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Human Heart LIM Protein Has Transcription Activation Ability Related to LIM Domain 1

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Abstract—Human heart LIM (hhLIM), a muscle-specific expressed transcriptional coactivator of cardiac hypertrophy related gene, stimulates transcriptional activation of the *ANF* gene promoter in H9C2 cells. We revealed that the N-terminal half of hhLIM mediated this activation, in which the LIM domain 1 and protein kinase C phosphorylation site are important, especially the LIM domain 1. Further, mutagenesis of the conserved Cys in the LIM domain 1 abolished its ability to activate cardiac hypertrophy. These findings suggest that hhLIM is a typical LIM family member with powerful transcription activation.

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The LIM domain (CX2CX16-23HX2CX2CX2-CX16-21CX2(C/H/D), where X denotes any amino acid), a cysteine-rich zinc-finger motif found in a large family of proteins, is now recognized as a key component of the regulatory machinery of the cell [1]. Recent studies indicate that proteins containing LIM domains have diverse cellular roles as regulators of gene expression, cytoarchitecture, cell adhesion, cell motility, and signal transduction [2]. Human heart LIM (hhLIM), a newly revealed LIM protein, contains two LIM domains and is expressed mainly in the heart [3]. We have previously reported that hhLIM plays a role in regulation of cardiomyocyte growth and cell size [4]. Nevertheless, little is known about the mechanism of action of hhLIM in cardiac hypertrophy. Here we used H9C2 cell line, which is a clonal myogenic cell line derived from embryonic rat ventricle that can serve as a surrogate for cardiac or skeletal muscle *in vitro*, as a tool and characterized the LIM domain of hhLIM, which could activate hypertrophy marker gene expression and mediate cardiac hypertrophy.

Abbreviations: ANF) atrial natriuretic factor; hhLIM) human heart LIM protein; PCR) polymerase chain reaction; PKC) protein kinase C; TK) thymidine kinase.

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MATERIALS AND METHODS

Cell culture, transfection, and reporter gene assay. H9C2 cell lines were normally maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin under a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Transfection was performed using Lipofectamine (Gibco BRL, USA), according to the manufacturer's instructions. After 48 h, cells were harvested and luciferase activities as well as Renilla luciferase activities were measured with the Dual-Luciferase Reporter system (Promega, USA) according to the manufacturer's protocol.

Western blot analysis. The cells transfected with different plasmids were lysed, and lysates were electrophoretically separated and immunoblotted with antibodies specific for ANF (atrial natriuretic factor), skeletal α-actin, GATA-4, Nkx2.5, and GAPDH (glyceraldehyde phosphate dehydrogenase). GAPDH was used for normalization by protein [5, 6].

Subcellular localization of exogenous hhLIM-GFP. For analysis of subcellular localization and the effect of hhLIM-GFP protein in cells, exponentially growing H9C2 cells were transfected with pEGPN1-hhLIM or pEGPN1-mLIM1 plasmid (3 μg/well), respectively. After

30 h, cells were fixed with 3.7% paraformaldehyde for 15 min at room temperature and viewed using a normal fluorescence microscope (Olympus (Japan) fluorescence inverted microscope equipped with a digital camera) [7].

Site-directed mutagenesis of the LIM domain of hhLIM. Site-directed mutation of LIM domain 1 or the PKC (protein kinase C) phosphorylation site was carried by PCR (polymerase chain reaction) using oligonucleotide primers that contain the appropriate point substitutions at various amino acids. The reactions were carried out using a QuikChange site-directed mutagenesis kit (Stratagene, USA). Each mutation was verified by DNA sequence analysis. PCR primers used in site-directed mutagenesis of the LIM domain of hhLIM, which introduced two point mutations into each LIM domain, were: LIM1$^{(Cys10\rightarrow Ser,\ Cys13\rightarrow Ser)}$ (5′-GGAGGCGCAAAATCTGGAGCCTCTGAAAAGACCGTCTAC-3′), PKC$^{(Ser95\rightarrow Phe,\ Ser117\rightarrow Phe)}$ (5′-CGACCGACGCTACGTCTCAAGGCTCTTTCAAGACCCTTTGG-3′).

Statistical analysis. All data are expressed as mean ± S.E.M. Differences between two groups were assessed using analysis of variance followed by Student's *t*-test.

