>1.5-fold) and cardiac myocytes (>2.49-fold), a phenotype commonly associated with cardiac hypertrophy. RT-PCR and Western blot showed that transfection of hhLIM into C2C12 muscle cells and cardiomyocytes increased skeletal α-actin levels and triggered the expression of the embryonic-related gene BNP, which is associated with cardiac hypertrophy. Inhibition of hhLIM expression by antisense transcripts blocked the induction of skeletal α-actin and BNP expression by endothelin-1. These data indicated that hhLIM played a role in regulation of cardiomyocyte growth and cell size in response to hypertrophic stimuli through its modulation of skeletal α-actin and BNP expression. We also determined by confocal laser scanning microscopy and immunoprecipitation that hhLIM was associated with α-actin and localized in the cytoplasm in unstimulated cells, and was relocalized from the cytoplasm to the nucleus upon hypertrophic stimulation. These studies suggest that hhLIM protein is involved in cardiac hypertrophy.

Keywords: hhLIM gene; Cardiomyopathy; C2C12 cell; Skeletal α-actin; BNP

1. Introduction

Recent clinical studies have suggested that cardiac hypertrophy is an independent risk factor of cardiac morbidity and mortality [1]. Therefore, it is important to determine the mechanism of how cardiac hypertrophy develops. Hypertrophic growth is one of the ways the heart adapts to a variety of pathological stimuli, including hypertension, myocardial infarction, endocrine disorders, and perturbations in sarcomeric function due to altered expression or mutation of contractile protein genes. Cardiomyocyte hypertrophy can be induced by a variety of factors such as mechanical stress and vasoactive materials, including catecholamines, angiotensin II, endothelin-1, and cytokines [2]. We and others have reported that mechanical stress induces cardiomyocyte hypertrophy through vasoactive peptides such as angiotensin II and endothelin-1 [3]. In response to hypertrophic signals, cardiomyocytes activate a cellular response characterized by an increase in cell size and sarcomere assembly, induction of fetal cardiac genes, and repression of genes encoding the corresponding adult isoforms.

The LIM domain is a cysteine-rich zinc-finger motif found in a large family of proteins [3]. Proteins containing LIM domains have been linked with a variety of fundamental biological events including cytoskeletal organization, cell lineage specification and organ development [4]. Biochemically, the LIM domains are responsible for key interactions with co-activators, co-repressors, competitors, and other transcription factors, and are therefore of considerable importance for the regulation of associated transcriptional activities [4]. Recently, a new member of the LIM family, named hhLIM (also known as hLIM3, GenBank AF121260), was cloned by three elements PCR-select

Abbreviations: hhLIM, human heart LIM protein (myogenic factor LIM3); BNP, brain natriuretic peptide; GFP, green fluorescent protein; IGF, insulin-like growth factor

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cDNA subtraction [5]. hhLIM has a typical LIM domain. In recent years, accumulating results have continued to attribute essential functions to LIM proteins in a variety of different biological processes. But little is known about the new gene hhLIM. We have identified that hhLIM transcripts were detected in cardiac cell progenitors during embryogenesis, indicating that hhLIM gene is tightly linked to cardiomyocyte specification. We also found that hhLIM RNA level increased in pressure-induced cardiac hypertrophy and that hhLIM protein played a role as an effector of hypertrophic response. But it is not clear whether hhLIM acts directly as a necessary sufficient mediator of the response. In the present study we provide further evidence linking hhLIM to the hypertrophic response and show that hhLIM is sufficient for inducing cardiomyocyte hypertrophy in cultured myoblasts. In addition, ours is the first report that hhLIM protein location changes from cytoplasm to nucleus in response to stimuli inducing differentiation and hypertrophy.

2. Materials and methods

2.1. Cell culture

The muscle-derived cell line C2C12 myoblasts (ATCC, CRL1772) were cultured in growth medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum) (MB). To induce myogenic differentiation, subconfluent cultures were shifted to differentiation medium (Dulbecco's modified Eagle's medium supplemented with 2% horse serum) (MT).

2.2. RNA isolation and RT-PCR

Total cellular RNA was isolated by guanidine isothiocyanate [6]. Semi-quantitative RT-PCR was performed with gene-specific primer pairs for hhLIM and BNP cDNA. The sequences for hhLIM and BNP cDNA were obtained from GenBank AF121260, D16497). The specific primer sets for hhLIM and BNP were: hhLIM, forward primer 5′-GCTGTCTCAGCACAGACA-3′ and reverse primer 5′-ATGGCACAGCGGA-3′; BNP, forward primer 5′-GCTGCTGGAGCTGATAAG-3′ and reverse primer 5′-TTTGAGGTCTCTGCTGGA-3′. The level of transcription for the constitutive housekeeping gene, GAPDH, was quantitatively measured in each sample to control differences in RNA concentrations. The data were analyzed by Bio 1D software (Kodak).

2.3. Western blot analysis

Cells were harvested and protein extracts were prepared in RIPA buffer (1% NP40, 1% SDS, 150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 10% glycerol, 1 mM $NaVO_3$, 1 mM PMSF) as described previously [7]. Protein concentration was determined by a modified Lowry protein assay. Fifty-microgram protein per sample was separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and blocked in 5% milk/TTBS (500 mM NaCl, 0.05% Tween 20, 50 mM Tris–HCl, pH 7.5). Membranes were incubated overnight at 4 °C with a mouse monoclonal antibody specific to skeletal α-actin (Sigma), then incubated with an alkaline phosphatase-conjugated secondary antibody. Bands were detected by chemiluminescence (Visa ECF, Amersham Pharmacia Biotech).

