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ARTICLES

Protein–Protein Interaction of FHL3 with FHL2 and Visualization of Their Interaction by Green Fluorescent Proteins (GFP) Two-Fusion Fluorescence Resonance Energy Transfer (FRET)

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Abstract LIM domain proteins are found to be important regulators in cell growth, cell fate determination, cell differentiation and remodeling of the cell cytoskeleton. Human Four-and-a-half LIM-only protein 3 (FHL3) is a type of LIM-only protein that contains four tandemly repeated LIM motifs with an N-terminal single zinc finger (half LIM motif). FHL3 expresses predominantly in human skeletal muscle. In this report, FHL3 was shown to be a novel interacting partner of FHL2 using the yeast two-hybrid assay. Furthermore, site-directed mutagenesis of FHL3 indicated that the LIM2 of FHL3 is the essential LIM domain for interaction with FHL2. Green fluorescent protein (GFP) was used to tag FHL3 in order to study its distribution during myogenesis. Our result shows that FHL3 was localized in the focal adhesions and nucleus of the cells. FHL3 mainly stayed in the focal adhesion during myogenesis. Moreover, using site-directed mutagenesis, the LIM1 of FHL3 was identified as an essential LIM domain for its subcellular localization. Mutants of GFP have given rise to a novel technique, two-fusion fluorescence resonance energy transfer (FRET), in the determination of protein–protein interaction at particular subcellular locations of eukaryotic cells. To determine whether FHL2 and FHL3 can interact with one another and to locate the site of this interaction in a single intact mammalian cell, we fused FHL2 and FHL3 to different mutants of GFP and studied their interactions using FRET. BFP/GFP fusion constructs were cotransfected into muscle myoblast C2C12 to verify the colocalization and subcellular localization of FRET. We found that FHL2 and FHL3 were colocalized in the mitochondria of the C2C12 cells and FRET was observed by using an epi-fluorescent microscope equipped with an FRET specific filter set. J. Cell. Biochem. 80:293–303, 2001.

Key words: FHL3; FHL2; protein-protein interaction; LIM domain protein; GFP; FRET

LIM domain proteins are defined as proteins having a double zinc finger motif with a consensus amino acid sequence $CX_2CX_{16-23}HX_2(C/H)X_2CX_2CX_{16-23}CX_2(C/H/D)$ [Morgan and Madgwick, 1999]. It is believed that LIM domain proteins are found to be important regulators in cell growth, cell fate determination, cell differentiation and remodeling of the cell cytoskeleton. The Four-and-a-half LIM-only domain (FHL) protein family is a new member among the LIM-only domain proteins. FHL1 [Lee et al., 1999] and FHL3 [Lee et al., 1998a, 1998b; Morgan and Madgwick, 1996] are expressed in relatively high levels in human skeletal muscle [Morgan and Madgwick, 1996] while FHL2 [Chan et al., 1998] is expressed at a high level in human heart muscle and at a lower level in human skeletal muscle. FHL3 is expressed differentially during C2C12 myogen-

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esis. It was found to be downregulated during the first 10 days of myogenesis and there is a significant elevation of FHL3 after the 10 days of myogenesis [Morgan and Madgwick, 1999]. The change of expression level of FHL3 in C2C12 suggests that FHL3 is involved in muscle cell development and differentiation.

The available evidence suggests that LIM domain proteins may mediate protein–protein interaction. This property has been demonstrated by 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]. In addition, 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 be specific [Feuerstein et al., 1994; Schmeichel and Beckerle, 1994]. In order to examine the interaction partners of FHL3, the yeast two-hybrid assay was employed [Hobert et al., 1996]. Our results from yeast two-hybrid assays show that FHL3 interacts with FHL2, another member of four-and-a-half LIM only protein family. Using site-directed mutagenesis, the second LIM domain, LIM2, of FHL3 was identified as the principal LIM domain for their interaction. Other than hCDC47 and the androgen receptor, FHL3 is the third protein that was shown to interact with FHL2, which support the hypothesis that LIM domain protein functions as a large protein complex.

Mutants of GFP have given rise to a novel technique, two-fusion fluorescence resonance energy transfer (FRET), in the determination of protein–protein interaction at particular subcellular locations of eukaryotic cells [Mahajan et al., 1998]. FRET studies are based on two GFP mutants. S65T has a red shift excitation peak around 488 nm, and an emission peak of 511 nm, and it emits fourfold more intensely when compared with that of the wild type GFP. Another mutant, known as the blue fluorescent protein (BFP) which can be excited at 389 nm and gives a bright blue fluorescence at around 450 nm was also developed for FRET. The combination of GFP and BFP fluorophores can be used for FRET studies as the emission spectrum of BFP (as donor) overlaps the excitation of spectrum of GFP (as acceptor). If the distance between these two GFP mutants is small enough (10–50 Å), with the excitation of the donor BFP, the energy for exciting BFP can be transfered to the acceptor GFP nonradiatively and as a result of green fluorescence in the presence of UV only [Day, 1998; Mahajan et al., 1998; Mitra et al., 1996; Pollok and Heim, 1999; Tsien, 1998]. In this report, we demonstrated that the two LIM-only proteins, FHL2 and FHL3 interact with each other in the yeast two hybrid assay. To determine whether FHL2 and FHL3 interact with one another and to locate the site of this interaction in a single intact mammalian cell, we fused FHL2 and FHL3 to different mutants of GFP and studied their interactions using FRET. BFP/GFP fusion constructs were transfected into mouse myoblasts (C2C12) to verify the colocalization of these two fusion proteins at the mitochondria where we observe FRET under a FRET specific filter set.