RESULTS

Human heart LIM (hhLIM) is a transcriptional activator. Our previous studies demonstrated that overexpression of hhLIM in skeletal muscle cells caused an increase in cell volume. To determine whether hhLIM activates the expression of the *ANF* gene, a cardiac hypertrophic marker gene, we examined the effects of hhLIM on *ANF* promoter activity. Cardiomyocytes and H9C2 cells were cotransfected with pcDNA-hhLIM plasmid with pANF-luc, a promoter construct containing a (–638/+15 bp) *ANF* promoter-enhancer. As shown in Fig. 1a, hhLIM significantly activated *ANF* promoter activity by 2.9- or 3.5-fold, respectively. Cotransfecting various amounts of the pcDNA$_3$-hhLIM plasmid expression with the reporter plasmids into H9C2 cells resulted in the activation of luciferase activity in a dose-dependent manner (Fig. 1b). These results indicate that hhLIM can activate the transcription of the hypertrophy marker gene *ANF*.

hhLIM activates transcription through its LIM domain 1. hhLIM has two LIM domains and four phosphorylation sites (Fig. 2a). To identify which domains or sites are important for the activation activity of hhLIM, a series of truncated mutants were constructed and their ability to activate *ANF* promoter was measured. As shown in Fig. 2b, F3 fragment (aa 44-120) alone could not activate *ANF* promoter. The F5 and F6 fragments (aa 1-154) showed stronger activation activity than F4 fragment (aa 1-44) and full length hhLIM, suggesting that the region between residues 44 and 120 but not the C terminus are more important for its activation activity. Moreover, comparing the activities of F2 (aa 10-44) and F4 (aa 1-44) fragments distinguished by 10 amino acids, there is an apparent increase in transcriptional activity in F4 fragment where the sequence aa 1-10 is present. In addition, the F2 fragment (aa 10-44) is more effective in activation of *ANF* promoter reporter gene compared with F3 fragment (aa 44-120). In order to find which site is essential for hhLIM to activate *ANF* promoter, the LIM domain 1 (aa 10-44) and PKC phosphorylation site of hhLIM were

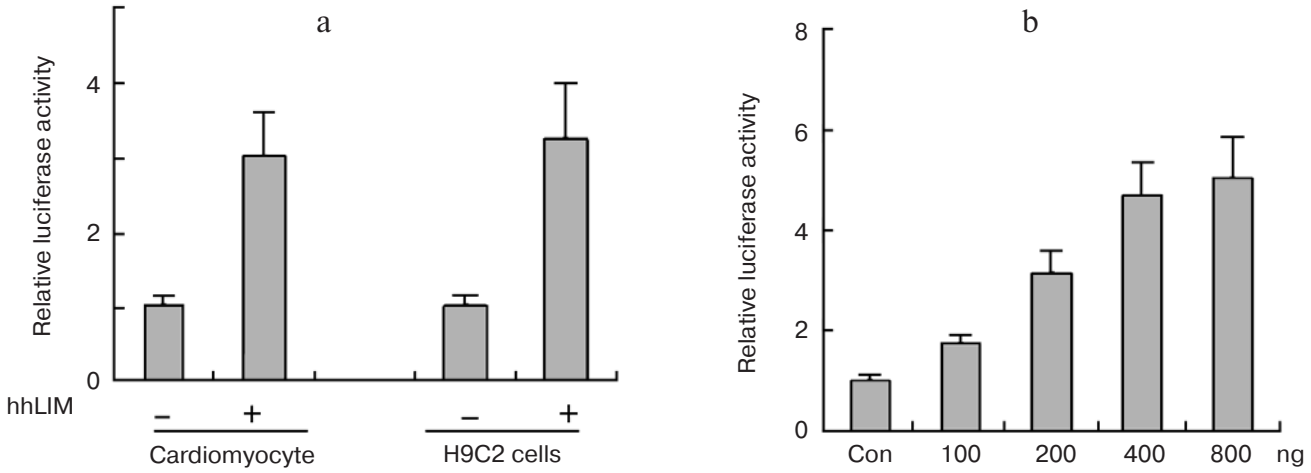

Fig. 1. a) *ANF* promoter activation by hhLIM. Cardiomyocytes and H9C2 cells were serially co-transfected with equal amounts of the pcDNA$_3$ (200 ng/well) or pcDNA$_3$-hhLIM (200 ng/well) along with the reporter plasmid pANF-luc (200 ng/well) and the internal control plasmid pRL-TK (100 ng/well) and assayed for luciferase activity. Results were obtained from three different transfection experiments after normalization for the internal control of thymidine kinase (TK) activity. b) Dose-dependent transcriptional activation of *ANF* promoter by hhLIM. H9C2 cells were co-transfected with increasing amounts of the pcDNA$_3$-hhLIM plasmids (100-800 ng/well) along with the reporter plasmids pANF-luc (200 ng/well) and pRL-TK (100 ng/well). The value represents the average of two independent experiments after normalization for the internal control of TK activity.