2.4. Plasmids and construction

The hhLIM expression plasmid (pX) was a gift from Dr. Chen Kuang-hui [5] that was constructed by subcloning the hhLIM cDNA into the $pcDNA_2$ vector. The hhLIM/luciferase reporter plasmid (phL) was constructed by ligation of PCR-amplified hhLIM upstream DNA fragment (− 537 to + 16 bp) to the promoterless luciferase reporter vector (pGL-Basic) (Promega) [7]. The hhLIM fusion plasmid (phF) was constructed by inserting a complete hhLIM cDNA fragment into pEGFP-C3 vector. An antisense hhLIM plasmid (phA) was constructed by inserting the hhLIM cDNA into pEGFP-N1 plasmid. pCMV-IGF-1 was a gift from Dr. Pietrzkowski (Jefferson Medical College) [8]. The BNP/luciferase reporter plasmid was a gift from Dr. Gardner (University of California) [9].

2.5. Transfection and determination of reporter activity in C2C12 cells

Plasmids were prepared for transfection using Wizard Sv 96 plasmid DNA purification system (Promega). C2C12 cells were grown up to 70% confluence in 60-mm culture dishes, and transiently transfected with 1 μg of hhLIM promoter plasmid (phL) together with 1 μg of β-galactosidase vector (pCMVgal, Promega) using ESCORT™ Transfection Reagent (Sigma). Cells were incubated for 16 h in DMEM + 10% FCS and then placed in DMEM containing 2% horse serum with or without endothelin-1 (1×10^{-7} M) for 48 h. Cells were then harvested and the luciferase and β-galactosidase activities were measured by a liquid scintillation counting-based assay following the manufacturer's instructions (Promega).

2.6. Cell size measurement and intracellular distribution of hhLIM protein

C2C12 cells were grown up to 70% confluence and transiently co-transfected with 1 μg of hhLIM expression plasmid (pX) and 0.5-μg pEGFP-N1. After transfection cells were placed in DMEM containing 2% horse serum for 48 h. For cell size measurement, 10 or 20 randomly chosen 400-fold magnification fields were visualized by phase contrast and photographed. From these photos, cell surface of 100 randomly selected individual cells was measured by planim-

etry. C2C12 cells were transfected with hhLIM-GFP fusion plasmid (phF) as described above and then incubated in DMEM containing 2% horse serum for 24, 48, and 72 h. Distribution of hhLIM protein was detected using fluorescent microscopy.

2.7. Immunofluorescence

Cells were fixed on the coverslips with 4% paraformaldehyde for 30 min at room temperature. These fixed cells were blocked with a solution of 1% BSA and then incubated overnight at 4 °C with antibodies against skeletal α-actin (1:100) (Sigma) and hhLIM (1:100) (a gift from Dr. Chen Kuang-hui, Peking University) [5]. FITC and TRITC conjugated secondary antibodies were used at 1:100 for 2 h at room temperature. Stained cells were observed with a Leica DMRE confocal laser-scanning microscope (Leica Microsystems, Heidelberg, Germany).

2.8. Co-immunoprecipitation of hhLIM and α-actin

Cells were collected and lysed in lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1 mM PMSF, 1% NP40, 0.5% deoxycholate) on ice for 30 min, and then centrifuged at $12000 \times g$ at 4 °C. To immunoprecipitate proteins, the supernatant was incubated with 20-μl anti-hhLIM antibody conjugated to agarose beads (1.8 mg antibody/ml) in a total volume of 0.5 ml and rocked continuously overnight at 4 °C. The beads were then pelleted, washed five times in lysis buffer, suspended in 2× Laemmli buffer, and subjected to Western blot analysis for α-actin as described above [10].

2.9. Primary cardiomyocyte cultures

Primary cardiomyocytes were cultured as described previously [2]. Briefly, 1-day-old mouse neonates were sacrificed by CO_2 inhalation, the heart was collected, atria were removed, and the ventricles were cut into four pieces and digested using 0.05% pancreatin and 0.1% collagenase. Then the cells were plated on gelatinized cell culture dishes and cultured overnight in DMEM supplemented with 15% FCS and penicillin/streptomycin (100 units/ml) [2].

2.10. Statistical analysis

Each experiment was performed in triplicate. All data presented are means ± S.E. Results were analyzed using a *t*-test for nonpaired and paired variates.

3. Results

3.1. hhLIM gene expression was augmented by endothelin-1

To assess the progression of hypertrophy of C2C12 cells (MT) induced by endothelin-1, the hypertrophic marker BNP and skeletal α-actin were analyzed by RT-PCR and Western blot analysis. As analyzed by Western blot, skeletal α-actin protein level in cultured C2C12 cells (MT) progressively increased with duration of endothelin-1 treatment (Fig. 1A). BNP gene expression could not be detected in untreated C2C12 cells (MT), but was significantly induced by endothelin-1 stimulation (Fig. 1B). These results show that endothelin-1 is a good hypertrophic stimulus. To