METHODS

Yeast Two-Hybrid Assay

FHL3 cDNA was amplified by using a pair of primers flanking the open reading frame (ORF) FHL3 (Forward: 5′-TAG GGC GAA TTC AGC GAG TCA TTT GAC TGT GCA -3′; Reverse: 5′-TAG GGC GGA TCC GGG CCC TGC CTG GAT ACA-3′). An *Eco*RI site and a *Sal*I site were present in the forward and reverse primers, respectively. After digestion with *Eco*RI and *Sal*I restriction enzymes, the PCR product was subcloned into the two-hybrid DNA-AD vector, pGAD424. For the cloning of hFHL1, hMLP, hCRP2 into the yeast two-hybrid DNA-BD vector, pAS2-1, the same cloning procedures were used as described previously. PCR of hFHL1 [Lee et al., 1999, 1998a; Morgan and Madgwick, 1999], hMLP [Arber et al., 1994] and hCRP2 [Karim et al., 1996] cDNA clones were performed by using a pair of primers flanking the open reading frame (ORF) of FHL1 (Forward: 5′-TAG GGC GAA TTC AAT GGC GGA GAA GTT TGA CTG CCA-3′; Reverse: 5′-TAG GGC GTC GAC CAG CTT TTT GGC ACA GTC GGG-3′), hMLP (Forward: 5′-TAG GGC GAA TTC AAT GCC AAA CTG GGG CGG A-3′; Reverse: 5′-TAG GGC GTC GAC TTC TTT CTT TTC CAC TTG TTG T-3′), hCRP2 (Forward: 5′-TAG GGC GAA TTC AAT GGC CTC CAA ATG CCC CAA G-3′; Reverse: 5′-TAG GGC GTC GAC CTA GGG CTG GAC CTT GCC TTC-3′). An *Eco*RI site and a *Sal*I site are present in the forward and reverse primers, respectively.

TABLE I. PCR Primers (Which Introduced Two Point Mutations into Each LIM Domain) Used in Site-Directed Mutagenesis of the LIM Domain of FHL3

LIM domain	Point mutations to introduce two amino acids changes from **cysteine** to **serine** (underlined)
LIM1 (FHL3M1)	5′-CAT TTC CAC GAG GGC TCC TTC CGC TCC TGC CGC TGC CAGGCG-3′
LIM2 (FHL3M2)	5′CA TGG CAT GCG CAC TCC TTC CTG TCC ATT GGC TGT GAA CAG CCA C-3′
LIM3 (FHL3M3)	5′-CCG TGG CAT CCA AA TCT CTG GTC TCT ACC GGA TGC CAG ACG-3′
LIM4 (FHL3M4)	5′-CAC TGG CAC CAC AAC TCC TTC ACC TCC GAC CGC TGC TCT AAC-3′

FHL2-pAS2-1 [Chan et al., 2000] and CLIM-pAS2-1 vectors [Kotaka et al., 2000] were kindly provided by Dr. K.K. Chan and Dr. Massyo Kotaka of our Laboratory.

Bait plasmid and either the MATCHMAKER AD plasmid or GAL-AD tested plasmid were cotransformed into yeast reporter strains, Y190 and Y187 using the lithium acetate (LiAc) method. Then the cells were resuspended in 1X TE buffer and plated out on synthetic triple dropout (SD) agar plates with 3-AT lacking Trp, Leu and His for yeast strain Y190 and double dropout SD agar plates lacking Trp and Leu for yeast strain Y187. The transformants were incubated at 30°C for 4–5 days, and restreaked onto triple dropout SD agar plates lacking Trp, Leu and His. The His^+ transformants of Y190 and Trp^+Leu^+ transformants of Y187 were 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 or liquid culture β-galactosidase assay with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate. 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 piece of filter paper.

Site-Directed Mutagenesis of FHL3 and Vectors Construction

Site-directed mutagenesis of each LIM domain was carried out by PCR using oligonucleotide primers (Table I) which contain the appropriate point substitutions of amino acids. Each mutation was verified by DNA sequence analysis. PCR were performed by using High Fidelity PCR system (Boehringer Mannheim, Inc.). Mutants of FHL3 were subcloned into pGAD424 and EGFP-C1 vectors (Clontech).

