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Interaction of the Heart-Specific LIM Domain Protein, FHL2, With DNA-Binding Nuclear Protein, hNP220

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Abstract Using a yeast two-hybrid library screen, we have identified that the heart specific FHL2 protein, four-and-a-half LIM protein 2, interacted with human DNA-binding nuclear protein, hNP220. Domain studies by the yeast two-hybrid interaction assay revealed that the second LIM domain together with the third and the fourth LIM domains of FHL2 were responsible to the binding with hNP220. Using green fluorescent protein (GFP)-FHL2 and blue fluorescent protein (BFP)-hNP220 fusion proteins co-expressed in the same cell, we demonstrated a direct interaction between FHL2 and hNP220 in individual nucleus by two-fusion Fluorescence Resonance Energy Transfer (FRET) assay. Besides, Western blot analysis using affinity-purified anti-FHL2 antipeptide antibodies confirmed a 32-kDa protein of FHL2 in heart only. Virtually no expression of FHL2 protein was detected in brain, liver, lung, kidney, testis, skeletal muscle, and spleen. Moreover, the expression of FHL2 protein was also detectable in the human diseased heart tissues. Our results imply that FHL2 protein can shuttle between cytoplasm and nucleus and may act as a molecular adapter to form a multicomplex with hNP220 in the nucleus, thus we speculate that FHL2 may be particularly important for heart muscle differentiation and the maintenance of the heart phenotype. J. Cell. Biochem. 84: 556–566, 2002.

Key words: human heart cDNA; zinc finger protein; yeast two-hybrid system; protein–protein interaction; molecular adapter

LIM domain proteins belong to a family of proteins that carry cysteine-rich zinc-binding motifs. LIM domain contains a 50–60 amino acid motif which possesses a specialized double-zinc finger domain (C_2HC and C_4) with a consensus cysteine-rich sequence [Liebhaber et al., 1990]. Proteins containing LIM domain(s) can be classified into four main groups according to the other additional domains they contain. They are: (1) LIM-only proteins; (2) LIM-homeodomain proteins; (3) Carboxyl terminal LIM domain proteins, e.g., paxillin and zyxin [Sadler et al., 1992; Turner and Miller, 1994; Macalma et al., 1996]; and (4) LIM-functional domain proteins, e.g., LIMK [Mizuno et al., 1994]. LIM domain proteins are found in a diverse range of species and were shown to have a broad spectrum of functions [Sanchez-Garcia and Rabbitts, 1994; Dawid et al., 1995, 1998; Taira et al., 1995]. Many muscle-specific LIM proteins have been identified [Arber et al., 1994; Morgan et al., 1995; Jain et al., 1996; Morgan and Madgwick, 1996], and one of them, muscle LIM protein (MLP), has been associated with a key role in muscle development [Arber et al., 1994, 1997]. LIM proteins are involved in cell identity, differentiation, and growth control [Dawid et al., 1995; Sanchez-Garcia and Rabbitts, 1994; Taira et al., 1995].

Besides, the LIM domain has been proposed to direct protein–protein interactions. LIM domains interact specifically with other LIM domains or with many other different proteins and protein domains. LIM domains are thought to function as protein interaction modules, mediating specific contacts between members

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of functional complexes and modulating the activity of some of the constituent proteins [Dawid et al., 1998]. This property has been demonstrated by the fact that LIM-only proteins can bind specifically to certain transcription factors [German et al., 1992; Valge-Archer et al., 1994; Wadman et al., 1994; Bach et al., 1995]. These proteins can interact with one another directly through their LIM domains. Such interactions have been demonstrated in vitro and in yeast two-hybrid systems, and appear to involve some specificitiy [Feuerstein et al., 1994; Schmeichel and Beckerle, 1994].

In order to study the relationship between the LIM domain proteins and the differentiation and growth regulation of the heart, we have cloned and characterized several novel human LIM domain proteins [Tsui et al., 1994, 1996; Chan et al., 1998; Lee et al., 1998, 1999; Kotaka et al., 1999]. One of them is a human heart cDNA that codes for a heart-specific four-and-a-half LIM-only protein 2 (FHL2) [Chan et al., 1998]. In order to characterize the FHL2 further, a yeast two-hybrid library screen was used to identify the interacting protein partners of the FHL2 protein in vivo. In this study, we identify a human DNA-binding nuclear protein, hNP220, as one of the interacting partners of FHL2. Using the two-fusion Fluorescence Resonance Energy Transfer (FRET) assay, we confirmed that FHL2 interacted with hNP220 in the nuclei of mammalian cells. Using affinity-purified anti-FHL2 antipeptide antibodies, we also report that the protein expression of FHL2 was found in heart only by Western blot analysis.