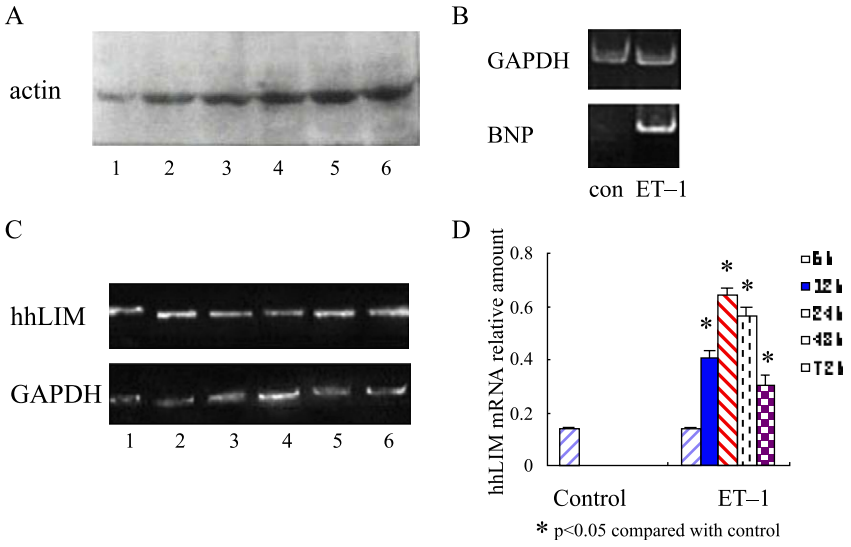

Fig. 1. Skeletal α-actin protein, BNP and hhLIM gene expression in C2C12 cells (MT) treated by endothelin-1. C2C12 cells (MT) were grown to 70% confluence and washed with Hanks balanced salt solution before incubation for 6, 12, 24, 48, and 72 h in DMEM containing 2% horse serum with or without 1×10^{-7} M endothelin-1. Western blot and RT-PCR analysis for α-actin (A), BNP (B) or hhLIM (C, D) were performed under Materials and methods. Samples were assayed in triplicate. 1–6: C2C12 cells (MT) treated by endothelin-1 for 0, 6, 12, 24, 48, and 72 h.

investigate the role of hhLIM as a hypertrophic regulator, we examined whether expression of hhLIM was altered by hypertrophic stimuli. As shown in Fig. 1C and D, treatment of cultured C2C12 cells (MT) with endothelin-1 for 6, 12, 24, 48, and 72 h increased hhLIM mRNA levels with a peak induction after 24 h (4.5-fold over control, $P<0.05$).

To examine the transcriptional responsiveness of hhLIM to a hypertrophic agonist, C2C12 cells (MT) were transiently transfected with a luciferase reporter containing the hhLIM promoter (phL). Transfection of the phL, together with endothelin-1 stimulation, caused a 1.56-fold increase in luciferase activity as compared to the control ($P<0.05$) (Fig. 2), showing that endothelin-1 can augment transcription of the hhLIM promoter.

3.2. Overexpression of hhLIM induced C2C12 cell hypertrophy in culture

The above data suggested that hhLIM is an important factor in the hypertrophic response of C2C12 cells (MT). However, we still did not know whether hhLIM plays a direct role in triggering cellular hypertrophy. To test this we studied whether hhLIM overexpression is sufficient to bypass upstream signaling events and initiate the hypertrophic response. Accordingly, C2C12 cells (MT) transfected with hhLIM expression plasmid (pX), which constitutively express hhLIM or pCMV-gal (as a control), were examined for morphological and biochemical features of hypertrophy 24 to 48 h later. The data demonstrated that hhLIM induced a striking hypertrophic response characterized by increased cell size in both the long and short cellular axes. At 48 h after transfection, hhLIM overexpression increased cell surface area by more than 150% as compared with β-gal ($P<0.05$) (Fig. 3A and B). Collectively, the data indicate that hhLIM is sufficient for hypertrophic induction in cultured C2C12 cells (MT).

We also set out to determine whether hhLIM is capable of activating expression of the hypertrophic markers, BNP and skeletal α-actin. Expression of BNP was undetected in C2C12 cells (MT) grown in 2% horse serum, but BNP mRNA in these differentiated cells was elevated 48 h after transfection of the hhLIM expression plasmid (pX) (Fig. 4A and B). Western blot analysis showed that overexpression of hhLIM caused a 50% increase in skeletal α-actin protein levels in C2C12 cells (MT) as compared with those transfected with empty vector alone (Fig. 4C and D). Although skeletal α-actin protein was undetectable in C2C12 cells (MB), transfection of hhLIM could induce this protein expression, to a certain extent, in these cells. These results showed that hhLIM alone, when overexpressed, was capable of inducing hypertrophy in C2C12 cells (MT) and might be doing so through transcriptional activation of hypertrophic factors.

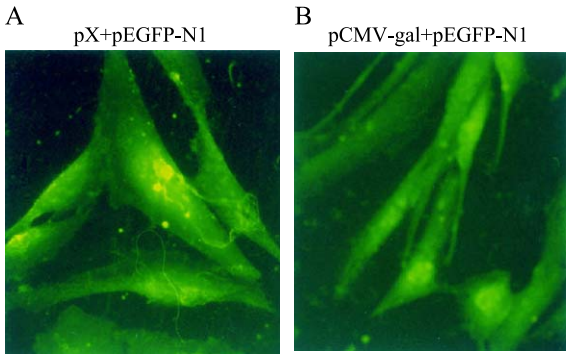

Fig. 3. Effect of hhLIM overexpression on C2C12 cells in size. C2C12 cells (MT) were co-transfected with hhLIM expression plasmid (pX) and pEGFP-N1 and the images were detected using fluorescent microscopy (A), pCMV-gal and pEGFP-N1 co-transfected the cells as a control (B). (For color see online version).