Construction of GFP Fusion Protein Expression Vector

The expression vectors FHL2-GFP and FHL3-BFP were constructed as follows. The coding region of FHL2 and FHL3 were amplified by the PCR with the following primers. For FHL2 amplification, primers (forward: 5′-TAG GGC GTC GAC ACT GAG CGC TTT GAC TGC C-3′; reverse: 5′-TAG GGC GGA TCC TGT GAG ATC ACA AGC AGC AAC-3′) containing *Sal*I and *Bam*HI sites were used, while for FHL3 amplification, primers (forward: 5′-TAG GGC GAA TTC T AGC GAG TCA TTT GAC TGT GCA-3′; reverse: 5′-TAG GGC GTC GAC TTA GGG CCC TGC CTG GAA ACA G-3′) containing *EcoR*I and *Sal*I sites were used. Both the *Sal*I–*Bam*HI and *EcoR*I–*Sal*I PCR fragments were cloned into the multiple cloning site of the CMV-promoter driven pEBFP-C1 and pEGFP-C1(Clontech) expression vector. The resulting constructs, FHL2-GFP and FHL3-BFP code for N-terminal GFP-tagged fusion proteins. The two different PCR amplified products were checked for the absence of PCR mutation using an ALF automated sequence analyzer (Pharmacia). As a control for the cellular distribution of unfused GFP, the original pEGFP-C1 vector (Clontech) was used. Two control vectors, cytochrome c-GFP and GFP-human papillomavirus (HPV)-16-E6 [Mahajan et al., 1998], for FRET study are kindly provided by Prof. Mahajan, N.P.

Tissue Culture and Transfection

Mouse C2C12 myoblast cell line was maintained in DMEM medium (Life Technologies, Inc.) supplemented with 10% (v/v) heat-inactivated, certified fetal bovine serum (Life Technologies Inc.), 1% antibiotics (penicillin, 100 units/ml; streptomycin sulfate, 100 μg/ml) at

37°C in 5% CO_2 in a humidified atmosphere. They were transfected with various plasmid constructs using Lipofectamine Plus Reagent (Life Technologies, Inc.). 1×10^5 Cells were grown on coverslips in a six-well plate, until cells were 60–70% confluent. One micrograms of DNA (1.5 μg of each DNA for double transfectants) and 4 μl of Plus Reagent were added to 125 μl DMEM medium lacking serum and antibiotics and incubated for 15 min at room temperature. Four microliters of lipofectamine reagent were added to 125 μl medium without serum and antibiotics to another tube and incubated for 15 min at room temperature. Two solutions were mixed and incubated for another 15 min at room temperature. Cellswere washed in PBS twice. One milliliter of serum free medium was added to the cells. The DNA-lipofectamine plus mixture was added to the cells and incubated for 3 h at 37°C. After 3 h incubation, cells were rinsed in PBS and fresh medium supplemented with 10% FBS and antibiotics was added followed by incubation for another 24 h before microscopic examination. For the study of subcellular localization of FHL3 in myotubes, stably transfected FHL3-GFP C2C12 myoblast was selected in the presence of 0.5 mg/ml of G418, respectively. 1×10^3 Cell were seeded on coverslips and then differentiation medium (DMEM medium supplement with 2% FBS) was replaced with the nutrient rich PRMI (10% FCS) for 6 days. The cells were washed in PBS and fixed in 3.7% paraformaldehyde before microscopic examinations.

Staining of Actin, Nucleic Acid and Mitochondria

To confirm the subcellular localization of the fusion protein inside the cells, Mitotracker (Molecular Probes, Inc.), rhodamine phalloidin (Molecular Probes, Inc.) and acridine orange (Molecular Probes, Inc.) were employed. For rhodamine phalloidin, 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. Cells were extracted with acetone at −20°C for 5 min. Cells were then rinsed again in PBS twice. Cells were incubated with one unit of rhodamine phalloidin in 200 μl PBS with 1% BSA for 20 min at room temperature. (250 μM) Acridine orange was added to the cells directly to visualize nucleic acids after images of GFP were captured. For Mitotracker, transfected cells were incubated with 250 nM of Mitotracker reagent for 30 min at 37°C. Cells were then rinsed in PBS twice and fixed in 3.7% paraformaldehyde in PBS for 15 min at 37°C.

Fluorescence Microscopy and FRET Microscopy

Both epi-fluorescence microscopy and laser confocal microscopy were used in the study of subcellular localization of GFP fusion proteins. 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 Mitotracker, rhodamine phalloidin and acridine orange, a 600EFLP filter was used for emission. For epi-fluorescence microscopy (Zeiss 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) (exposure 1–4 s) and were artificially colored using IpLab spectrum software. For FRET microscopy, epi-fluorescence microscopy was equipped with a XF89-BFP/GFP FRET filter set (365HT25 for excitation, dichroic mirror 400DRLP, 450DF65 for BFP emission, 535RDF45 for GFP and FRET emission) (Omega Optical, Inc.) for the detection of FRET in the co-transfected cells.

RESULTS

Identification of FHL2 as the Interacting Partner of FHL3 by Yeast Two-Hybrid Assay

GAL-BD vector constructs (pGAD-FHL3) were contransformed with GAL-AD vector constructs (pAS2-1-FHL1, pGBT9-FHL2, pAS2-1-hMLP, pAS2-1-hCRHP, pAS2-1-hCRP2 and pAS2-1-hCLIM) into *Saccharomyces cerevisiae* yeast strain Y190. Interaction of the fusion proteins in the Trp^+Leu^+ transformants was determined by a β-galactosidase in situ colony lift filter assay (Fig. 1a). In the two-hybrid experiment, yeast strain Y190 with only GAL-4 fusion proteins of FHL2 and FHL3 had deep blue *lac*Z phenotype upon β-galactosidase activity assay; it suggests that these fusion proteins interact with each other resulting in the activation of transcription of the β-galactosidase reporter gene. When the experiment was repeated using *Saccharomyces cerevisiae* yeast

