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Protein-Protein Interaction of FHL2, A LIM Domain Protein Preferentially Expressed in Human Heart, With hCDC47

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Abstract In the yeast two-hybrid library screening, the heart-specific FHL2 protein was found to interact with hCDC47. In vitro interaction study between FHL2 protein and hCDC47 was demonstrated. From the results of domain studies by the yeast two-hybrid assay, the second and third LIM domains in conjunction with the first half LIM domain of FHL2 were identified to be important in binding with hCDC47. Besides, in Northern blot hybridization of human cancer cell lines, the highest FHL2 mRNA expression was detected in colorectal adenocarcinoma SW480 and HeLa cell S3. Our results imply that FHL2 protein may associate with cancer development and may act as a molecular adapter to form a multicomplex with hCDC47 in the nucleus, thus it plays an important role in the specification or maintenance of the terminal differentiated phenotype of heart muscle cells. J. Cell. Biochem. 76:499–508, 2000.

Key words: heart cDNA; zinc finger protein; yeast two-hybrid screen; molecular adapter

The LIM domain protein is an important new family of protein that carries a novel cysteine-rich zinc-binding domain. Since zinc finger proteins can be classified based on different classes of consensus sequences, LIM domain protein family is a new class of zinc finger proteins [Sanchez-Garcia and Rabbitts, 1994; Dawid et al., 1998]. The LIM domain is a specialized double-zinc finger domain (C_2HC and C_4) with consensus cysteine-rich sequence [Liebhaber et al., 1990]. LIM domain proteins are found in a diverse range of species and were shown to have a broad spectrum of functions [Dawid et al., 1995, 1998; Sanchez-Garcia and Rabbitts, 1994; Taira et al., 1995]. Nowadays, 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 (LIM-HD) proteins; 3) Carboxyl terminal LIM domain (C-LIM) proteins, e.g., paxillin and zyxin [Macalma et al., 1996; Sadler et al., 1992; Turner and Miller, 1994]; and 4) LIM-functional domain (LIM-FD) proteins, e.g., LIMK [Mizuno et al., 1994]. Many muscle-specific LIM proteins have been identified [Arber et al., 1994; Jain et al., 1996; Morgan et al., 1995; 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., 1994; 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 and with many different proteins and protein domains. LIM domains are thought to function as protein interaction modules, mediating specific contacts between members of functional complexes and modulating the activity of some of the constituent proteins [Dawid et al., 1998]. This property has been demonstrated by

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the fact that LIM-only proteins can bind specifically to certain transcription factors [Bach et al., 1995; German et al., 1992; Valge-Archer et al., 1994; Wadman et al., 1994] These proteins can bind 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 specificity [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 [Chan et al., 1998; Lee et al., 1998; 1999; Kotaka et al., 1999; Tsui et al., 1994, 1996]. One of them is a human heart cDNA which codes for a heart-specific four-and-a-half LIM-only protein 2 (FHL2) [Chan et al., 1998]. In order to further characterize the FHL2, the yeast two-hybrid library screening was used to identify the interacting protein partners of the FHL2 protein in vivo. In this study, we report the identification of hCDC47 as one of the interacting partners of FHL2. hCDC47 cDNA has been isolated from a HeLa cell cDNA library and hCDC47 protein has 49% amino acid identity with the budding yeast CDC47 protein, indicating its conserved function from yeast to mammals [Dalton and Whitbread, 1995; Fujita et al., 1996; Kiyono et al., 1996]. hCDC47 is a human homologue of yeast CDC47 and a member of the minichromosome maintenance (MCM) protein family which has been implicated in the regulatory machinery causing DNA to replicate only once in the S phase [Fujita et al., 1996; Takizawa et al., 1995]. The interaction between FHL2 and hCDC47 are described in this paper. We also report the human cancer cell line expression of FHL2 as revealed by Northern hybridization.