MATERIALS AND METHODS

Yeast Two-Hybrid Library Screening

Saccharomyces cerevisiae strains HF7c (*HIS3* and *lacZ* reporters) and SFY526 (*lacZ* reporter), two-hybrid GAL4 DNA-binding domain (DNA-BD) fusion vector, pGBT9 and GAL4 activation domain (AD) fusion vectors, pGAD10 and pGAD424 obtained from the MATCHMAKER Two-Hybrid System (Clontech) were used for the yeast two-hybrid screen. *FHL2* cDNA was subcloned into the DNA-BD vector, pGBT9 as described previously [Chan et al., 2000]. Nasopharyngeal cancer (NPC) cell cDNA library (a gift from Dr. Kwok-Wai Law) were constructed in the two-hybrid AD vector, pGAD10, and were used in the screening test with the "bait" protein FHL2. Before performing the yeast two-hybrid library screen, the DNA-BD/FHL2 hybrid construct, pGBT9-FHL2, alone was verified for transcriptional activation in yeast in vivo by the yeast TransAct assay (Clontech). The pGBT9-FHL2 was transformed into *S. cerevisiae* strain, Y187, using small-scale yeast transformation by the lithium acetate (LiAc) method. Trp^+ transformants were tested for expression of *lacZ* reporter gene by the blue/white screening of the colony lift β-galactosidase filter assay. Control transformations were also performed in parallel.

In the yeast two-hybrid library screening, pGBT9-FHL2 plasmid and the MATCHMAKER AD/cDNA library plasmid were cotransformed into yeast reporter strain, HF7c, using the LiAc method. All procedures were carried out according to the manufacturer's instructions (Clontech). Cotransformants were plated out on synthetic triple dropout (SD) agar plates lacking Trp, Leu, and His. The transformants were incubated at 30°C for 6–7 days. The transformants were restreaked and tested for the expression of the *lacZ* reporter gene by colony lift β-galactosidase filter assays. Putative positive library plasmids were rescued from the transformants. Interaction was further confirmed by cotransformation of library plasmid of interest and pGBT9-FHL2 into the yeast Y187. The inserts of putative positive clones were identified by PCR and automated DNA sequencing.

Yeast Two-Hybrid Interaction Assay of Deletion Mutants of FHL2

To identify the domain requirements for FHL2 interaction, a quantitative yeast two-hybrid assay to measure in vivo binding efficiency between library plasmid and several deletion mutants of FHL2 was performed. For the cloning of different deletion mutants of *FHL2* into pGBT9, half-LIM (residues 1–40), 1H-LIM (residues 1–101), 2H-LIM (residues 1–161), and 3H-LIM (residues 1–221) were amplified by PCR using the full-length *FHL2* cDNA clone as described previously [Chan et al., 2000]. Each of the FHL2 mutants (Half-LIM, 1H-LIM, 2H-LIM, 3H-LIM) and full length FHL2 were co-expressed with the library plasmid in both yeast reporter strains HF7c and SFY526. The interactions were quantitated by the liquid culture β-galactosidase assay with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate.

Quantitative liquid culture assay with ONPG as substrate of β-galactosidase activity was performed by assaying five separate transformants, with each in triplicate. Z buffer with 50 mM β-mercaptoethanol was added into the yeast culture, and immediately the timer was started after ONPG in Z buffer was added. The reaction mixture was stopped until a yellow colour was developed at 30°C. The elapsed time in minute(s) was recorded. The reaction mixture was centrifuged at 14,000*g* for 10 min to pellet cell debris. The absorbance of the supernatant at 420 nm was measured. Units of β-galactosidase activity were calculated as

$$\text{Units} = \frac{1,000(\text{OD}_{420})}{(\text{elapsed time in min})\,(0.1\text{ ml}\times\text{concentration factor})\,(\text{OD}_{600}\text{ of culture})}$$

Subcellular Localization Study and GFP-Two Fusion FRET Microscopy

The FHL2-GFP and hNP220-BFP hybrids were constructed by cloning into pEGFP-C1 and pEBFP-C1 vectors, respectively (Clontech). PCR primers used to amplify the constructs are shown as follows: (FHL2-GFP: forward 5′-TAGGGCGTCGACACTGAGCGCTTTGACTGCC-3′ and reverse 5′-TAGGGCGGATCCTGTGAGATCACAAGCAGCAAC-3′; hNP220-BFP: forward 5′-TAGGGCCCCGGGCACTCCTCGTGGTGTTGAGGGAGAA-3′ and reverse 5′-TAGGGCCCCGGGTCACCTAGAGCTTCTTTCTTCAGCC-3′). Primers for FHL2 amplification contained an *Eco*RI site and a *Sal*I site while primers for hNP220 amplification contained two *Sma*I sites. These GFP and BFP hybrid constructs were transiently transfected into HepG2 cells with lipofectamine Plus Reagent (Life Technologies, Inc.). After 24 h transfection, transfected cells were rinsed in PBS twice and fixed in 3.7% paraformaldehyde in PBS for 10 min at room temperature followed by washing in PBS. Epi-fluorescence microscopy and laser confocal microscopy were used in the FRET study. The cells expressing GFP fusion proteins were imaged using a 488 nm laser line excitation and a 530DF30 filter for emission (Molecular Dynamics Confocal Microscopy). Images were analyzed using Silicon Graphics IRIS INDIGO XS24. For epi-fluorescence microscopy (Zess Axioskop fluorescence microscopy), cells expressing GFP fusion protein was viewed with either a GFP filter set or an FITC filter set (Omega Optical, Inc.). Images were captured by cooled CCD camera (Photometeric, Inc.) and were coloured using IpLab spectrum software. For FRET microscopy, epi-fluorescence microscopy was equipped with an XF89-BFP/GFP FRET filter set (365HT25 for excitation, dichronic mirror 400DRLP, 450DF65 for BFP emission, 535RDF45 for GFP and FRET emission) for the detection of FRET in the cotransfected cells.