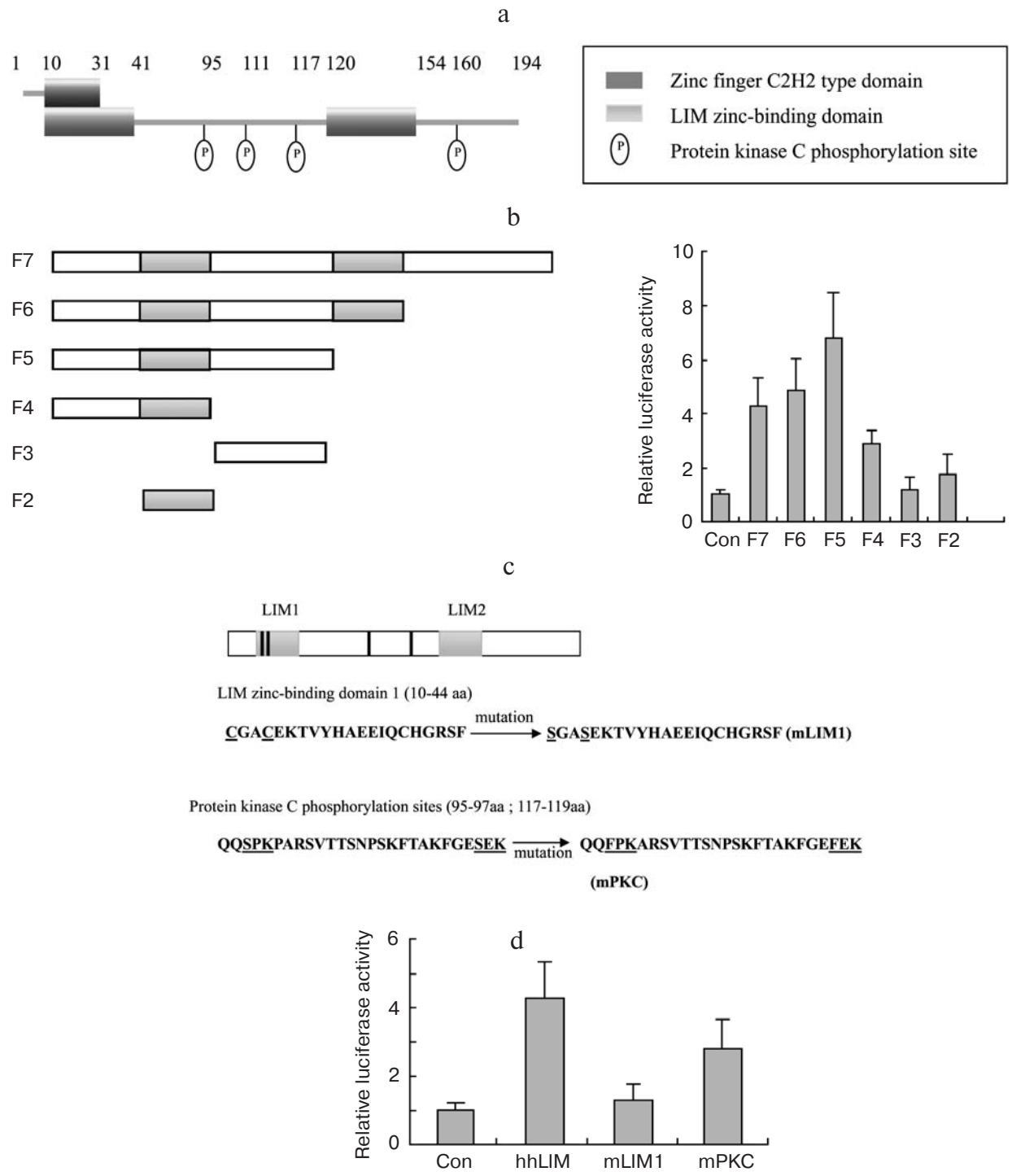

Fig. 2. Relationship between structure and activation activity of hhLIM. a) Structure and phosphorylation sites of hhLIM protein. The illustration mainly refers to the PROSITE motif search on website (http://cubic.bioc.columbia.edu/predictprotein). b) Transcriptional activation action of hhLIM and its various deletion mutants. hhLIM and its various deletion mutants were constructed and transiently expressed in H9C2 cells along with *ANF* reporter. hhLIM and its truncated proteins tested are schematically depicted on the left. Normalized relative luciferase activities are represented on the right. Results were obtained from five different transfection experiments after normalization for the internal control of TK activity. c) Scheme of mutagenesis. LIM domain 1 (Cys10→Ser, Cys13→Ser) and the PKC phosphorylation sites (aa 95-97 and 117-119) were mutated by PCR as described in materials and methods. d) LIM domain 1 of hhLIM is necessary for transcription activation of *ANF* promoter. H9C2 cells were transiently transfected with the wild or mutated hhLIM expression plasmid (200 ng/well), along with pANF-Luc plasmid (200 ng/well) and pRL-TK plasmid (100 ng/well). Results were obtained from three different transfection experiments after normalization for the internal control of TK activity.

mutated using a site-specific PCR mutation technique to create the mutations of Cys→Ser at positions 10 and 13 of hhLIM (named mLIM1) and Ser→Phe at positions 95 and 117 on PKC phosphorylation sites (named mPKC) (Fig. 2c). Transcription activation assay showed that the ability of mLIM1 to activate *ANF* promoter decreased by 30%, which reveals that the transcription activation ability of hhLIM is related to its LIM domain 1. At the same time, mPKC showed modest decrease in activation of *ANF* promoter, indicating a role of hhLIM in activating *ANF* expression, which revealed that hhLIM has a potential relationship with the PKC signaling pathway.

LIM domain 1 of hhLIM mediates cardiac hypertrophy. Since LIM domain 1 of hhLIM was important for activating *ANF* gene expression, the question arises whether it also mediates cardiac hypertrophy. H9C2 cells infected with mLIM1 expression plasmid showed a slight increase in cell size compared with the cells infected with hhLIM expression plasmid, indicating that mLIM1 did not provoke cardiomyocyte hypertrophy (Fig. 3a).