MATERIALS AND METHODS

The Yeast Two-Hybrid System

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 assay. FHL2 cDNA was subcloned into the DNA-BD vector, pGBT9 as described previously [Chan et al., 1998]. For the cloning of different domains of FHL2 gene into pGBT9, the same cloning procedures were used. Different domains, 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 the template. PCR were performed by using four pairs of primers flanking different domains of FHL2 cDNA. First pair of primers flanking the cDNA encoding the first half LIM domain (i.e., Half-LIM or an extra zinc finger; Forward:5′-TAGGGCGAATTCATGACTGAGCGCTTTGACTGCCA-3′; Reverse: 5′-TAGGGCGTCGACTCAGCAGGTGTTGGCGAACAGGGT-3′) was used. Second pair of primers flanking the cDNA encoding the one and a half LIM domain (1H-LIM; Forward: 5′-TAGGGCGAATTCATGACTGAGCGCTTTGACTGCCA-3′; Reverse: 5′-TAGGGCGTCGACTCAGCACTTGGATGAGTACTCGTT-3′) was used. Third pair of primers flanking the cDNA encoding the two and a half LIM domains (2H-LIM; Forward: 5′-TAGGGCGAATTCATGACTGAGCGCTTTGACTGCCA-3′; Reverse: 5′-TAGGGCGTCGACTCAGCACTGCATGGCATGTTGTTT-3′) was used. Fourth pair of primers flanking the cDNA encoding the three and a half LIM domains (3H-LIM; Forward: 5′-TAGGGCGAATTCATGACTGAGCGCTTTGACTGCCA-3′; Reverse: 5′-TAGGGCGTCGACTCAACACTTCTTGGCATACAAGTC-3′) was used. All primers have an end clamp (TAGGGC) which facilitated cleavage by restriction enzymes. An *Eco*RI site and a *Sal*I site, shown as the underlined bases, are present in the forward and reverse primers, respectively. After digestion with *Eco*RI and *Sal*I restriction enzymes, the PCR products with different domains of FHL2 (Half-LIM, 1H-LIM, 2H-LIM, and 3H-LIM) were cloned into pGBT9. All constructs were verified by automated DNA sequencing.

HeLa cell cDNA library (Clontech) and 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 assay with the "bait" protein FHL2.

Before performing the yeast two-hybrid library screening, the DNA-BD/FHL2 hybrid construct, pGBT9-FHL2 alone was verified for transcriptional activation in yeast in vivo by yeast

TransAct assay (Clontech). The pGBT9-FHL2 was transformed into *Saccharomyces cerevisiae* strain Y187 using small-scale yeast transformation by the lithium acetate (LiAc) method. The yeast reporter strain Y187 contains an integrated *lacZ* construct that is regulated by the Wild-type GAL1 promoter. The transformants were selected in SD agar plates lacking Trp. Then, 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 strains, HF7c and SFY526 using the lithium acetate (LiAc) method. All procedures were carried out according to the instructions of the manufacturer (Clontech). Cotransformants were plated out on synthetic triple dropout (SD) agar plates lacking Trp, Leu, and His for yeast strain HF7c and double dropout SD agar plates lacking Trp and Leu for yeast strain SFY526. The transformants were incubated at 30°C for 4–5 days. The His^+ transformants of HF7c and Trp^+Leu^+ transformants of SFY526 were restreaked and tested for the expression of the *lacZ* reporter gene by colony lift β-galactosidase filter assay using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as a chromogenic substrate.

To characterize or identify the domain requirements for this interaction, we used a quantitative yeast two-hybrid assay to measure in vivo binding efficiency between AD/cDNA library plasmid and several different LIM domain constructs of FHL2. Each of the different LIM domains constructs of FHL2 (Half-LIM, 1H-LIM, 2H-LIM, 3H-LIM) and full length FHL2, that were prepared to fuse with DNA-BD, were coexpressed with AD/cDNA library fused with AD in both yeast reporter strains HF7c and SFY526. The interactions of these recombinant proteins were tested by the ability of cotransformants to grow on SD agar plates without Leu, Trp, and His, and to generate blue color (β-galactosidase activity) in colony lift β-galactosidase filter assays. Besides, the interactions were further quantitated by liquid culture β-galactosidase assay with o-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate.

Colony Lift β-Galactosidase Filter Assay

In situ colony lift filter assay for blue/white screening of β-galactosidase activity was performed by lifting out the colonies from the agar plates onto a filter paper. Then the filter paper with colonies was placed onto another filter paper presoaked with Z buffer/X-gal solution (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 0.1 mM $MgSO_4$, 50 mM β-mercaptoethanol, 0.33 mg/ml X-gal). Results were recorded as positive if blue color was developed after incubation at 30°C for 5 min to 2 h. Assays were repeated three times. Library clones activated the *lacZ* and *His3* reporter genes only in the presence of pGBT9-FHL2 were chosen for PCR and automated DNA sequencing.

