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# Interaction of hCLIM1, An Enigma Family Protein, With α-Actinin 2

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**Abstract** Enigma proteins are proteins that possess a PDZ domain at the amino terminal and one to three LIM domains at the carboxyl terminal. They are cytoplasmic proteins that are involved with the cytoskeleton and signal transduction pathway. By virtue of the two protein interacting domains, they are capable of protein-protein interactions. Here we report a study on a human Enigma protein hCLIM1, in particular. Our study describes the interaction of the human 36kDa carboxyl terminal LIM domain protein (hCLIM1), the human homologue of CLP36 in rat, with α-actinin 2, the skeletal muscle isoform of α-actinin. hCLIM1 protein was shown to interact with α-actinin 2 by yeast two-hybrid screening and immunochemical analyses. Yeast two-hybrid analyses also demonstrated that the LIM domain of hCLIM1 binds to the EF-hand region of α-actinin 2, defining a new mode of LIM domain interactions. Immunofluorescent study demonstrates that hCLIM1 colocalizes with α-actinin at the Z-disks in human myocardium. Taken together, our experimental results suggest that hCLIM1is a novel cytoskeletal protein and may act as an adapter that brings other proteins to the cytoskeleton. J. Cell. Biochem. 78:558–565, 2000. © 2000 Wiley-Liss, Inc.

**Key words:** LIM domain; PDZ domain; α-actinin 2; yeast two-hybrid screening

Members of the carboxyl terminal LIM domain proteins have LIM domains clustered at the carboxyl terminal [Dawid et al., 1998]. LIM domains are defined by the cysteine-rich consensus sequence ($CX_2CX_{17-19}HX_2CX_2CX_2CX_{16-20}CX_2C/D/H$) forming two closely associated zinc fingers [Dawid et al., 1995, 1998; Sanchez-Garcia and Rabbitts, 1994; Gill, 1995; Taira et al., 1995]. Studies have indicated that LIM domains are capable of protein-protein interactions and are found in a variety of proteins with different cellular functions [Dawid et al., 1998; Sanchez-Garcia and Rabbitts, 1994; Taira et al., 1995]. Because of the protein interacting capabilities, LIM domains are therefore thought to be important for targeting proteins to specific subcellular locations and for mediating the assembly of multiprotein complexes [Dawid et al., 1998]. Carboxyl terminal LIM domain proteins have been shown to be primarily cytoplasmic and are associated with the cytoskeleton [Dawid et al., 1998]. Some of the carboxyl terminal LIM domain proteins are shown to be involved in protein trafficking [Kuroda et al., 1996; Wu et al., 1996].

Enigma proteins are an emerging subclass of carboxyl terminal LIM proteins first identified by Xia et al. [1997]. Enigma proteins are proteins that possess a PDZ domain at the amino terminal and one to three LIM domains at the

Abbreviations used: ALP, actinin-associated LIM protein; CLP36, carboxyl terminal LIM domain protein of 36 kDa; E, embryonic day; ENH, Enigma homologue; GFP, green fluorescent protein; hCLIM1, human 36 kDa carboxyl terminal LIM domain protein; mCLIM1, mouse 36 kDa carboxyl terminal LIM domain protein; ORF, open reading frame; PBT, phosphate buffered saline with 0.1% Tween-20; PVDF, polyvinyldifluoride; TBS, Tris-buffered saline; TBST, 0.1% Tween 20 in TBS; ZASP, Z-band alternatively spliced PDZ-motif protein.