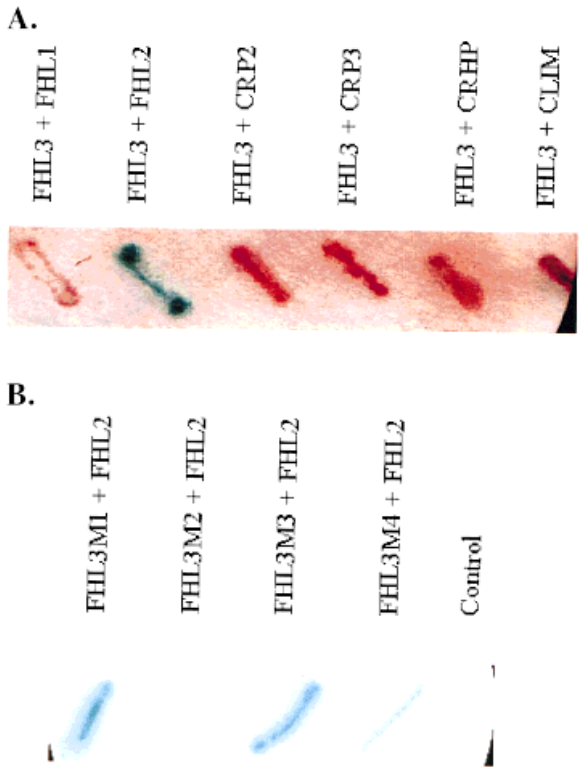

Fig. 1. (**A**) Small-scale yeast two-hybrid assay to verify that FHL3 is the interacting partner of FHL2. (**B**) Identification of LIM2 of FHL3 as the principal interacting domain of FHL2.

strain Y187 instead of Y190, the same result was obtained.

Identification of the Essential LIM 2 Domain of FHL3 for Interaction with FHL2 by Site-Directed Mutagenesis

By using the yeast two-hybrid assay, the interaction of FHL2 with different LIM domains of FHL3 were characterized and the interacting domain of FHL3 was identified. Each of the different LIM domain mutants of FHL3 (LIM1$^{(Cys65\rightarrow Ser65 \text{ and } Cys68\rightarrow Ser68)}$, LIM2$^{(cys126\rightarrow Ser126 \text{ and } Cys129\rightarrow Ser129)}$, LIM3$^{(Cys185\rightarrow Ser185 \text{ and } Cys188\rightarrow Ser188)}$ and LIM4$^{(Cys248\rightarrow Ser248 \text{ and } Cys251\rightarrow Ser251)}$), that were prepared to fuse with GAL-AD, were coexpressed with FHL2 proteins fused with GAL-AD in yeast reporter strains Y187. The interactions of these proteins were tested by the ability of cotransformants to generate blue colour (β-galactosidase activity) in colony lift β-galactosidase filter assays (Fig. 1b). For the interaction of FHL3 and FHL2, it showed that the binding between these two proteins was terminated when the second LIM domain-LIM2 domain of FHL3 was mutated; therefore, the LIM2 of FHL3 was identified to be essential for binding with FHL2. Negative control was done by cotransformation of different LIM domain mutants of pGAD424-FHL3 with pGBT9 vector (without insert).

FHL3 Localizes at the Focal Adhesion and Nucleus of Cells

Classically, subcellular studies are performed by indirect immunofluorescence. Here, we report the use of GFP fusion proteins to monitor the subcellular localization of FHL3 in living cells. The subcellular localization patterns of FHL3 was determined by transiently expressing GFP tagged versions of the protein in different cell types. GFP fusion protein constructs were generated, encoding GFP fusion proteins with FHL3. Purified expression constructs were transfected transiently into C2C12 myoblasts. After 24-h posttransfection, fusion proteins expressed in the cells were viewed using confocal laser scanning microscopy. FHL3 localized at the focal adhesions and the nucleus of the C2C12 myoblasts (Fig. 2). Confocal laser scanning microscopy imaging confirmed localization of FHL3 to focal adhesions (Fig. 2A and C in green). Actin filaments were visualized by rhodamine-conjugated phalloidin (Fig. 2B and C in red). FHL3 was also localized at the nucleus of C2C12 cells (Fig. 2D–F).

FHL3 Localize at Focal Adhesion of C2C12 Myotubes

Stable FHL3-GFP transfected C2C12 myoblasts were treated with differentiation medium (DMEM medium supplemented with 2% FCS) for 6 days. When myoblasts are supplemented with a low level of FCS, it would undergo myogenesis and become differentiated into myotubes within ≈6 days [Hashimoto and Ogashiwa, 1997]. Formations of myotubes were observed under a light microscope. The differentiated cells with GFP fluorescence were observed and the subcellular localization of FHL3 can be determined using fluorescence microscopy. FHL3 localized at focal adhesions only (arrow) but not in the nucleus during C2C12 differentiation (Fig. 3).