Liquid Culture β-Galactosidase Assay With ONPG as 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 color was developed at 30°C. The elapsed time in minute was recorded. The reaction mixture was centrifuged at 14,000 r.p.m. 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:

$$\text{units} = \frac{1000(OD_{420})}{(\text{elapsed time in min}) \cdot (0.1\ \text{ml} \times \text{concentration factor}) \cdot (OD_{600}\ \text{of culture})}$$

Subcloning and Protein Expression and Purification

The PCR product of FHL2 was subcloned into the T7 expression vector, pET-32a(+) (Novagen). The recombinant plasmid pET-32a(+)-FHL2 was transformed into *Escherichia coli* BL21(DE3)pLysS. The synthesis of FHL2 fusion protein with His·Tag in *E. coli* was induced by the addition of IPTG at a concentration of 1mM. The bacterial crude extract was electrophoresed on a 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel. The FHL2 fusion protein with His·Tag was purified using Ni-NTA

affinity chromatography by the QIAexpress purification system (QIAGEN). Purified fusion FHL2 protein was digested by recombinant enterokinase (rEK) by the Enterokinase Cleavage Capture Kit (Novagen). The reaction was mixed and incubated at room temperature (20–21°C) overnight. The cleavage of the protein was analysed by 15% SDS-PAGE.

PCR of AD/Library clones were performed by using a pair of primers flanking the open reading frame (ORF) of C-terminal hCDC47 (residues 592–719). The PCR products bearing the 5′*Eco*RI and 3′*Sal*I sites were subcloned into the polylinker region of T5 expression vector, pQE-30 (QIAGEN). The recombinant plasmid was transformed into *E. coli* strain M15 (pREP4). The production of hCDC47 protein fragment with 6X His-tag in *E. coli* was induced by the addition of IPTG at a concentration of 2 mM. The 6X His-tagged hCDC47 protein fragment was purified using Ni-NTA affinity chromatography by the QIAexpress purification system (QIAGEN). The bacterial crude extract and eluted protein were electrophoresed on a 15% SDS-polyacrylamide gel.

In Vitro Interaction Study

The FHL2 protein was covalently coupled into a HiTrap NHS-activated affinity column (1 ml; Pharmacia). The purified hCDC47 protein fragment (1 mg/ml) was dissolved in an alkaline buffer (124 mM glycine, 20 mM Tris-HCl, pH 8.6) consists of either 5 mM $ZnCl_2$ (in the presence of zinc), or 10 mM EDTA (in the absence of zinc). The hCDC47 protein solution was loaded into the column coupled with FHL2 and equilibrated at room temperature for 30 min. The column was then washed with 5 ml alkaline buffer, and fractions of 1 ml each were collected. Finally, the column was further washed with 3 ml elution buffer (0.1 M glycine-HCl, pH 2.7). Again each 1 ml fraction was collected. The eluted fractions were analyzed by reversed-phase chromatography on a microbore column. The peak of interest was analyzed by the Peptide Sequencer (Hewlett Packard).

Northern Hybridization

Human cancer cell lines multiple tissue Northern blot was purchased from Clontech. The integrity of the poly A^+ RNAs of the Northern hybridization was examined by denaturing gel electrophoresis and probing with a radioactively labeled human β-actin cDNA control probe (Unpublished data, quality control sheet supplied from Clontech). A pair of primers flanking the coding region of the FHL2 was designed to give a PCR product. A radioactive random-primed probe was made using the purified PCR product of FHL2 as the template. The blot was prehybridized for 30 min and hybridized for 1 h at 65°C using ExpressHyb hybridization solution (Clontech). The blot was then washed in 2× SSC with 0.05% SDS twice and again in 0.1× SSC with 0.1% SDS at room temperature to remove nonspecific annealing. Autoradiography was performed at −70°C overnight.

RESULTS

Identification of hCDC47 by Using the Yeast Two-Hybrid System

Before the yeast two-hybrid library screening, the yeast TransAct assay (Clontech) was performed to assure that the FHL2 protein fused with DNA-BD alone did not activate transcription in vivo. White colonies were detected from the colony lift β-galactosidase filter assay for the transformants with pGBT9-FHL2 plasmid only (data not shown). It indicates that FHL2 alone does not function in transcriptional activation and FHL2 does not initiate the transcription of the integrated *lacZ* reporter gene in yeast.