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carboxyl terminal. The PDZ domains of the class members are identified by the replacement of G-L in the G-L-G-F signature sequence of the PDZ domain by P/S-W [Guy et al., 1999]. This family of proteins can further be divided into two groups by the number of LIM domains the members possess [Guy et al., 1999]: hCLIM1, CLP36 [Wang et al., 1995], RIL [Kiess et al., 1995], and actinin-associated LIM protein (ALP) [Xia et al., 1997] each has one LIM domain; while Enigma [Gill and Wu, 1994; Guy et al., 1999], Enigma homologue (ENH) [Kuroda et al., 1996] together with the recently identified proteins Cypher [Zhou et al., 1999] and Z-band alternatively spliced PDZ-motif protein (ZASP) [Faulkner et al., 1999] have three LIM domains.

hCLIM1 was the human homologue of CLP36 and it is most abundantly expressed in heart and skeletal muscles [Kotaka et al., 1999]. CLP36 was shown to be down-regulated in hepatocytes when subjected to hypoxia [Wang et al., 1995]. RIL expression was shown to be down-regulated in H-ras transformed cells [Kiess et al., 1995], the LIM domain of RIL was capable of binding to its own PDZ domain and to the PDZ domain of the protein tyrosine phosphatase PTP-BL [Cuppen et al., 1998]. ALP binds to the Z-disk protein α-actinin 2 through its PDZ domain and colocalizes with α-actinin 2 at the Z-disks of skeletal muscle [Xia et al., 1997]. LIM2 and LIM3 of Enigma recognize the tyrosine kinase Ret and insulin receptor, respectively [Wu et al., 1996], and the PDZ domain of Enigma was found to interact with β-tropomyosin [Guy et al., 1999]. All of the LIM domains of ENH bind to Protein Kinase C β-1 [Kuroda et al., 1996]. The PDZ domain of ZASP interacts with α-actinin 2 and the two proteins colocalize at the Z-disks of heart and skeletal muscle [Faulkner et al., 1999]. Cypher interacts with α-actinin 2 and Protein Kinase C. The interaction with α-actinin 2 was mediated through the PDZ domain of Cypher, while the interaction with Protein Kinase C is mediated via the LIM domains [Zhou et al., 1999].

Though we previously reported that hCLIM1 is most abundantly expressed in heart and skeletal muscle [Kotaka et al., 1999], little is known about the function of hCLIM1 and its rat homologue, CLP36, except that the expression of CLP36 is down-regulated when cells are subjected to hypoxia [Wang et al., 1995]. We therefore would like to determine the possible roles of hCLIM1 by identifying the protein partners of hCLIM1 with the use of the yeast two-hybrid system. Yeast two-hybrid analyses indicate that hCLIM1 interacts with α-actinin 2, the muscle isoform of α-actinin. Immunochemical studies indicate that the two proteins occur as a protein complex in vivo in rat heart tissues. Immunofluorescent staining indicates that hCLIM1 protein colocalizes with α-actinin at the Z-disks and also localizes at the intercalated disks in human myocardium.

## MATERIALS AND METHODS

### Yeast Two-Hybrid Screening

Nucleotides encoding the full length hCLIM1 protein were amplified by PCR with a pair of hCLIM1 specific primers (Forward: 5′TAG GGC GAA TTC ACC ACC CAG CAG ATA GAC CTC 3′; Reverse: 5′ TAG GGC GTC GAC CTG GAG AAC AGT GGT CAC ATC 3′) from hCLIM1-pBlueScript SK-plasmid [Kotaka et al., 1999]. An *Eco*RI site is present in the forward primer and a *Sal*I site in the reverse primer as shown underlined. After digestion with *Eco*RI and *Sal*I, the PCR product was subcloned into the GAL4 DNA'2dbinding domain plasmid, pAS2-1 (Clontech, Palo Alto, CA). The hCLIM1-pAS2-1 construct was cotransformed into yeast strain Y190 with a library of human skeletal muscle cDNAs cloned into a GAL4 activation-domain plasmid pGAD10 (Clontech). The cotransformant mixture was plated onto synthetic dextrose plates containing 25 mM 3-amino-1,2,4-triazole, lacking tryptophan, leucine, and histidine. After a 10-day incubation at 30°C, interaction was determined by β-galactosidase colorimetric filter assay. Positive clones were rescued and the yeast colonies retaining the library plasmid of interest that have lost the hCLIMl-pAS2-1 construct were counterselected by plating the positive clones onto synthetic dextrose plates lacking leucine, containing 10 mM cycloheximide. Interaction was further confirmed by mating the counterselected yeast Y190 containing the library plasmid of interest with the yeast Y187 transformed with the hCLIMI-pAS2-1 construct. The mated yeast were plated out on synthetic dextrose plates as for the cotransformants described above and interaction was confirmed as for the cotransformants. The in-

serts of positive colonies were identified by PCR and automated sequencing.