LIM1 of FHL3 is Responsible for its Localizaiton to the Nucleus and Focal Adhesions

Different mutants of FHL3 were then subcloned into GFP mammialian expression vector

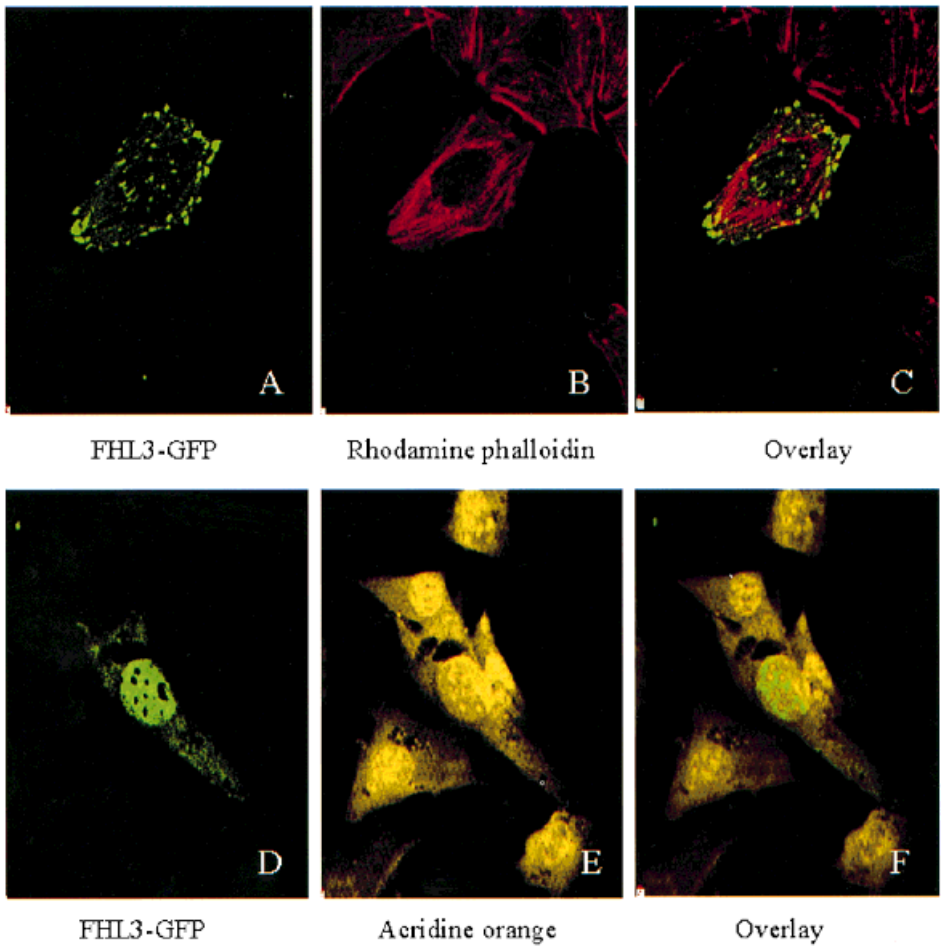

Fig. 2. Dual subcelluar localization of FHL3-GFP fusion proteins were found in C2C12 myoblasts. Upper panel showing that FHL3 is localized in the focal adhesion plagues (**A**, **B** and **C**, arrows) and lower panel showing that Fhl3 is localized in the nucleus (**D**, **E** and **F**, arrows).

to determine their localization. Mutation of LIM2$^{(Cys126\rightarrow Ser126 \text{ and } Cys129\rightarrow Ser129)}$ (FHL3M2), LIM3$^{(Cys185\rightarrow Ser185 \text{ and } Cys188\rightarrow Ser188)}$ (FHL3M3) and LIM4$^{(Cys248\rightarrow Ser248 \text{ and } Cys251\rightarrow Ser251)}$ (FHL3M4) (Fig. 5A, B and C) did not affect its localization in nucleus and focal adhesion. However, mutation of LIM1$^{(Cys65\rightarrow Ser65 \text{ and } Cys68\rightarrow Ser68)}$ (FHL1M1) of FHL3, which destablize the structure of LIM1 of FHL3 by replacing two cysteine residues with two serine residues in LIM1, results in a loss of its ability to locate in the nucleus and focal adhesions (Fig 4A). Our result suggested that LIM1 of FHL3 is responsible for its localization.

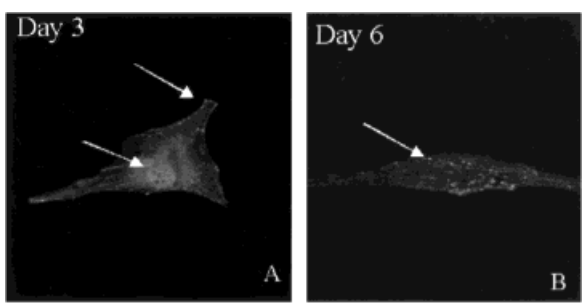

Fig. 3. Subcellular localization of FHL3 during myogenesis of C2C12 myoblast. Stably transfected C2C12 myoblasts with FHL3-GFP were treated with differentiation medium (described previously) for 6 days. The cells were examined in Day 3 and Day 6. FHL3 were mainly seen at the focal adhesion plaques of the differentiating myoblasts (**A** and **B**, arrows) but not in the nucleus (B, arrow).