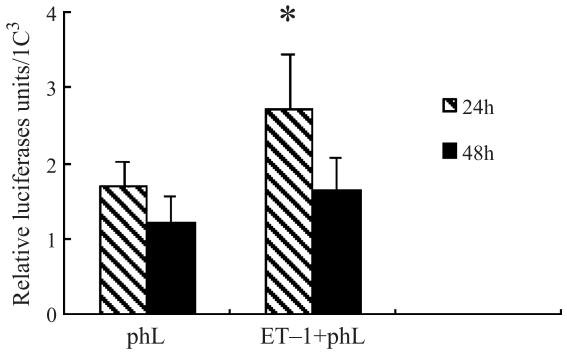

Fig. 2. Effects of endothelin-1 on hhLIM promoter activation. C2C12 cells (MT) were transfected with constructs (phL) possessing the 5′ flanking sequence of hhLIM gene ligated to luciferase, plated for 24 or 48 h with endothelin-1 treatment. *$P<0.05$ compared with group transfected with phL.

3.3. Intracellular distribution of hhLIM protein in C2C12 cells

To determine the subcellular distribution of hhLIM, cells were incubated in DMEM containing 2% horse serum for 24, 48, and 72 h after transfection with GFP-hhLIM fusion plasmid (phF). The results indicated that cells expressing GFP alone showed a diffuse fluorescence pattern with uniform intensity throughout the cells (Fig. 3B), consistent with its known distribution in both cytoplasmic and nuclear compartments. Although C2C12 cells (MT) expressing the GFP-hhLIM fusion protein initially displayed a similar diffuse pattern of fluorescence, the fluorescent signal became more concentrated in the cytoplasm as the time of post-transfection increased (Fig. 5A–C). These results indicated that hhLIM protein was localized in both the cytoplasm and the nucleus in exponentially growing C2C12 cells (MT).

We also wanted to confirm these results in cells that naturally express hhLIM. For this purpose, anti-hhLIM immunofluorescence assay was performed on C2C12 cells (MT) before and after treatment with endothelin-1 for different durations. In the absence of stimulation, hhLIM

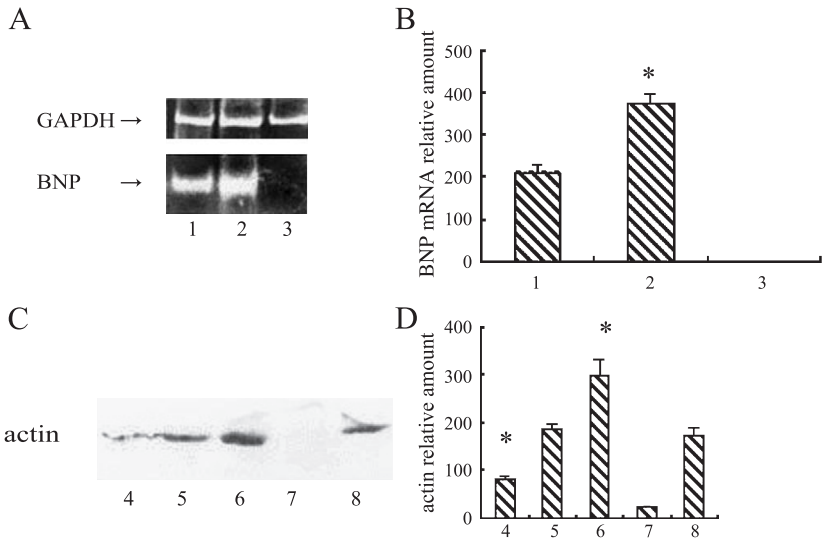

Fig. 4. BNP mRNA and skeletal α-actin expression in C2C12 cells. C2C12 cells (MB) were grown up to 70% confluence and transiently co-transfected with 1 μg of hhLIM expression plasmid (pX) and 0.5-μg pEGFP-N1. After transfection, cells were placed in DMEM containing 2% horse serum for 48 h. BNP mRNA (A, B) and skeletal α-actin (C, D) in C2C12 cells were analyzed by RT-PCR and Western blot analysis. 1, 6: pcD2-hhLIM (pX)-transfected MT; 2: pcD2-transfected MT treated by endothelin-1; 3, 8: pcD2-transfected MT; 4: pcD2-hhLIM (pX)-transfected MB; 5: pCMV-IGF-1-transfected MT; 7: pcD2-transfected MB. *$P<0.05$ compared with pcD2-transfected C2C12 cells (MB and MT).

protein was diffusely distributed throughout the cytoplasm (results not shown). Interestingly, when C2C12 cells (MT) were induced by endothelin-1, hhLIM protein was found to accumulate in the nuclei, to the greatest extent at 24 h after treatment and to a somewhat reduced level after longer treatments (Fig. 5D and E). These results show that hhLIM enters the nucleus and that temporal nuclear accumulation of hhLIM after hypertrophic stimuli corresponds with timing of BNP induction, suggesting that hhLIM may be relevant for activating cell hypertrophy.