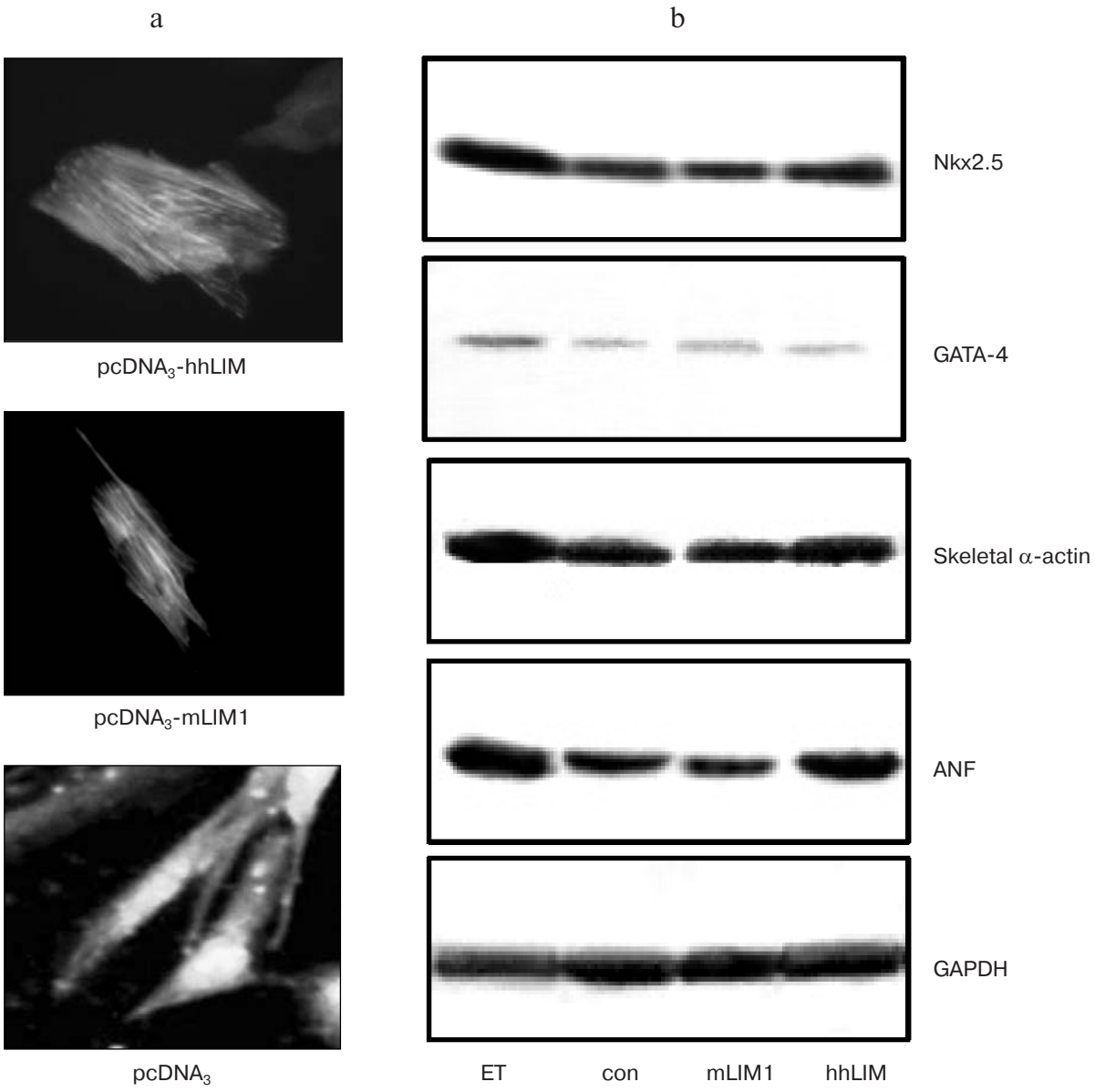

Fig. 3. Effect of hhLIM and mLIM1 on H9C2 cells. a) Subcellular localization of hhLIM and mLIM1 and the effect on cell hypertrophy. H9C2 cells were transiently transfected with hhLIM or mLIM1expression plasmids. Intracellular localization of hhLIM and the effect on hypertrophy was examined by fluorescence microscopy. b) Expression of cardiac hypertrophy marker genes in H9C2 cells infected with hhLIM or mLIM1 expression plasmids. H9C2 cells were transiently transfected with hhLIM or mLIM1 expression plasmids. Expression of cardiac hypertrophy marker genes was detected by Western blot. Positive control was H9C2 cells treated by endothelin-1 (ET). Each lane contains 40 μg of total protein. All samples were run in 8% SDS-PAGE and reacted with specific antibodies.

Moreover, the overexpression of hhLIM markedly induced the expression of cardiac hypertrophy marker genes Nkx2.5, skeletal α-actin, and *ANF* by approximately 2-fold, respectively, whereas infection with pcDNA-mLIM1 did not initiate the expression of hypertrophic marker genes (Fig. 3b). Together, these data indicate that the LIM domain 1 of hhLIM is required for cardiac hypertrophy.

DISCUSSION

ANF is a cardiac-restricted endocrine peptide that is a widely used marker for cardiac hypertrophy. The transcriptional regulation of *ANF* has been explored extensively, and it has been documented that multiple transcription factors can bind to the promoter