### Deletion Yeast Two-Hybrid Analyses

The nucleotides encoding the PDZ domain of hCLIM1 (a.a. 1-128) and the LIM domain of hCLIM1 (a.a. 129-329) were amplified by PCR with two pairs of specific primers (5′ TAG GGC GAA TTC ACC ACC CAG CAG ATA GAC CTC 3′ and 5′ TAG GGC GTC GAC GGT AAA GGG CAT GGC ACT TC 3′; 5′ TAG GGC GAA TTC GCC TCG CCT GCC TCC AGC AC 3′ and 5′ TAG GGC GTC GAC CTG GAG AAC AGT GGT CAC ATC 3′, respectively) and subcloned into the GAL4 DNA-binding domain plasmid, pAS2-1 (Clontech) as described above. The constructs namely, PDZ-pAS2-1 and LIM-pAS2-1, were transformed into yeast strain Y187.

The nucleotides encoding the fourth spectrin-like repeat and the carboxyl terminal EF hand region (a.a. 607-894) and the EF hand region alone (a.a. 720-894) of α-actinin 2 were amplified by PCR with two pairs of specific primers (5′ TAG GGC GAA TTC CTC CGG ACC AAG TGG GAC AAG GTG 3′ and 5′ TAG GGC GTC GAC AAG CTC AGC ATC ACA GAT CGC 3′; 5′ TAG GGC GAA TTC ATT CGT GTT GGA TGG GAG CTG CTG 3′ and 5′ TAG GGC GTC GAC AAG CTC AGC ATC ACA GAT CGC 3′, respectively). The PCR products were subcloned into the GAL4 activation domain plasmid, pGAD424 (Clontech) as described above. The constructs, namely SE-pGAD424 and EF-pGAD424, were then transformed into yeast strain Y190. Interaction was determined by mating the transformed Y187 with transformed Y190 as described above.

### Antibody Production, Immunoprecipitation, and Immunoblotting

For antibody production, the recombinant 6 × His-tagged hCLIM1 fusion protein expressed in *Escherichia coli* [Kotaka et al., 1999] was used as the immunogen. Rabbits were immunized with the purified 6 × His-tagged hCLIM1 fusion protein emulsified with equal volume of Freund's complete adjuvant (Sigma, St. Louis, MO) for the first injection, and Freund's incomplete adjuvant (Sigma) for the subsequent booster injections. hCLIM1 antiserum was collected and applied to Hi-Trap Protein G column (Pharmacia Biotech, Uppsala, Sweden) for purification. Monoclonal antibody to α-actinin, EA-53, (Sigma) was also used.

The immunoprecipitation kit (Protein A; Boehringer Mannheim, Germany) was used for immunocoprecipitation with slight modifications. Briefly, rat heart tissues was homogenized in 5 ml lysis buffer containing 50 mM Tris-HCl, pH 7.5; 150 mM NaCl; and Complete protease inhibitor (Boehringer Mannheim). Supernatant of the homogenized suspension was precleared with 50 μl of Protein A Agarose. Polyclonal hCLIM1 antibodies were added to the precleared supernatant, and samples were incubated at 4°C for 1 h. Protein A Agarose was then used to precipitate the antibodies. Protein A pellets were washed once with buffer containing 20 mM NaCl. Immunoprecipitated proteins were denatured with loading buffer and resolved by SDS-PAGE. For immunoblotting, protein extracts resolved by SDS-PAGE were transferred to polyvinyldifluoride (PVDF) membranes. Membranes were incubated overnight at 4°C with either rabbit anti-hCLIM1 antibody or mouse anti-α-actinin antibody in TBST (0.1% Tween 20 in TBS) containing 5% skim milk. The membranes were then incubated with either goat anti-rabbit antiserum conjugated with horseradish peroxidase (Promega, Madison, WI) or goat anti-mouse antiserum conjugated with horseradish peroxidase (Dako, Denmark) in TBST. The labeled bands were visualized with Enhanced Chemiluminescence (Amersham, Arlington Heights, IL).