In the yeast two-hybrid library screening, approximately 4 million cDNA library clones were screened. After series of screening to eliminate false positives, the putative positive AD/library plasmids were isolated from yeast. Two putative positive AD/library plasmids were further investigated and confirmed to be true positives. The inserts of these putative positive clones were amplified by PCR and sequenced by automated DNA sequencing. These clones were identified as hCDC47 with a correct reading frame (GenBank/EMBL accession number U55716) [Fujita et al., 1996]. Sequence analysis of these true positive clones indicated that it encoded 128 amino acid residues corresponding to the C-terminal of hCDC47. Thus, FHL2 can interact with the C-terminal of hCDC47 and the C-terminal (residues 592–719) of hCDC47 is necessary and sufficient to mediate interaction with FHL2 in yeast cells. Specificity of the interaction between FHL2 and hCDC47 was further tested by retransforming the AD/library 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, the AD/library plasmid contained hCDC47 and pGBT9-FHL2, has the positive result that 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).

Identification of the Interacting Domain of FHL2 Using the Yeast Two-Hybrid System

To identify the domain requirements for this interaction, we used a quantitative yeast two-hybrid assay to measure the in vivo binding efficiency between hCDC47 and several different LIM domain constructs of FHL2.

The interactions between different LIM domains of FHL2 and hCDC47 were tested by the ability of cotransformants to grow on SD agar plates without Leu, Trp, and His, and to generate blue color (β-galactosidase activity) in colony lift β-galactosidase filter assays (Fig. 2). It showed that the fusion of DNA-BD with FHL2 containing all LIM domains bound strongly to hCDC47. 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 substrate and the results were shown on Figure 2B. The β-galactosidase activity is diminished to approximately 50% of that of full-length FHL2 when the truncated FHL2 contains two and a half LIM domains (2H-LIM) only. And the β-galactosidase activity is also diminished to approximately 40% of that of full-length FHL2 when the truncated FHL2 contains only the first half LIM (Half-LIM) domain or the first one and a half LIM domains (1H-LIM; Fig. 2B). Therefore, the second and third LIM domains in conjunction with the first half LIM domain of FHL2 were identified to bind with hCDC47

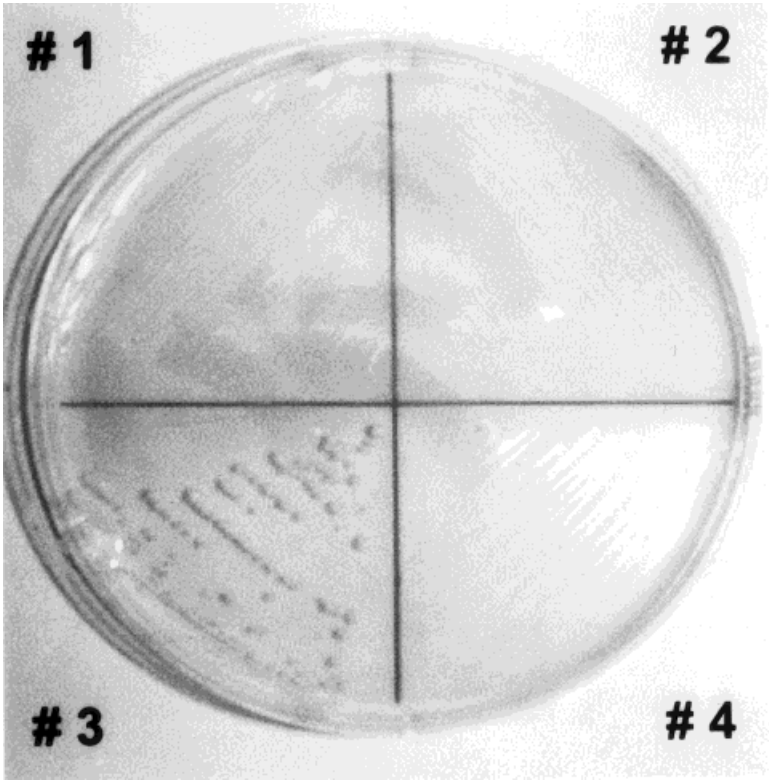

Fig. 1. Results of the *HIS* reporter gene expression by selection of yeast cotransformants on the SD agar plates lacking Leu, Trp, and His. Transformation #3 showed the positive result while the rest are negative controls. **#1**: AD/library plasmid only, **#2**: pGBT9 plasmid (DNA-BD only without insert)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