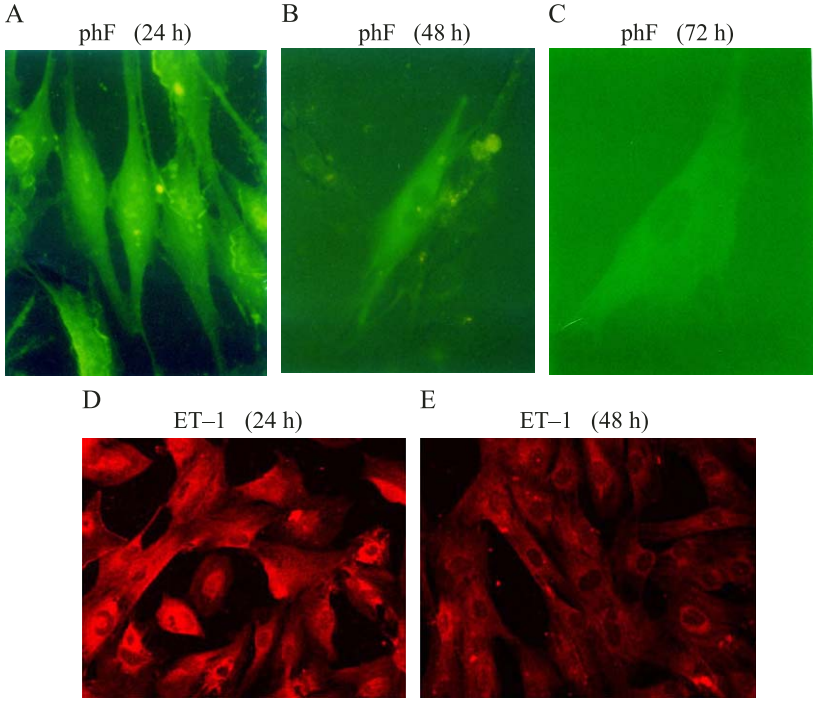

Fig. 5. Subcellular localization of hhLIM protein in C2C12 cells. C2C12 cells were transfected with GFP-hhLIM fusion protein plasmid (phF). GFP fluorescence of GFP-hhLIM fusion protein in C2C12 cells after 24 (A), 48 (B), and 72 (C) h, respectively, was displayed by fluorescent microscopy. C2C12 cells were grown on coverslips in six-well plates in the presence of DMEM containing 2% horse serum for 48 h and then switched to fresh DMEM containing 2% horse serum plus 1×10^{-7} M endothelin-1. Subcellular localization of endogenous hhLIM protein in C2C12 cells (MT) after treatment with endothelin-1 for 24 h (D) and 48 h (E) was detected with immunofluorescence analysis. (For color see online version).

3.4. Interaction between α-actin and hhLIM in C2C12 cells

Many previous reports have shown that some LIM proteins play a role in fundamental biological processes, such as cytoskeletal organization. From the above data, we know that hhLIM protein was distributed in the C2C12 cytoplasm at 48 h after transfection. To test whether the cytoplasmic hhLIM could interact with α-actin, we carried out co-immunoprecipitation using an anti-hhLIM antibody-linked to agarose beads, and the resulting precipitate was analyzed by Western blot analysis using an anti-α-actin antibody. As expected, α-actin was found to co-precipitate with hhLIM (Fig. 6A and B). α-Actin is expressed in the cells at a very high level and is notoriously known to bind to many proteins nonspecifically. In order to testify the specificity of interaction between hhLIM and α-actin, 3–6-fold excess of negative control (BSA) was added 15 min before addition of anti-hhLIM antibody in competition assay. The results showed that excess amount of BSA could not affect the specific interaction between hhLIM and α-actin. When we examined whether endogenous hhLIM protein induced by endothelin-1 could also interact with α-actin by immunofluorescence and confocal microscopy, it was found that hhLIM did associate with α-actin (Fig. 6C), showing that their interactions do have physiological relevance.

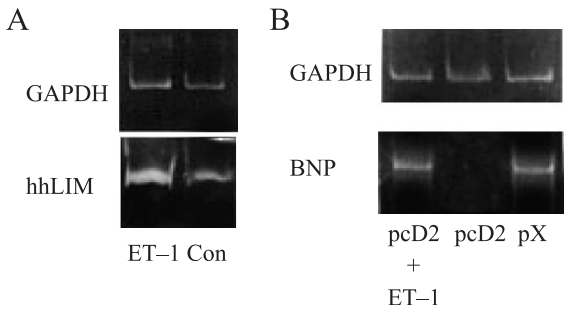

Fig. 7. hhLIM and BNP mRNA expression in cardiac myocytes. Primary cardiomyocytes were treated by endothelin-1 for 48 h, hhLIM gene expression was analyzed by RT-PCR (A). Cardiomyocytes were grown to 70% confluence and transfected with 1 μg of hhLIM expression plasmid (pX). After transfection, cells were incubated in DMEM containing 10% FCS for 48 h. BNP gene expression was analyzed by RT-PCR (B).