### Immunohistochemistry

Human myocardial tissues were obtained from three hearts with normal left ventricles unsuitable for heart transplantation. This study was approved by the Ethics Committee in Germany and clinical data have been published previously [Hein et al., 1994]. Tissue samples were mounted with Tissue Tek (OCT Compound, Miles, Inc., Elkhart, IN), flash frozen in liquid nitrogen and stored at −80°C until further use. Cryosections of 10 μm were prepared, air dried and fixed in methanol-acetone mixture (1:1) for 10 min at −20°C. The preparations were sequentially exposed to 0.1% carboxylated bovine serum albumin (Aurion, The Netherlands) and 100 mM glycine in phosphate-buffered saline (PBS) for 10 min in each step.

The cryosections were incubated sequentially for 1 h each step with 1) primary polyclonal anti-hCLIM1 diluted 1:5 in PBS, 2) FITC conjugated to donkey anti-rabbit IgG (Dianova, Hamburg, Germany) in 1:100 dilution, 3) primary monoclonal antibody against

α-actinin, EA-53 (Sigma) (1:200) or vinculin, hVIN-1 (Sigma; 1:40), 4) TRITC conjugated to goat anti-mouse IgG (Chemicon International) diluted 1:100. All incubations were done at room temperature in a moist chamber. Repeated washes with PBS were done after each step of the immunolabeling procedure. The preparations were mounted in Mowiol (Hoechst, Frankfurt am Main, Germany). The following controls were used in the double-labeling procedure as described previously [Kostin et al., 1998a]: 1) omission of both primary antibodies, 2) comparison with single labeling patterns obtained with each antibody, 3) alternating the detection system in single-labeling experiments (e.g., using mouse monoclonal followed by anti-rabbit secondary antibodies), and 4) reversing the order of primary antibodies.

The sections were examined by laser scanning confocal microscopy LEICA TCS 4D, equipped with an argon/krypton mixed gas laser, which allows an improved signal separation of FITC from TRITC fluorescence. Series of confocal optical sections (from five to ten) were taken through the depth of tissue samples at 0.51 mm intervals by using either a Leica Neofluar ×40/1.0 or Leica Planapo ×63/1.4 objective lens. Each recorded image was taken using dual-channel scanning and consisted of 512 × 512 pixels. After data acquisition the images were transferred to a Silicon Graphics Indy workstation (Silicon Graphics) for restoration and three-dimensional reconstruction using "Imaris?" the multichannel image processing software (Bitplane, Zurich, Switzerland).

## RESULTS

### Interaction of the LIM Domain of hCLIM1 With α-Actinin 2

In this study, we used the yeast two-hybrid system to identify the potential protein partners for hCLIM1. We screened $2 \times 10^6$ clones from a human adult skeletal muscle cDNA library (Clontech) and obtained 142 putative positive clones. Only 18 putative positive AD/library plasmids were further characterized and confirmed to be true positives. These clones were sequenced and 17 of them were found to encode different fragments of human α-actinin 2 [Beggs et al., 1992], extending to the end of the coding region, with a correct ORF (Fig. 1).