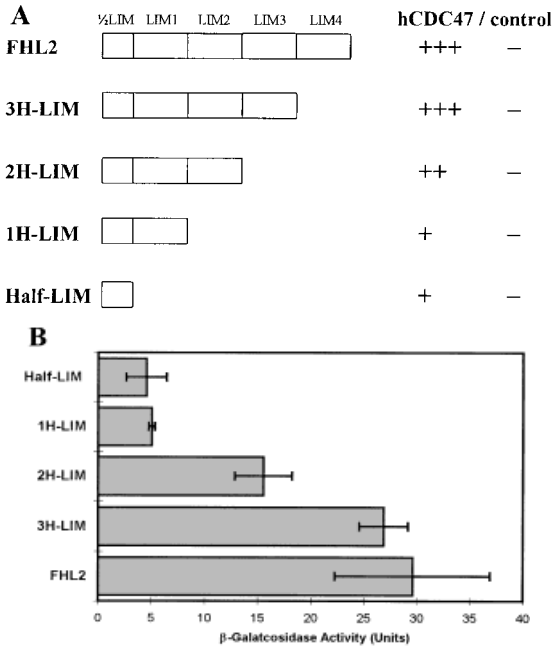

Fig. 2. **A**: Domain studies of FHL2 by the yeast two-hybrid assay. The interactions were tested by the ability of cotransformants to grow on SD agar plates lacking Leu, Trp, and His, and to generate blue color (β-Galactosidase activity) in filter lift assays. After developing for 10 h, the cotransformants were classified into positive colonies showing either dark blue (+++), blue (++), or pale blue (+) and negative colonies showing white (−). **B**: β-Galactosidase activity of the transformants were measured by liquid culture assay with ONPG as substrate. It showed the results of different LIM domains of FHL2 and hCDC47 coexpressed in yeast. The mean ± SD (n = 6) for each interacting pair is shown.

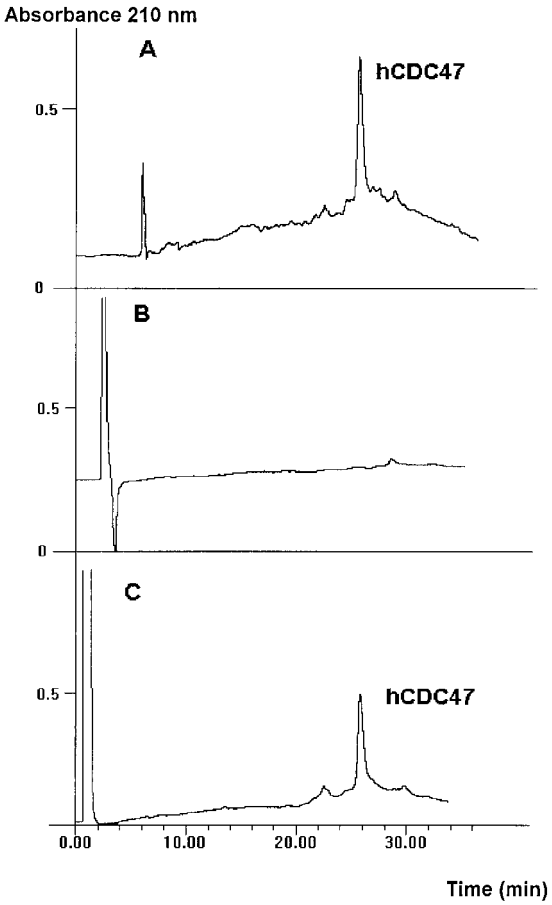

Fig. 3. Study of the interaction between FHL2 and hCDC47 by HPLC. **A**: Fraction of hCDC47 protein before loading into FHL2 affinity column was loaded onto the microbore reversed-phase column (RPC). **B**: Alkaline buffer washed fraction was loaded onto RPC. **C**: Eluted fraction was loaded onto RPC.

whereas the first LIM and the fourth LIM domains may be less important for binding to hCDC47.