Visualization of the LIM–LIM Interaction between FHL2 and FHL3 by GFP-Two Fusion Fluorescence Resonance Energy Transfer (FRET)

Our results show that FHL3-BFP fusion protein was relocalized from focal adhesions and nucleus into mitochondria when it is cotransfected with FHL2-GFP fusion protein into the same single intact mammalian cell (rat

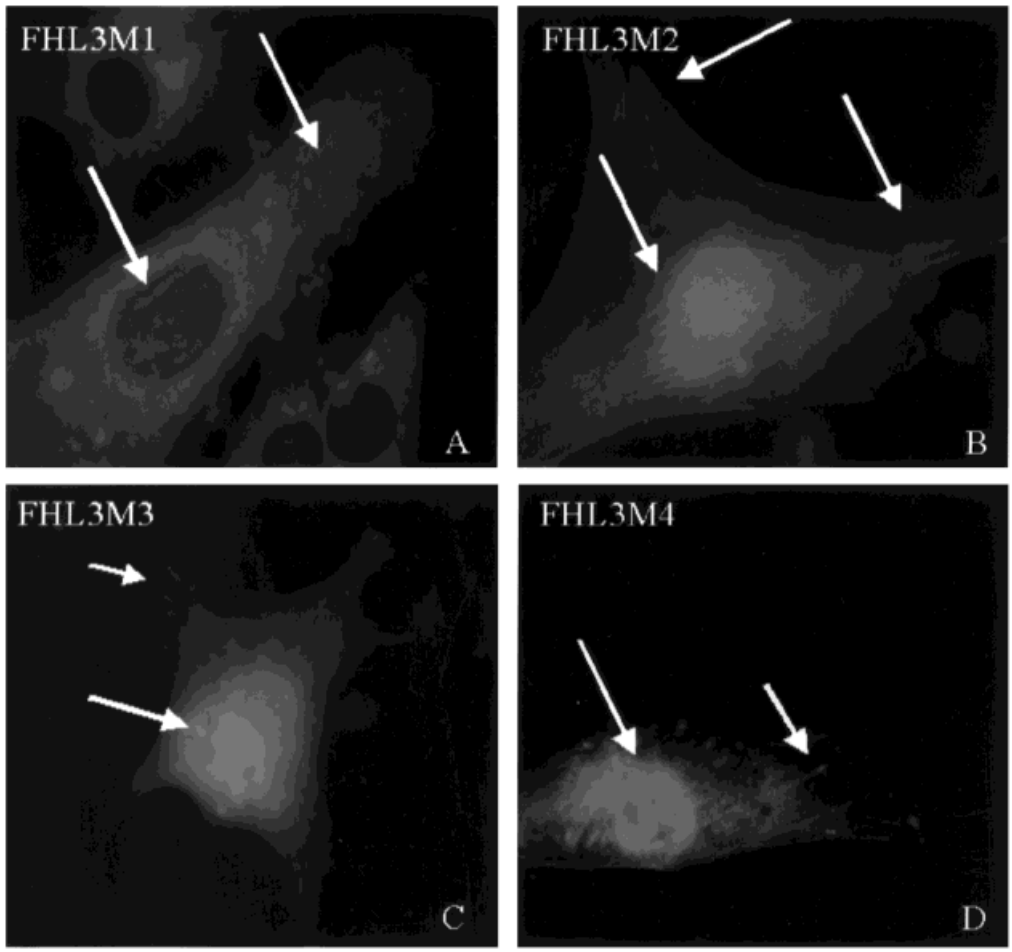

Fig. 4. Identification of LIM1 as the essential LIM domain for the localization of FHL3 in focal adhesion and nucleus by PCR site-directed mutagenesis. Mutation of the first LIM domain of FHL3 (FHL3M1) results in a lost of the ability of its specific localization in myoblast (**A**). Mutation of other LIM domains (FHL3M2, FHL3M3 and FHL3M4) of FHL3 did not affect its specific localization in the focal adhesion and the nucleus (**B**, **C** and **D**, arrows).

C2C12 myoblast). FRET was observed in the mitochondria of those cotransfected cells under a fluorescence microscope equipped with a BFP/GFP FRET filter set (Omega Optical) (Fig. 5). Two control vectors [Mahajan, 1998], for FRET study were kindly provided by Prof. Mahajan, N.P. for the FRET study. Cotransfection of control vectors with FHL3-BFP or FHL2-BFP shows that neither of them alone gives FRET under the FRET filter set (data not shown).

DISCUSSION

Our studies identified that FHL3 is another interacting partner of FHL2, a LIM only protein which has been shown previously to interact with a minichromosome maintainence protein hCDC47 [Chan et al., 2000] and the androgen receptor of epithelial cells of the prostate [Muller et al., 2000]. It was proposed that LIM domain proteins are multiple binding and adapter modules, and functional modifiers in protein interactions. Some of the LIM proteins appear to function solely as adapters to bring other components together in a complex [Dawid et al., 1998]. The interaction of FHL2 with FHL3, hCDC47 and androgen receptor further supported the hypothesis that FHL2 serves as an adapter module to form a functional multicomplex.