Antipeptide Antibody Production

For the production of anti-FHL2 antibodies, specific antisera were raised by immunizing a rabbit with an acetylated FHL2 synthetic peptide, residues 232–247 (Ac-GGTKYISFEERQWHND) which was purchased from Research Genetics. The synthetic peptide was coupled to a carrier protein keyhole limpet hemocyanin (KLH). FHL2 (0.1 mg) synthetic peptides were mixed with KLH solution (5 mg/ml) for 30 min. EDAC (10 mg/ml) was added to the mixture and stirred for 1–2 h at room temperature. The peptide conjugate was purified with a G-50 Sephadex desalting column (Amersham Pharmacia Biotech).

For immunization, the peptide conjugate was mixed with an equal volume of Freund's complete adjuvent (Sigma) for the first injection, and Freund's incomplete adjuvent (Sigma) for the subsequent booster injections. The emulsified peptide conjugate was injected at multiple intradermal sites distributed over the back of a shaved rabbit. An injection was made every month for a total of five times and blood was drawn after each injection except for the first. The blood was allowed to clot and was centrifuged. The clot was discarded and the FHL2 antiserum was applied to a HiTrap Protein G affinity column (Amersham Pharmacia Biotech) for purification.

Western Blot Analysis

Different rat tissues were isolated from Spraque–Dawley rats, and the human diseased heart tissue was obtained from a patient with dilated cardiomyopathy. After the tissues were isolated, they were rinsed with ice-cold PBS. The tissues were immediately homogenized with ice-cold PBS and the pellet was washed three times with pre-chilled ether. The washed samples were freeze-dried overnight. The dried homogenized tissues were lysed in a

minimal volume of buffer, consisting of 8 M urea, 0.1 M sodium phosphate, 0.01 M Tris-HCl, 10 mM DTT at pH 8.0. Supernatant was collected and the total protein concentration (100 μg/lane) was normalized using the Folin–Lowry method [Dunn and Harris, 1989] before loading onto a 12% SDS–polyacrylamide gel.

The total proteins were separated on a 12% SDS–polyacrylamide gel and were visualized with Coomassie Blue staining. Proteins on SDS–polyacrylamide gel were transferred onto a PVDF membrane (Millipore) at 100 V for 1 h. Non-specific binding sites of the PVDF membrane with bound protein was blocked with 5% non-fat milk powder in TBST (20 mM Tris-HCl pH 7.6, 137 mM sodium chloride, 0.05% Tween-20) for 2 h at room temperature. The membrane was probed with anti-FHL2 specific antibodies (1:300) in TBST and incubated at 4°C overnight. Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG antibodies (Promega) were used as the secondary antibodies (1:3,000) and the membrane was incubated at room temperature for 1 h with secondary antibodies. After washing with TBST, the signal was detected by enhanced chemiluminescence (ECL) detection reagents (Amersham, Life Science), and exposed onto BioMax MR films (Kodak).

RESULTS

Identification of hNP220 as an Interacting Partner of FHL2

In the yeast two-hybrid library screening, approximately four million cDNA library clones were screened. After a series of screening to eliminate false positives, the putative positive clones were isolated from yeast. Two putative positive clones were further investigated and confirmed to be true positives. The inserts of the two putative positive clones were amplified by PCR and sequenced by automated DNA sequencing. The two clones were identified as *hNP220* with a correct reading frame (GenBank/EMBL accession number D83032) [Inagaki et al., 1996]. Sequence analysis of the two true positive clones indicated that they both encoded 391 amino acid residues corresponding to the C-terminal of hNP220. Thus, FHL2 can interact with the C-terminal of hNP220 and the C-terminal (residues 1588–1978) of hNP220 is sufficient to mediate interaction with FHL2 in yeast cells. Specificity of the interaction between FHL2 and hNP220 was further tested by retransforming the pGAD10-hNP220 plasmid with the pGBT9-FHL2, or with the control plasmids provided by MATCHMAKER two-hybrid system (Clontech) into the yeast reporter strains, HF7c and SFY526 (Fig. 1). Only transformation #3, pGAD10-hNP220 plasmid and pGBT9-FHL2, gives the positive result in which transformants grew on the SD agar plates lacking Leu, Trp, and His (Fig. 1) and blue colonies were obtained from the colony lift β-galactosidase filter assay (data not shown).