Expression and Purification of Protein in *E. coli*

To further confirm the results of the interaction between FHL2 and hCDC47, an in vitro interaction study was performed (Fig. 3). FHL2 cDNA was expressed in *E. coli* BL21(DE3)pLysS. The purified FHL2 fusion protein was digested and analyzed in 15% SDS-PAGE. The size of the digested protein is consistent with the calculated molecular mass of FHL2 protein, which is approximately 32 kDa (data not shown).

hCDC47 cDNA (encodes residues 592–719) was expressed in *E. coli* M15 (pREP4). The purified 6X His-tagged hCDC47 protein fragment was analyzed in 15% SDS-PAGE. The size of the expressed protein is consistent with the calculated molecular mass of the 6× His-tagged hCDC47 protein fragment, which is approximately 15 kDa (data not shown).

In Vitro Interaction Study

The results of the in vitro interaction study shows that no peak was detected in the alkaline buffer washed fraction (i.e., no hCDC47 protein; Fig. 3B). A peak (hCDC47 protein) was detected in the eluted fraction and in the fraction of hCDC47 protein before loading (Fig. 3A,C). Moreover, the results show that there was interaction between FHL2 and hCDC47 only in the presence of zinc. In the absence of zinc, negative results were obtained (data not shown). The peak of interest was sequenced to confirm that it is hCDC47 by a peptide sequencer (data not shown).

FHL2 mRNA Expression Level in Different Cancer Cell Lines

From the human cancer cell line multiple tissue Northern blot, HeLa cell S3 and colorectal adenocarcinoma SW480 showed the highest FHL2 mRNA level (Fig. 4) whereas chronic myelogenous leukemia K-562, lung carcinoma A549, and melanoma G361 were lower in the signal representing FHL2 mRNA level. Only a very low signal could be detected in lymphoblastic leukemia MOLT-4. Virtually no signal could be detected in promyelocytic leukemia HL-60 and Burkitt's lymphoma Raj (Fig. 4). The integrity and equal loading of the RNAs of the Northern hybridization was confirmed by denaturing gel electrophoresis and probing with a radioactively labeled human β-actin cDNA control probe (unpublished data, quality control sheet supplied from Clontech).

DISCUSSION

In the yeast two-hybrid library screening and in vitro interaction study, FHL2 was identified to interact with hCDC47. It has become increasingly clear that LIM domains are multiple binding and adapter modules, and functional modifiers in protein interaction. Some LIM proteins appear to function solely as adapters to bring other components together in a complex [Dawid et al., 1998]. FHL2 and hCDC47 are found to participate in protein-protein interaction and formation of multicomplex. hCDC47 protein is present in the nucleus of cultured human cells, and together with other MCM proteins forms a tetramer complexed with histone H3, H4 and occupies the central region in the nucleosome structure [Fujita et al., 1996, 1997; Dalton and Whitbread, 1995; Ishimi et al., 1996].

In addition, the coding region between the FHL2 and the DRAL (downregulated in rhabdomyosarcoma LIM protein) is highly similar [Chan et al., 1998]. Previous studies have shown that DRAL was localized in the nucleus as well as in cytoplasm [Genini et al., 1996, 1997]. It has been found that LIM domain proteins can be nuclear, cytoplasmic, or can shuttle between compartments [Dawid et al., 1998]. Therefore, it might be possible that FHL2 interacts with hCDC47 together with other MCM proteins in the nucleus, and involved in the regulation of mammalian DNA replication together through their association-dissociation with the nuclear structure. 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 [Cuppen et al., 1998; Hammarstrom et al., 1996; Perez-Alvarado et al., 1996]. Interestingly, muscle-specific LIM protein (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 of MLP interacting with the muscle regulatory factors (MRFs) and enhancing the formation of MRF-DNA complexes, whereas another LIM domain primarily interacts with cy-