By using the yeast two-hybrid assay, we were also able to identify the domains of hCLIM1 and α-actinin 2 responsible for mediating the interaction. The interaction between these two proteins is abolished when the LIM domain of hCLIM1was deleted. However, the interaction retained when the LIM domain of hCLIM1 alone was present. Thus, the LIM domain of hCLIM1 was identified to interact with α-actinin 2 (Fig. 1). For the interaction of the LIM domain of hCLIM1 and different domains of α-actinin 2, the interaction between these two proteins was detected even when only the carboxyl terminal EF hand region of α-actinin 2 (a.a. 720–894) is present. Therefore, the carboxyl terminal EF hand region of α-actinin 2 alone was identified to be sufficient to mediate the interaction with hCLIM1 (Fig. 1).

### Colocalization of hCLIM1 and α-Actinin 2 at the Z-disks of the Human Myocardium

To determine whether the interaction with α-actinin 2 could localize hCLIM1 to the cytoskeleton, we produced an affinity-purified polyclonal antibody. We evaluated the specificity of the antibody by Western blot analysis of crude tissue extracts. As expected, the antibody recognized a prominent band of 36 kDa in the heart (Fig. 2A). The antibody was subsequently used to perform immunoprecipitation studies, so as to determine whether hCLIM1 and α-actinin 2 occur together in a protein complex in cardiac muscles. We found that the anti-hCLIM1 antibody coimmunoprecipitated α-actinin from the tissue extracts from cardiac muscles (Fig. 2).

Colocalization of hCLIM1 and α-actinin 2 was examined by performing double immunostaining on the longitudinal sections of the human myocardium. Confocal examination of longitudinal cryosections from human myocardium showed that specific labeling of hCLIM1 appeared in a clear cross-striated sarcomeric pattern. In order to determine precisely the localization of hCLIM1, we used the immunolabeling for specific sarcomeric proteins, known to be present in well-defined regions of the sarcomere [Kostin et al., 1998b]. Double immunolabeling for α-actinin, a typical marker of Z-disk, with hCLIM1 showed a precise colocalization of both proteins at the Z-disks (Fig. 3A–C). In addition to the sarcomeric Z-disk distribution, immunofluorescent staining for hCLIM1 was particularly conspicuous at the intercalated disks. Double labeling for vinculin, which is one of the components of cell-cell adhe-