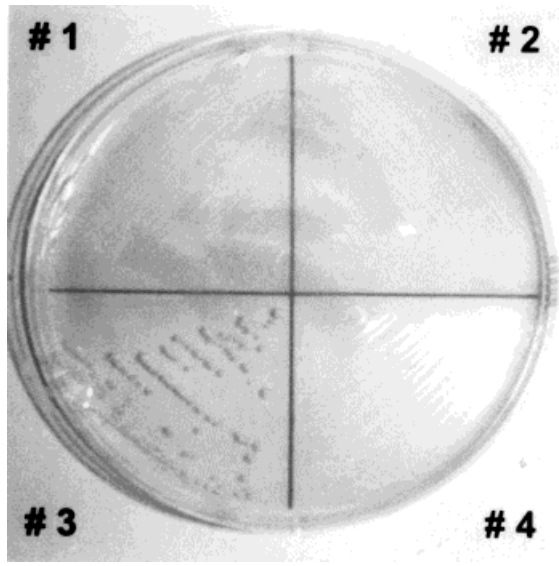

Fig. 1. Results of the *HIS* reporter gene expression by selection of yeast cotransformants on the SD agar plates lacking Leu, Trp, and His. Transformation #3 showed the positive result while the rest are negative controls. **#1**: AD/library plasmid (AD-NP220) only; **#2**: pGBT9 plasmid (DNA-BD only without insert) and AD/library plasmid, **#3**: AD/library plasmid and pGBT9-FHL2 (DNA-BD-FHL2); and **#4**, the AD/library plasmid and pGBT9 plasmid (with insert encoding an unrelated human lamin C protein)(DNA-BD-LAM).

Studies on the Interaction of FHL2 Deletion Mutants With hNP220

To identify the domain requirements for the interaction of FHL2 with NP220, we used a quantitative yeast two-hybrid assay to measure the in vivo binding efficiencies between hNP220 and several deletion mutant constructs of FHL2 (Half-LIM, 1H-LIM, 2H-LIM, 3H-LIM). The interactions between different FHL2 mutants and hNP220 were tested by the ability of cotransformants to grow on SD agar plates without Leu, Trp, and His, and to generate blue colour (β-galactosidase activity) in colony lift β-galactosidase filter assays. It

showed that the fusion of DNA-BD with full length FHL2 bound strongly to hNP220. The binding was reduced substantially by the truncation of LIM domains from C-terminal (Fig. 2A). Besides, the interactions were further quantitated by liquid culture β-galactosidase assay with ONPG as the substrate, and the results were shown on Figure 2B. The β-galactosidase activity is diminished to approximately 50% of that of the full-length FHL2 when the truncated FHL2 contains three-and-a-half LIM domains (3H-LIM) only. The β-galactosidase activity is also diminished to approximately 20% of that of full length FHL2 when the truncated FHL2 contains only two-and-a-half LIM domains without the third and fourth LIM domains (2H-LIM) (Fig. 2B). Binding to hNP220 was eliminated by the deletion of the second LIM domain. Therefore, the second LIM domain in conjunction with the third and the fourth LIM domains were identified to bind with hNP220, whereas the first half LIM (Half-LIM) domain or the first one and a half LIM domains (1H-LIM) is insufficient for binding with hNP220.

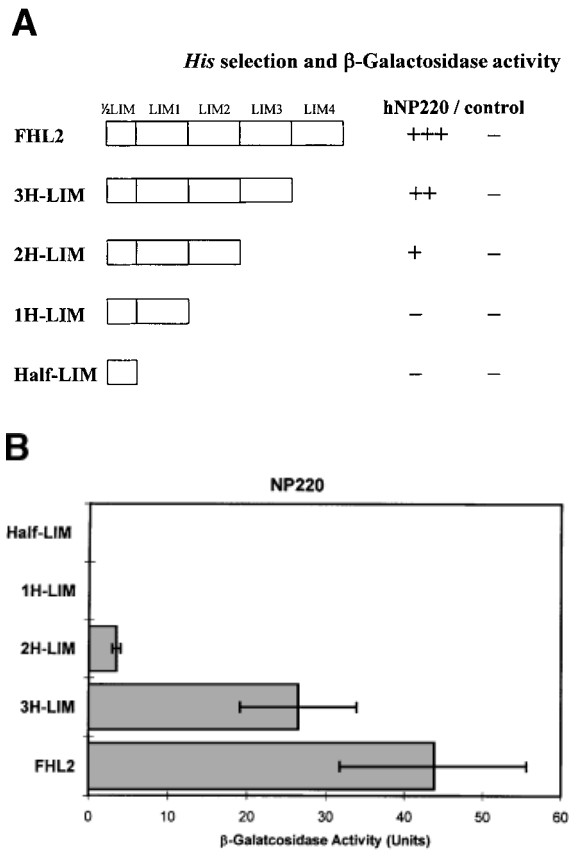

Fig. 2. **Panel A**, Domain studies of FHL2 by the yeast two-hybrid assay. The interactions were tested by the ability of cotransformants to grow on SD agar plates lacking Leu, Trp, and His, and to generate blue colour (β-Galactosidase activity) in filter lift assays. After developing for 10 h, the cotransformants were classified into positives colonies showing either dark blue(+++), blue(++), or pale blue(+) colours or negative colonies showing a white (–) colour. **Panel B**, β-Galactosidase activity of the transformants were measured by liquid culture assay with ONPG as the substrate. The results of different LIM domains of FHL2 and hNP220 coexpressed in yeast were shown. The mean ± S.D. (n = 6) for each interacting pair is shown.