3.5. Overexpression of hhLIM induced cardiac myocyte hypertrophy in culture

To further investigate the role of hhLIM as cardiac hypertrophic regulator, we examined whether endothelin-1 stimulation affects expression of endogenous hhLIM in cardiac myocytes. As analyzed by RT-PCR, treatment of cultured cardiomyocytes with endothelin-1 for 48 h up-regulated hhLIM gene expression (2.12-fold over control,

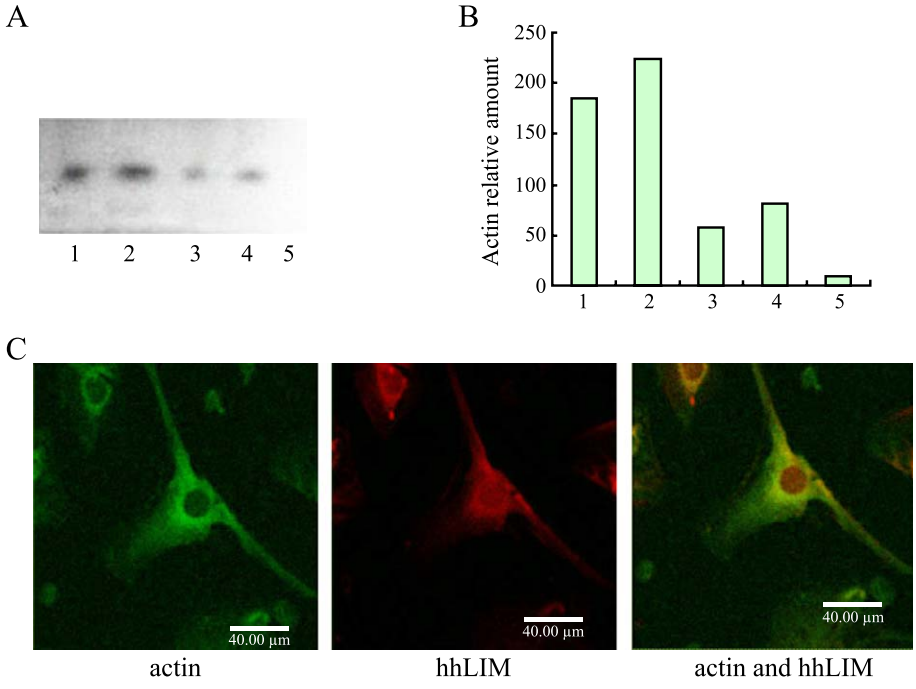

Fig. 6. Co-immunoprecipitation of hhLIM and skeletal α-actin in C2C12 cells and localization of α-actin and hhLIM in C2C12 cells (MT) treated by endothelin-1. C2C12 cells were grown up to 70% confluence, and transiently transfected with 1 μg of hhLIM expression plasmid (pX). After transfection, cells were placed in DMEM containing 2% horse serum or 10% FCS for 48 h. The whole lysate was subjected to immunoprecipitation with anti-hhLIM antibody conjugated to agarose beads. Beads were pelleted, washed, and subsequently analyzed by Western blot analysis using anti-α-actin antibody, pcD2 plasmid-transfected cells as a control (A, B). Double immunofluorescence of hhLIM and α-actin is overlaid in C2C12 cells treated by endothelin-1 for 24 h (C). 1: pcD2-transfected MT; 2: pcD2-hhLIM (pX)-transfected MT; 3: pcD2-hhLIM (pX)-transfected MB; 4: pCMV-IGF-1-transfected MT; 5: pcD2-transfected MB. (For color see online version).

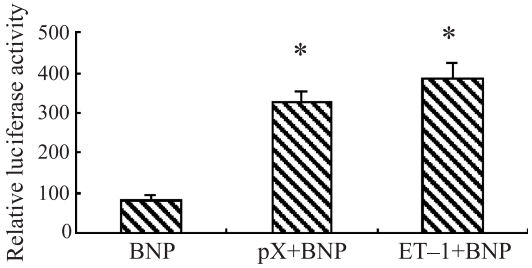

Fig. 8. Effect of hhLIM overexpression on BNP promoter activation. Luciferase activities of cardiac myocytes co-transfected with BNP promoter reporter and hhLIM expression plasmid (pX) were analyzed by a liquid scintillation counting-based assay. Cardiac myocytes transfected with BNP promoter reporter and induced with endothelin-1 as a positive control. *$P<0.05$ compared with BNP promoter reporter transfected cardiac myocytes.

$P<0.05$) (Fig. 7A). Expression of the hypertrophic marker BNP gene was undetected in untreated cardiac myocytes, but was dramatically induced by endothelin-1 stimulation (Fig. 6B). These data demonstrated that endothelin-1 stimulation of cardiomyocytes could increase expression of both hhLIM and BNP, thus linking these two genes to hypertrophic response.

To investigate whether hhLIM is sufficient to bypass upstream signaling events and is capable of initiating the hypertrophic response, cardiomyocytes transfected with hhLIM expression plasmid (pX) were examined for morphological and biochemical features of hypertrophy 48 h after transfection and compared to those transfected with the control vector, pCMV-β-gal. The data demonstrated that hhLIM overexpression induced a striking hypertrophic response characterized by increased cell size in both the long and short cellular axes (data not shown). Overexpression of hhLIM also induced expression of BNP (Fig. 7B) and skeletal α-actin protein (data not shown).