FHL3 consists of four LIM domains and a zinc finger at the N-terminus of the protein. All four LIM domains have a similar structure, including striking similarities between the first and second zinc fingers of each domain. Our results on site-directed mutagenesis, suggested that the second LIM domain plays a central

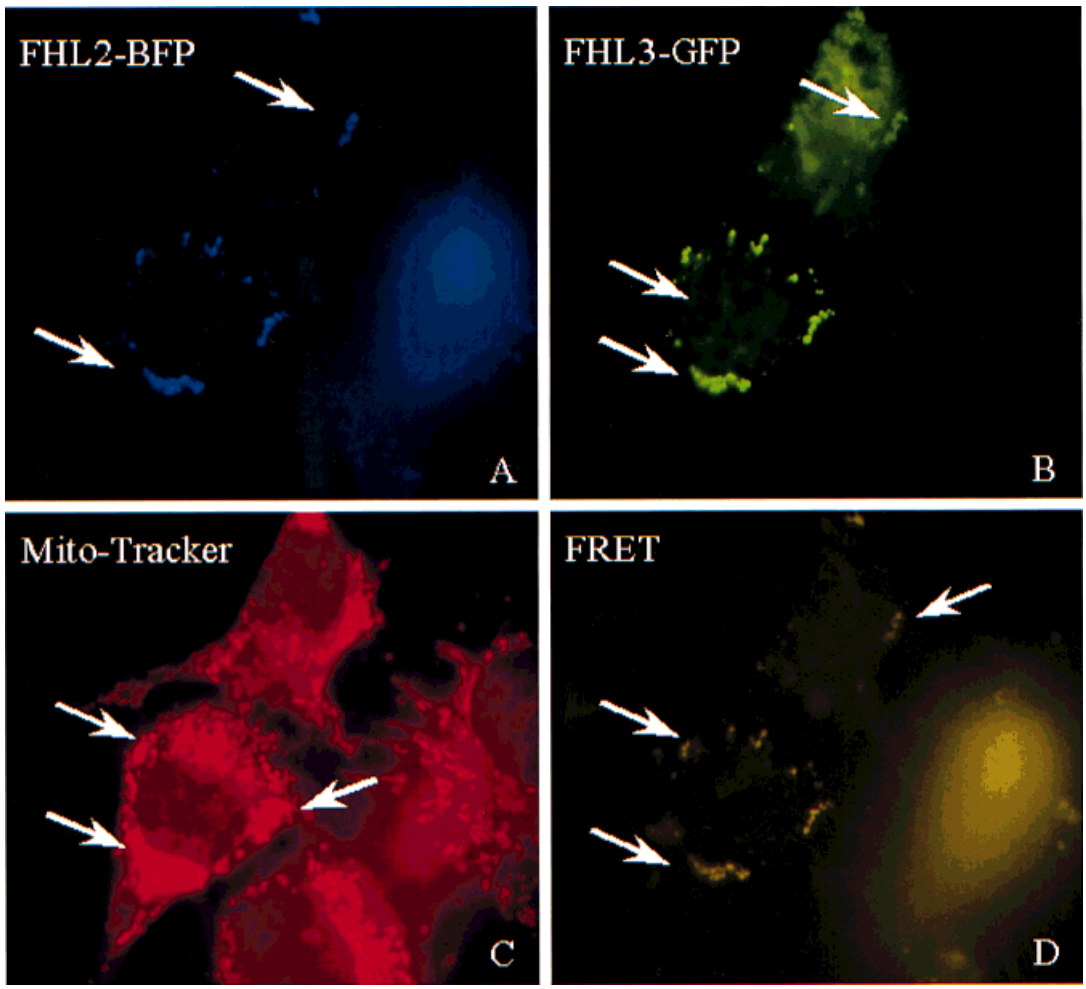

Fig. 5. Visualization of LIM–LIM interaction of FHL3 and FHL2 in mitochondria by GFP two-fusion fluorescence energy transfer. FHL3-BFP (**A**, arrows) and FHL2-GFP (**B**, arrows) were cotransfected into C2C12 myoblasts. They were colocalized in the mitochondria where FRET was observed by using a FRET specific fluorescent microscope (**D**, arrows).

role in interacting with FHL2 and the first LIM domain of FHL3 plays a central role for its localization in the nucleus and focal adhesions. Thus, our result demonstrated that each LIM domain might function independently. Our results are similar to that reported for muscle LIM protein (MLP), another LIM-only protein. MLP has a dual subcellular localization. When MLP is localized in the nucleus, it interacts with MyoD via the first LIM domain-LIM1 of MLP. When MLP is localized in the cytoskeleton, it interacts with actin via another LIM domain-LIM2 of MLP [Arber et al., 1994].

FHL3 is localized in the nucleus and focal adhesions. Our results using PCR site-directed mutagenesis demonstrated that the first LIM domain is essential for its dual localization. We believe that FHL3 might interact with different kinds of proteins like other LIM-only protein as an adapter module to facilitate its function in either the focal adhesion or the nucleus. For example, FHL2 and FHL3 may interact in a way similar to that observed for CRP3/MLP. Focal adhesions are specialized sites of adhesion developed by many cells in culture. They mediate the attachment of cells to the extracellular matrix and regulate the morphology, migratory properties, growth and differentiation of the cells. Many proteins have been identified at focal adhesions. Some of these proteins have predominantly a structural role, such as the cytoskeletal protein vinculin and α-actinin, whereas others are involved in signal transduction such as focal adhesion kinase, in transmembrane linkage to the extracellular matrix such as intergrins [Burridge and Chrzanowska-Wodnicka, 1996]. Most of the focal adhesion proteins are house-keeping pro-

TABLE II. Summary of the Result of the Colony Lift β-Galactosidase Filter Assay of the Trp^+Leu^+ Cotransformant Yeast Cells[a]

		β-galactosidase activity					
GAL-AD	*GAL-BD*	FHL1	FHL2	HCRP2	HMLP	hCRHP	hCLIM
FHL3		−	+	−	−	−	−

[a]Positive and negative β-galactosidase activities are represented by a plus symbol '+' and a minus symbol '−', respectively.