Visualization of FHL2 and hNP220 Interaction by Green Fluorescent Protein (GFP)-Two Fusion FRET Assay

To verify whether FHL2 interacts with hNP220 in living mammalian cell culture, GFP-two fusion FRET study was conducted. Our results showed that FHL2-GFP fusion protein was localized in both the nuclei and the cytoplasms of HepG2 cells (Fig. 3A), while hNP220-BFP fusion protein was predominantly localized in the nuclei of HepG2 cells (Fig. 3B). When FHL2-GFP was cotransfected with hNP220-BFP into the same cell, FHL2-GFP fusion protein was mainly localized in the nucleus (compare Figs. 3A and 4A). The nuclear localization of hNP220-BFP was not affected in the absence (Fig. 3B) or in the presence of FHL2-GFP (Fig. 4B). Co-localization of FHL2 and hNP220 was found in the nuclei of cells where FRET was observed under a fluorescence microscope that was equipped with a BFP/GFP FRET filter set (Fig. 4C). A control vector, GFP-human papillomavirus (HPV)-16-E6, for the FRET study were kindly provided by Prof. Mahajan, N.P. Cotransfection of the control vector, (HPV)-16-E6, with FHL2-BFP or hNP220-BFP showed that neither combination gives FRET under the FRET filter set (data not shown).

Expression Pattern of FHL2 Protein

From the result of Western blot analysis, our affinity-purified anti-FHL2 antipeptide antibody is shown to be able to detect a single 32-kDa band, which corresponds to the FHL2 intact protein without any obvious degradation product (Fig. 5A). From the tissue distribution of the FHL2 protein amongst different rat tissues, the expression of the FHL2 protein was detectable in the heart only. Virtually no expression of FHL2 protein was detected in brain, liver, lung, kidney, testis, skeletal muscle, and spleen (Fig. 5A). This result agrees with

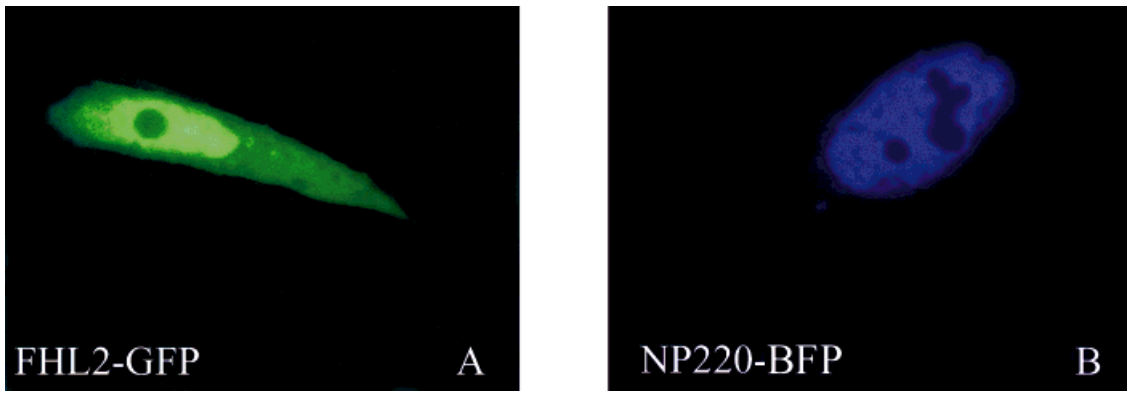

Fig. 3. Subcellular localization of FHL2-GFP and hNP220 fusion proteins was studied in HepG2 cells. **A**: FHL2-GFP was localized in both the nucleus and the cytoplasm; **B**: hNP220-BFP was predominantly localized in the nuclei of cells.

the Northern blot analysis results and both of them indicated that the FHL2 is preferentially expressed in the heart [Chan et al., 1998]. Besides, the expression of FHL2 protein was also detected in the human diseased heart tissues (dilated cardiomyopathy) by the Western blot analysis using our anti-FHL2 antibody (Fig. 5A). This difference in the FHL2 protein expression level is not due to an increase in protein degradation of the total cellular proteins, since Coomassie Blue staining of the total protein pattern remains unchanged (Fig. 5B).