To examine the transcriptional responsiveness of BNP gene to hhLIM protein, cardiac myocytes were transiently co-transfected with a luciferase reporter directed by the BNP promoter (− 1595 bp) and hhLIM expression plasmid (pX). As shown in Fig. 8, their co-transfection resulted in 3.96-fold activation of luciferase activity as compared to untransfected controls ($P<0.05$), suggesting that hhLIM could

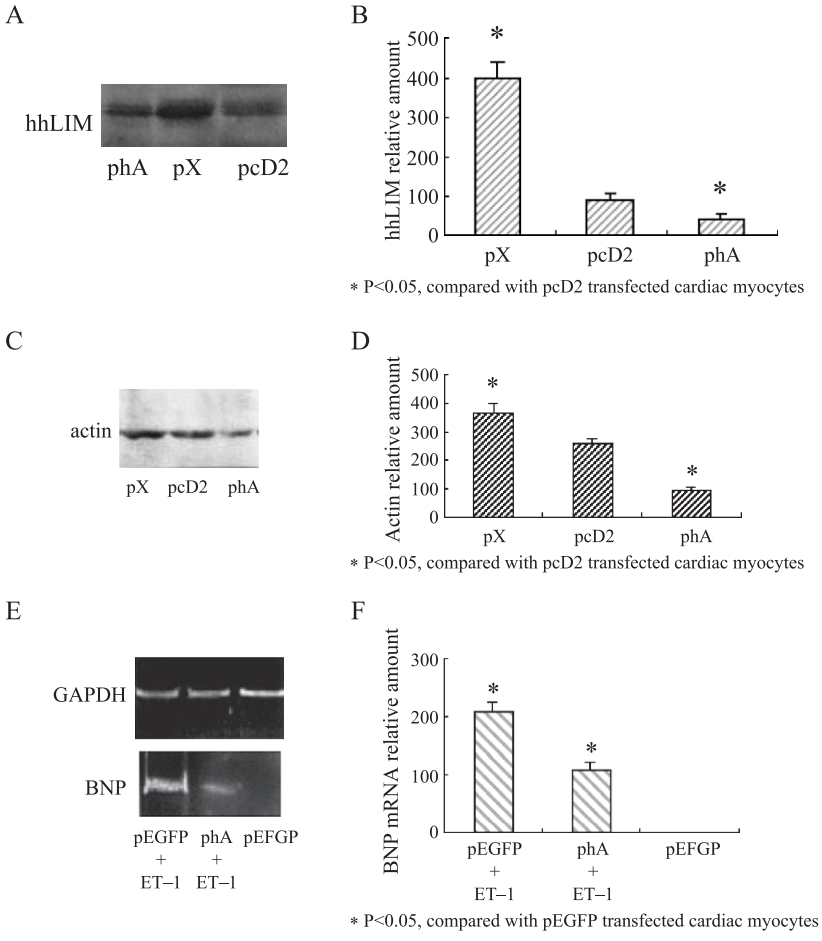

Fig. 9. BNP mRNA and skeletal α-actin expression in cardiac myocytes transfected with an antisense hhLIM expression plasmid. Primary cardiomyocytes were grown to 70% confluence and transfected with 1 μg of hhLIM expression plasmid (pX) and 1 μg of hhLIM antisense plasmid (phA), respectively. BNP gene expression and skeletal α-actin protein level were analyzed by RT-PCR and Western blot.

augment BNP promoter activity. Endothelin-1 stimulation also augmented expression of the BNP promoter to a same extent as hhLIM overexpression. In addition, individual expression vector for hhLIM or Nkx2.5 could enhance BNP reporter expression in cardiac myocytes. Co-transfection of hhLIM and Nkx2.5 produced additive luciferase expression (data not shown). Collectively, these results indicate that hypertrophic stimuli induce hhLIM expression and its trans-activation capability, and this activation correlates with induction of the downstream hypertrophic effectors BNP and skeletal α-actin.

3.6. Antisense hhLIM blocked the hypertrophic response in culture

To determine whether endogenous hhLIM act as essential hypertrophic transcriptional effectors, antisense hhLIM expression plasmid (phA) was designed complementary to the translation site of the hhLIM gene, and transfected into primary cardiac myocytes. It was demonstrated that hhLIM antisense transcripts decreased hhLIM protein levels by approximately 50% (Fig. 9A and B). Cardiomyocytes transfected with antisense hhLIM expression plasmid (phA) or pEGFP empty vector (as a control) were stimulated with endothelin-1 for 24 h. Endothelin-1 increased cell surface and BNP and α-actin expression in cultures transfected with control plasmid, suggesting that the control plasmid alone did not affect hypertrophic response. In contrast, overexpression of antisense hhLIM blocked the increase in cell surface and BNP and α-actin expression induced by endothelin-1 (Fig. 9C–F). Taken together, these data indicate that inhibition of endogenous hhLIM expression can block cardiomyocyte hypertrophy and suggest that hhLIM is a necessary hypertrophic transducer.

4. Discussion

Cardiac hypertrophy is defined as increase in ventricular and/or sepal wall thickness caused by increased wall tension or enhanced neural-hormonal stimulation. Although a compensatory response initially normalizes wall stress and augments pump function, prolonged cardiac hypertrophy is a significant risk factor for the development of future heart failure [2]. Both the increased wall tension and neural-hormonal stimuli are thought to directly activate various membrane-bound receptors and signal transduction cascades within cardiomyocytes, resulting in the activation of immediate early genes such as c-jun, c-fos, c-myc, erg-1, ANF, BNP, and β-MHC and skeletal α-actin [11]. A number of transcription factors have been implicated as direct mediators of hypertrophic gene expression including AP1, SP1, GATA-4, Nkx2.5 and hhLIM protein [12].

The LIM domain defines a unique double zinc finger structure that is highly conserved among proteins present in organisms representing an extensive range of evolution. They are thought to function as versatile protein modules, capable of acting within diverse cellular contexts and in multiple subcellular compartments [13]. Many LIM domains have been shown to participate in direct protein–protein interactions. Nuclear LIM proteins have long been recognized as playing important roles in the control of gene expression and cell fate determination. Although there is little evidence to support direct DNA-binding activity of LIM domains, nuclear magnetic resonance structural analysis of a LIM domain indicates that it is related to the zinc fingers of the GATA family of transcription factors [4]. Recently, a new member of LIM family named hhLIM was cloned; it has a typical LIM domain [5].