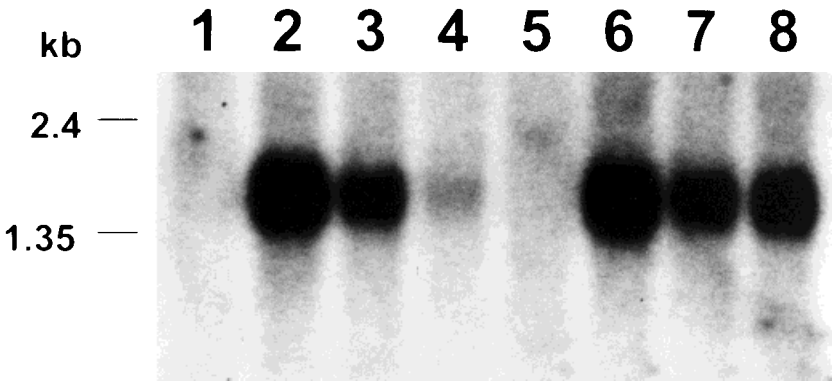

Fig. 4. FHL2 mRNA distribution amongst the various human cancer cell lines. Northern hybridization of human mRNA in which FHL2 was used as a probe. **Lane 1**: promyelocytic leukemia HL-60; **lane 2**: HeLa cell S3; **lane 3**: chronic myelogenous leukemia K-562; **lane 4**: lymphoblastic leukemia MOLT-4; **lane 5**: Burkitt's lymphoma Raji; **lane 6**: colorectal adenocarcinoma SW480; **lane 7**: lung carcinoma A549; **lane 8**: melanoma G361.

toplasmic 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 and third LIM domain in conjunction with the first half LIM domain of FHL2 were identified to bind with hCDC47. Thus, from the heart specific expression of FHL2, which is analogous to MLP, it with hCDC47 may form a multicomplex with other proteins through different LIM domains or hCDC47. They may appear to regulate other muscle regulatory factors and become involved in heart muscle differentiation and many cellular processes. Such speculation awaits further investigation in finding more protein partners of FHL2.

It has been proposed that FHL2 might bind to DNA indirectly through the formation of multiprotein complex. The reporter and yeast two-hybrid assays 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 in haematopoietic cells [Osada et al., 1995]. Such oligomeric complex exists in erythroid cells, binds to DNA and can function in transcriptional activation [Wadman et al., 1997]. 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]. We have shown that FHL2 can bind with DNA replication regulatory protein, hCDC47. Therefore, pending that the localization of hCDC47 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 hCDC47 or other transcriptional activators may be formed. This complex may bind DNA and promote transcriptional activation of muscle-specific gene and regulate DNA replication by association-dissociation from the nuclear structure during different developmental stages of the heart, thus it might promote myogenesis. Besides, this interaction with FHL2 molecular adapter and other transcriptional factors may be important in regulation of the DNA helicase activity of the hCDC47 and the initiation of the DNA replication [Ishimi, 1997]. 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.

It is possible that FHL2 gene may be involved in cancer development. Many LIM-only proteins are related to cancer development. For example, the oncogenic LIM protein rhombotin (RBTN1) and rhombotin-2 (RBTN2), both of which contain two tandem repeats of LIM domains, were identified as a result of their activation by chromosomal translocations that occurred in a subset of childhood T cell acute leukemias [Boehm et al., 1991]. FHL3, a member of the FHL family protein, was mapped to distal end of the short arm of chromosomal 1 and the region is deleted in several human malignancies [Lee et al., 1999]. From our results of the human cancer cell line and multiple tissue Northern blot, HeLa cell S3, and colorectal adenocarcinoma SW480 showed the highest FHL2 mRNA level whereas the levels in the rest of human cancer cell lines were lower or even undetectable. It is interesting that in lung carcinoma A549 and colorectal adenocarcinoma SW480, FHL2 mRNA was detected but in normal tissues such as lung and colon, no signal of FHL2 mRNA could be detected in the previous studies [Chan et al., 1998].

In addition, previous studies have shown that DRAL (which has nearly identical DNA sequence as FHL2) is expressed in human primary myoblasts but downregulated in the embryonal-rhabdomyosarcoma (RMS) cell line RD [Chan et al., 1998; Genini et al., 1996, 1997]. Other than down-regulation of FHL2 in rhabdomyosarcoma, many other LIM-only proteins are downregulated in transformed cells [Kiess et al., 1995; Shibanuma et al., 1994, 1997]. For hCDC47 protein expression and localization in normal human tissues, positive nuclei were found in the proliferative components, but not in differentiated cells [Hiraiwa et al., 1997]. Besides, malignant tumors from several other organs all exhibited a similar increase in nuclear hCDC47-positive cells, as compared to their

normal counterparts. Together, these data indicate that dysregulated expression of hCDC47 in neoplasms contributes to their proliferation and hCDC47 may play a role for normal and neoplastic cell growth in vivo [Hiraiwa et al., 1997]. FHL2 can interact with hCDC47 and we have shown that there are differences in the expression level of FHL2 mRNA between many human cancer cell lines and normal tissues. We speculate that FHL2 may also have the property of other LIM-only proteins which are associated with cancer development. Further investigation is still needed to reveal the possible involvement of FHL2 in human malignancies.

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