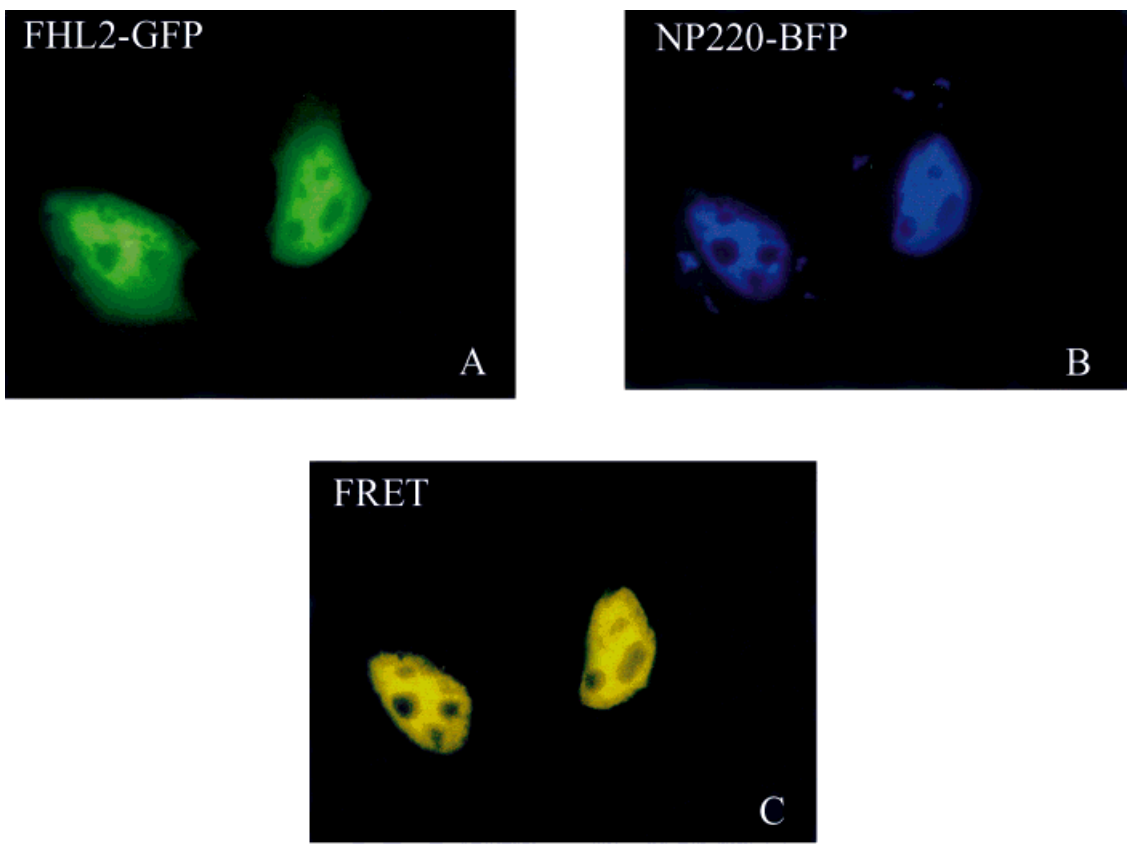

Fig. 4. Visualization of FHL2 and hNP220 interaction by GFP two-fusion FRET. FHL2-GFP and hNP220-BFP were cotransfected into HepG2 cells. **A**: A GFP filter set was used to visualize the presence of FHL2-GFP. **B**: A BFP filter set was used to visualize the presence of hNP220-BFP. **C**: FHL2-GFP and hNP220-BFP were co-localized in the same nuclei where FRET was observed under a fluorescent microscope with a BFP/GFP FRET filter set.