of the *ANF* gene to control its expression [8, 9]. The *ANF* promoter contains multiple *cis*-regulatory elements that are important for the binding of some transcription factors. It has been shown previously that the zinc finger transcription factor GATA-4 and others can directly bind to the *ANF* promoter and regulate its expression in response to hypertrophic signals [10-12]. In this work, we showed that hhLIM appears to be a potent transactivator of the *ANF* promoter in H9C2 cells. Although LIM proteins can activate reporter gene expression, the site important for activation is not yet clear. We mapped the location of the activation domain of hhLIM. It was found that the region localized to the N-terminus of hhLIM can activate *ANF* promoter reporter gene. Deletion of the C-terminus of hhLIM had no effect on its transcriptional activity. The N-terminus of hhLIM (aa 44-120), which contains one LIM domain and PKC phosphorylation site, is the most important region for transcriptional activity. In order to find which site is essential for hhLIM to activate *ANF* promoter, the LIM domain 1 (aa 10-44) and PKC phosphorylation site of hhLIM were mutated. Mutagenesis of the conserved Cys in the LIM domain abolished its activation activity. At the same time, mutagenesis of Ser in the PKC phosphorylation site just decreased the activity. This result revealed that the transcriptional activation of hhLIM is dependent on LIM domain 1 between aa 10-44.