It is well known that endothelin-1 is a strong hypertrophic agonist. Cardiac hypertrophy marker genes including BNP and α-actin were significantly up-regulated by endothelin-1. The present results demonstrate that hhLIM mRNA expression and the transcription of hhLIM promoter-enhancer luciferase gene were up-regulated by endothelin-1, suggesting that hhLIM is an effector of hypertrophic response. Although the results of the present studies clearly implicate an important role for hhLIM in hypertrophic signaling, direct experimental evidence linking hhLIM transcription activity to the initiation and/or maintenance of cardiac hypertrophy had not been demonstrated. C2C12 cells have been found to be suitable for stable transfection with foreign cDNA and to express high levels of exogenous proteins. So, C2C12 cells are useful for assessing the functional effects of exogenous proteins. Here we utilized hhLIM expression plasmids to examine the direct effects of hhLIM on the hypertrophic response in cultured cardiomyocytes and C2C12 cells (MT). Through these experiments, we showed that overexpression of hhLIM was capable of activating transcription at the BNP promoter, inducing BNP gene expression, promoting the accumulation of α-actin, and initiating the hypertrophic response in cultured C2C12 cells and cardiomyocytes. These results suggest that hhLIM protein is involved in cell hypertrophy. There are a number of possible mechanisms whereby hhLIM could alter the fate of cells. Thus, the LIM domains of hhLIM could, theoretically, directly bind DNA and modulate transcription of specific genes. From the results of co-immunoprecipitation and immunofluorescence assays, we found that hhLIM is a member of a family of cytosolic LIM domain-containing proteins that interacts with the cytoskeletal protein α-actin. In the absence of endothelin-1 stimulation, hhLIM was diffusely distributed throughout the cytoplasm, suggesting that hhLIM is a kind of cytoskeleton component. However, endothelin-1 stimulation of C2C12 cells (MT) resulted in a redistribution of hhLIM from the cytoplasm to the nucleus. Reporter gene assay showed that hhLIM was a transcriptional co-activator of BNP promoter. Together these results suggest that hhLIM protein is involved in cardiac hypertrophy in response to stimuli such as endothelin-1.

In order to further identify the role of hhLIM in cardiac hypertrophy, we generated an antisense hhLIM expression plasmid to analyze the effects of hhLIM gene silencing on hypertrophic response in cultured neonatal cardiomyocytes. Antisense hhLIM overexpression blocks endothelin-induced cardiomyocyte hypertrophy as determined by cell surface area, BNP expression, and skeletal α-actin accumulation. Overexpression of GFP alone did not down-regulate any of these parameters, showing that these results were specific to hhLIM gene silencing. These data indicate that a functional hhLIM-dependent transcriptional pathway is required for the efficient induction of the hypertrophic response in cultured cardiomyocytes.

Altogether, the present results demonstrate that hhLIM-dependent transcription is necessary for cardiomyocyte hypertrophy induced by endothelin-1. Recently, a number of mechanisms have been proposed whereby hhLIM might induce cardiac-gene expression. First, hhLIM transcripts are up-regulated by hypertrophic stimuli, which have the same expression pattern as the cardiac hypertrophy gene BNP. Their similar expression pattern and participation in the functional process suggest that hhLIM may contribute to cardiac hypertrophy. Second, we identified that hhLIM could interact with other cardiac-expressed transcription factors such as SRF, MEF-2, GATA-4 and Nkx2.5, each of which is involved in regulating hypertrophic gene expression [7,14,15]. Here we show that overexpression of hhLIM is capable of activating transcription of the BNP promoter and promoting accumulation of BNP mRNA and α-actin protein. These results suggest that hhLIM might contribute to the signaling pathway triggered by endothelin-1, stimulate expression of BNP and skeletal α-actin, and induce cardiac hypertrophy. However, these data only show that hhLIM is necessary for integrated signaling in response to hypertrophic stimuli, but do not preclude the involvement of other transcriptional regulatory factors in cellular hypertrophy. Indeed, a series of intracellular signaling pathways and transcriptional factors have been shown to regulate the hypertrophic response [15,16], suggesting that cardiac hypertrophy is a complex process that requires the simultaneous orchestration of multiple parallel effectors to induce a productive response. Determining how individual effector proteins fit into this signaling web will be important for understanding how the hypertrophic response is regulated.

In conclusion, we found that hhLIM gene expression in cultured cardiomyocytes and C2C12 cells was regulated by endothelin-1. hhLIM protein entered the nucleus when cardiomyocytes and C2C12 cells (MT) are stimulated with endothelin-1, and nuclear accumulation of hhLIM protein might be associated with the cell hypertrophy. In the cytoplasm, hhLIM interacts with skeletal α-actin and participates in cytoskeletal organization. Overexpression of hhLIM gene is sufficient to induce a great increase in cardiac myocyte area and trigger the expression of cardiac hypertrophic marker gene BNP and skeletal α-actin. Inhibition of hhLIM expression by antisense transcripts blocked endothelin-induced expression of skeletal α-actin and BNP genes. Altogether, our study suggests that an hhLIM-dependent transcriptional pathway is necessary to induce the hypertrophic response in cultured cardiomyocytes and C2C12 cells (MT).

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