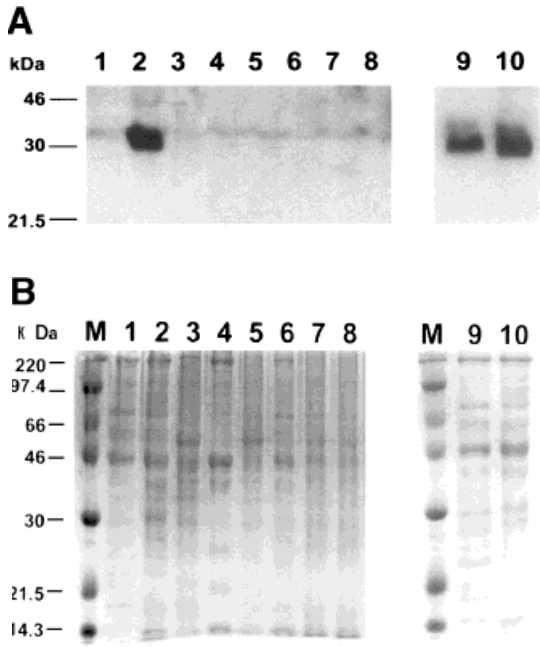

Fig. 5. **Panel A**, detection of the FHL2 protein at various rat tissues and human diseased heart tissue by Western blot analysis. **Lane 1**: Brain, **Lane 2**: Heart, **Lane 3**: Kidney, **Lane 4**: Liver, **Lane 5**: Lung, **Lane 6**: Testis, **Lane 7**: Skeletal muscle, **Lane 8**: Spleen, **Lane 9**: Human diseased heart, and **Lane 10**: Human heart. The FHL2 protein was detected using an anti-FHL2 antibody. **Panel B**, Coomassie Blue staining of the normalization of the rat tissue total protein and human diseased heart protein. **Lane M**: Rainbow^{TM} coloured protein molecular weight markers (Amersham, Life Science), Lane 1: Brain, Lane 2: Heart, Lane 3: Kidney, Lane 4: Liver, Lane 5: Lung, Lane 6: Testis, Lane 7: Skeletal muscle, Lane 8: Spleen, Lane 9: Human diseased heart, and Lane 10: Human heart.

DISCUSSION

This study demonstrated the interaction between a four-and-a-half LIM protein 2 (FHL2) and a human DNA-binding nuclear protein (hNP220). FHL2 is a LIM only protein which has been previously identified as the interacting partner of integrin alpha subunits [Wixler et al., 2000], a minichromosome maintenance protein hCDC47 [Chan et al., 2000] and a four-and-a-half LIM protein FHL3 [Li et al., 2001]. Recently, FHL2 was also shown to function as a co-activator in association with androgen receptors [Muller et al., 2000]. It was proposed that LIM domain proteins are multiple binding adapters and functional modifiers in protein–protein interactions. Studies revealed that some of the LIM domain proteins function solely as adapters to bring other components together in a complex [Dawid et al., 1998]. The FHL2 interacting partners previously identified further support the hypothesis that FHL2 serves as an adapter module to form a functional multiple complexes.

Human NP220 has an ORF encoding 1978 amino acids with a calculated molecular mass of 220 kDa. hNP220 protein is a large DNA-binding protein, which binds to double-stranded DNA fragments by recognizing clusters of cytidines [Inagaki et al., 1996]. The domain essential for DNA binding is localized in the C-terminal half of hNP220, and close to it are nine repeats of the consensus acidic sequences. hNP220 is a hydrophilic protein without any large hydrophobic domain, and shares three types of domains (MH1, MH2, and MH3) with the acidic nuclear protein, matrin 3, which was found as a major protein of the nuclear matrix. hNP220 also has a zinc finger motif and an arginine/serine-rich domain commonly found in pre-mRNA splicing factors [Matsushima et al., 1996, 1997]. Thus, hNP220 is a novel type of nucleoplasmic protein with multiple domains [Inagaki et al., 1996; Okumara et al., 1998].

The possibility of two interacting interfaces on a single LIM domain has also been put forward by Arber and Caroni [1996]. From structural data it can be deduced that distinct binding interfaces may reside in the two independent zinc-finger modules, each with a different amino acid composition in their loop [Hammarstrom et al., 1996; Perez-Alvarado et al., 1996; Cuppen et al., 1998]. Interestingly, MLP, which is a LIM-only protein with two LIM domains and a novel essential regulator of myogenesis, is highly expressed in the human heart. Its expression is enriched in striated muscle and occurs concomitantly with terminal muscle differentiation [Arber et al., 1994]. One of the LIM domains, LIM1, of MLP appears to play a role in nuclear localization, interacting with the muscle regulatory factors (MRFs) and enhancing the formation of MRF–DNA complexes, whereas another LIM domain, LIM2, primarily interacts with cytoplasmic proteins involved in maintaining the cellular architecture [Arber and Caroni, 1996; Stronach et al., 1996; Kong et al., 1997]. In our results of the identification of the interacting domains of FHL2, we have shown that the second LIM domain together with the third and the fourth LIM domains were identified to bind with hNP220. Thus, from the heart specific expression of FHL2, which is analogous to the muscle-specific LIM protein (MLP), we believe that FHL2 forms a

multicomplex with hNP220 or other proteins through the LIM domains and thus become involved in heart muscle differentiation and many cellular processes. Such speculation awaits further investigation in finding more protein partners of FHL2 and their biological functions.