We consider that the entire hhLIM protein contains two main domains. One LIM domain, protein–protein interaction and transcriptional regulatory region, is at the N-terminus, and the other is at the C-terminus to interact with actin. Researchers have already found a relationship between LIM protein and other factors. For example, Rath et al. have reported that LMCD1/Dyxin restricts GATA-6 function by its inhibiting DNA binding [13]. Akazawa et al. have reported that Cal promotes cardiac differentiation by association with Nkx2.5 [14]. Lu et al. have reported that MLP promotes expression of the acetylcholine receptor γ-subunit gene cooperatively with the myogenin–E12 complex [15]. In conclusion, similarities in transcriptional activation activity to previously defined LIM proteins suggest that hhLIM is a member of the same subclass of the zinc-finger transcription factor family. Probably, the mutation of the LIM domain 1 at aa 11-13 disrupts the protein–protein interaction between hhLIM and other factors. This mutant suggests the existence of a common pathway for the progression of dilated cardiomyopathy from mutation to a diseased heart. Continual advances in the field of cardiac cell biology provide clues toward ascertaining the possible mechanisms involved in this progressive process.

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REFERENCES

1. Kadrmas, J. L., and Beckerle, M. C. (2004) *Nat. Rev. Mol. Cell Biol.*, **5**, 920-931.
2. Zheng, B., Wen, J. K., and Han, M. (2003) *Biochemistry (Moscow)*, **68**, 650-657.
3. Chen, H., Zhou, Z., Zhang, J. F., and Zhou, A. R. (2000) *Chin. J. Biochem. Mol. Biol.*, **16**, 295-300.
4. Zheng, B., Wen, J. K., Han, M., and Zhou, A. R. (2004) *Biochim. Biophys. Acta*, **1690**, 1-10.
5. Li, J. J., Han, M., Wen, J. K., and Li, A. Y. (2007) *Biochem. Biophys. Res. Commun.*, **356**, 13-19.
6. Han, M., Wen, J. K., Zheng, B., Cheng, Y., and Zhang, C. (2006) *Am. J. Physiol. Cell Physiol.*, **291**, C50-C58.
7. Jiang, G. J., Pan, L., Huang, X. Y., Han, M., Wen, J. K., and Sun, F. Z. (2005) *Biochem. Biophys. Res. Commun.*, **335**, 351-355.
8. Andreas, F., Jurgen, K., Burkhard, K., Holger, D., Manfred, M., Bettina, H., Christoph, E., and Manfred, G. (2005) *Mol. Cell. Biol.*, **25**, 8960-8970.
9. Rafael, T., Irfan, S., Adisa, K., Mona, N., and Andrew, F. R. (2004) *J. Biol. Chem.*, **279**, 52087-52094.
10. Liang, Q. G., Wind, L. J., Witt, S. A., Kimball, T. R., Markham, B. E., and Molkentin, J. D. (2001) *J. Biol. Chem.*, **32**, 30245-30253.
11. Maretoshi, H., Koh, O., Tatsuya, M., Teruhisa, K., Hiromichi, W., Toru, K., and Koji, H. (2004) *J. Biol. Chem.*, **279**, 37640-37650.
12. Brown, C. O., Chi, X., Garcia-Gras, E., Shirai, M., Feng, X., and Schwartz, R. J. (2004) *J. Biol. Chem.*, **279**, 10659-10669.
13. Rath, N., Wang, Z., Lu, M. M., and Morrisey, E. E. (2005) *Mol. Cell. Biol.*, **25**, 8864-8873.
14. Akazawa, H., Kudoh, S., Mochizuki, N., Takekoshi, N., Takano, H., Nagai, T., and Komuro, I. (2004) *J. Cell Biol.*, **164**, 395-405.
15. Lu, P. Y., Taylor, M., Jia, H. T., and Ni, J. H. (2004) *Cell Mol. Life Sci.*, **61**, 2386-2392.