TABLE III. Domain Studies of FHL3 by the Yeast Two-Hybrid Assay[a]

		β-galactosidase activity				
GAL-BD	*GAL-AD*	FHL3M1	FHL3M2	FHL3M3	FHL3M4	pGAD424
FHL2		+	−	+	+	−

[a]The interactions were tested by the ability of cotransformants to generate blue color (β-galactosidase activity) in filter lift assays. '+' is an estimated arbitrary unit of colour intensity. '−' represents negative β-galactosidase activity. Negative control was done by contransformation of different LIM domains mutants of pGAD424-FHL3 with the pGAD424 vector (without insert).

teins such as integrins [Geiger et al., 1992], whereas others are cell-type specific such as dystrophin [Kramarcy and Sealock, 1990] and aciculin [Belkin and Burridge, 1994]. Focal adhesion LIM domain proteins, e.g. Pinch [Hobert et al., 1999], Zyxin [Macalma et al., 1996], Paxillin [Turner et al., 1991] and Hic-5 [Thomas et al., 1999], are transient or signaling components rather than a structurally essential component of cell adhesion sites. FHL3 may be capable of localizing at the nucleus and interacting with other proteins to modulate particular regulatory events like other focal adhesion LIM domain proteins. On the other hand, Morgan et al. demonstrated that FHL3 is expressed differentially during the C2C12 myogenesis. It was found to be downregulated during the first 10 days of myogenesis and then significantly elevated after the 10 days of myogenesis. FHL3 may be involved in the regulation of myogenesis in focal adhesion and serves as signaling components by interacting with different kinds of proteins. FHL2 is one of the possible partners in this event since focal adhesion is closely related to cell cycle progression and cellular differentiation in muscle cells. Moreover, focal adhesion proteins, such as integrin, that span the plasma membrane, serve to connect the extracellular matrix and cytoplasmic actin cytoskeleton at focal adhesions during myogenesis. The cellular proliferation and gene reprogramming in response to cellular adhesion are likely to be mediated by integrins [Juliano and Haskill, 1993; Juliano and Varner, 1993; Miyamoto et al., 1995; Slack and Higgins, 1996]. Our results also suggested that FHL3 does not stay in the nucleus after C2C12 myogenesis but localized only at the focal adhesion plaques of myotubes. It is interesting that CRP3/MLP are also relocalized during myogenesis from the nucleus to actin filaments. CRP/MLP has two LIM domains, the first LIM domain interacts with bHLH transcription factor MyoD in the nucleus [Arber and Caroni, 1996] and enhances the activity of MyoD, thus regulating muscle myogenesis [Arber et al., 1994]. Cytoplasmic CRP3/MLP associated with actin filament by its second LIM domain [Arber and Caroni, 1996] and is involved in facilitating the assembly of the myofibril apparatus during muscle maturation. The dual localization suggests that CRP3/MLP may provide a regulatory checkpoint for insuring appropriate transcriptional activity to support sufficient numbers of functional sacromere [Kong et al., 1997]. The redistrubution of FHL3 during myogenesis is similar to CRP3/MLP, which provides another evidence that FHL3 is involved in this complicated mechanism.

Recently, Mahajan et al. demonstrated that the interaction of Bcl-2 and Bax in mitochondria by using green fluorescent protein (GFP)

two-fusion fluorescence resonance energy transfer (FRET). Their experiments suggested that protein–protein interactions could be determined at particular subcellular locations of living eukaryotic cells. To determine whether FHL2 and FHL3 interact in living mammalian cell culture, GFP-FRET was employed. Colocalization of FHL3 and FHL2 was found in the mitochondria where FRET was observed under a FRET specific fluorescence microscope. Results gave supportive evidence on their interaction as demonstrated by the yeast two-hybrid assay. Mitochondrial biogenesis plays an important role during differentiation of C2C12. Proteins like citrate synthase, isocitrate dehydrogenase, and 3-hydroxyacyl-CoA, showed linear, four- to sixfold increase in enzymatic activities during mitochondrial biogenesis [Moyes et al., 1997]. It is no doubt that the expressions of these proteins are highly regulated and many transcription factors are involved. Interaction of FHL2 and FHL3 in mitochondria may be important in mitochondrial biogenesis by transmitting signals from the extracellular matrix at the focal adhesion plaques to the mitochondria. However, it is uncertain which signal transduction pathway is involved. On the other hand, mitochondria are believed to be involved in the process of apoptosis. Therefore, the overexpression of these two LIM-only proteins, which results in their interaction in the mitochondria, may help regulate the protein expression necessary for apoptosis. Since FHL3 is down regulated during myogenesis, we hypothesize that the decreased level of FHL3, can regulate cells to escape from the apoptosis pathway while cells are put under serum starvation at the beginning of myogenesis. However, cells that cannot enter myogenesis pathway, will undergo apoptosis [Bialik et al., 1999] by overexpressing some regulatory proteins.

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