Previous studies indicated that a multicomplex involving LMO2 (previously known as RBTN2 or TTG2) which is a LIM-only protein with two LIM domains, basic helix-loop-helix (bHLH) protein TAL1, E47, and GATA-1 could form a complex in haematopoietic cells [Osada et al., 1995]. Such a complex exists in erythroid cells and binds to DNA. Recently, this oligomeric DNA-binding complex, which also involves LIM-binding protein (Ldb1), specifically recognizes a unique bipartite E-box-GATA motif, consisting of an E-box, followed 9 bp downstream by a GATA site. Thus, the oligomeric complex can function in transcriptional activation [Wadman et al., 1997]. Moreover, other studies have shown that full-length and the C-terminal half of hic-5 (hydrogen peroxide-inducible clone-5) protein, including four LIM domains, binds to DNA in a zinc-dependent manner in vitro [Nishiya et al., 1998]. hNP220 protein is a large DNA-binding protein that binds to double-stranded DNA fragments by recognizing clusters of cytidines [Inagaki et al., 1996]. Therefore, it is possible that FHL2 might bind to DNA through the formation of multiprotein complex. We have shown that FHL2 can bind with DNA-binding nuclear protein, hNP220. Therefore, pending that the localization of hNP220 are in the nucleus, we further speculate that oligomeric DNA-binding complex involving heart-specific FHL2 as a bridging molecule or a multifunctional adapter in conjunction with hNP220 or other transcriptional activators that may be formed as a consequence. This complex may bind DNA and promote transcriptional activation of muscle-specific genes during different developmental stages of the heart, thus promoting myogenesis. Interestingly, MLP-deficient mice developed dilated cardiomyopathy with hypertrophy, heart failure, and disruption of cardiomyocyte cytoarchitecture after birth. The data suggest that MLP play a crucial and specific role in the organization of cytosolic structure in cardiomyocytes [Arber et al., 1997; Towbin, 1998]. Similarly, human FHL2 is preferentially expressed in the adult heart [Chan et al., 1998] as MLP, and thus the formation of multicomplex may play an important role in the specification or maintenance of the terminal differentiated phenotype of heart muscle cells.

Previous studies showed that hNP220 localized in the interchromatin space of various human cell lines [Inagaki et al., 1996]. The function of hNP220 might be related to both binding of RNA and DNA, and might be one of the mammalian factors regulating the gene-specific alteration in RNA splicing in response to tissue-specific or developmentally controlled signals [Inagaki et al., 1996]. Moreover, the structural aspects of mouse NP220 resemble splicing factors and splicing regulators transformer that activate a specific 3′-splicing site of specific genes in response to differentiation-dependent signals. Our subcellular localization and GFP-two fusion FRET studies demonstrated that in the absence of hNP220, FHL2 localized in both the cytoplasm and the nucleus of a cell. In the presence of hNP220, FHL2 interacted with hNP220 and their interaction mainly took place in the nucleus of a cell. These results indicated that FHL2 can be relocalized into the nuclei and interact with nuclear hNP220. Therefore, FHL2 might be able to shuttle between cytoplasm and nucleus and act as a molecular adapter, which interacts with hNP220 in the nucleus.

The coding region between the FHL2 and the DRAL, mouse homolog of FHL2, is highly similar, and previous studies have shown that DRAL is expressed in human primary myoblasts but down-regulated in the embryonal-rhabdomyosarcoma (RMS) cell line RD [Genini et al., 1996, 1997]. In recent study, Northern blot analysis revealed a differential regulation for FHL2 with a significant decrease in explanted hearts from patients with dilated cardiomyopathy ($n = 9$) in comparison to normal myocardium ($n = 3$) [Zimmermann et al., 1999], while FHL2 is not highly expressed in normal tissues other than heart, numerous cancer tissues or tumor cell lines have increased FHL2 levels [Chan et al., 2000]. We are interested to study whether our antibody could be used to detect FHL2 in human heart or human tumor tissues. Recently RT-PCR result showed that hNP220 is expressed in human fetal heart [Eichmuller et al., 2001]. Our Western blot results showed that the expression of FHL2 protein could be detected in the human diseased heart (dilated cardiomyopathy) using our antibody. Therefore, the antibody we prepared will

be useful to study whether FHL2 may play a crucial role in the development and differentiation of human muscles and to analyze FHL2 in heart diseases.

The expression patterns of several LIM domain proteins have been studied, given an indication of their potential functions. Many of them are tissues- or muscle-specific. Vertebrate LIM-Homeodomain genes are widely expressed, especially in the nervous system [Dawid et al., 1995]. LIMK, which belongs to LIM-functional domain protein, is predominantly expressed in neural tissues such as the spinal cord and the brain [Proschel et al., 1995]. For the LIM-only proteins, cysteine-rich protein (CRP) expression is prominent in smooth muscle derivatives and is correlated with muscle development in avian embryos [Crawford et al., 1994]. Besides, smooth muscle LIM protein (SmLIM) is expressed preferentially in arterial smooth muscle cells, and in response to external cues such as vascular injury that promote smooth muscle cell proliferation and dedifferentiation, SmLIM mRNA is down-regulated [Jain et al., 1996]. Similarly, MLP is also highly expressed in the human heart. Its expression is enriched in striated muscle and occurs concomitantly with terminal muscle differentiation [Arber et al., 1994].

Another protein, hic-5, has a similar primary structure as FHL2, and hic-5 shows some cytostatic effect on cellular senescence and terminal differentiation [Shibanuma et al., 1994, 1997]. Conclusively, it suggests that LIM domain proteins in general might be particularly important factors for the specification or maintenance of the muscle or cell phenotype. From the heart specific expression of FHL2, which is analogous to muscle-specific LIM protein (MLP) and has similar primary structure as hic-5, we speculate that FHL2 may be particularly important for heart muscle differentiation and the maintenance of the heart phenotype.

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