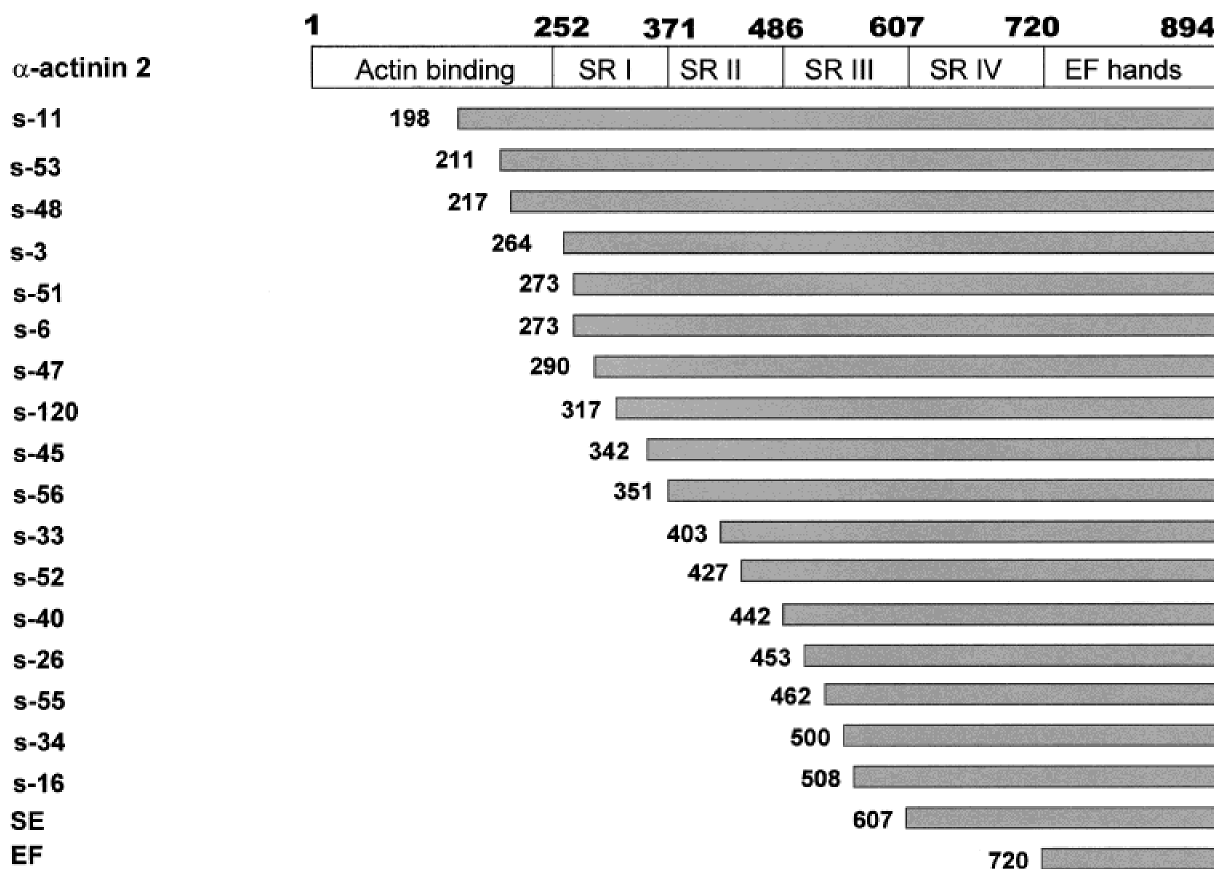


**Fig. 1.** Schematic representation of the α-actinin 2 fragments found to interact with hCLIM1 using the yeast two-hybrid system. The interactions were tested by the ability of diploid yeast cells to grow on SD $Trp^{-}Leu^{-}His^{-}$ agar plates containing 25 mM 3-amino-1,2,4-triazole, and the activation of the *lacZ* gene of the diploids in colony lift β-galactosidase filter assay. The domains of α-actinin 2 are shown at the top. The different fragments of α-actinin 2 that interacted with hCLIM1 and the LIM domain of hCLIM1 (a.a. 129-329) were displayed in the remaining part of the figure. The numbers at the side of the fragments indicate the starting amino acid of the fragments.

sion junction, and hCLIM1 showed both proteins were confined to transverse plicate regions of the intercalated disks, corresponding to the positions of the fascia adherens (Fig. 3D–F).

## DISCUSSION

The present study demonstrated the interaction between hCLIM1, a PDZ domain containing LIM domain protein, and α-actinin 2. We have previously shown that hCLIM1 is the human homologue of the rat LIM domain protein CLP36 and is most abundantly expressed in cardiac and skeletal muscles [Kotaka et al., 1999]. hCLIM1 belongs to the Enigma family of proteins that possess a PDZ domain at the amino terminal and one to three LIM domains at the carboxyl terminal. All family members studied were shown to localize at the actin filaments of various cell types [Xia et al., 1997; Cuppen et al., 1998; Zhou et al., 1999; Faulkner et al., 1999; Guy et al., 1999], and some were shown to interact with α-actinin 2. ALP, Cypher and ZASP were known to interact with α-actinin 2 via their PDZ domains to different regions of α-actinin 2 [Xia et al., 1997; Zhou et al., 1999; Faulkner et al., 1999]. ALP interacts with the third spectrin-like repeat of α-actinin 2 [Xia et al., 1997], while ZASP interacts with the carboxyl terminal region of α-actinin 2 [Faulkner et al., 1999]. Although hCLIM1 also interacts with the carboxyl terminal region of α-actinin 2, the interaction is mediated via the LIM domain of hCLIM1, contrary to the interaction of ALP, cypher 1, and ZASP which interact via their PDZ domains.

Though this is the first report on the interaction of LIM domain of the Enigma proteins with cytoskeletal proteins, interaction of LIM domains with cytoskeletal proteins, in particular α-actinin, were observed in other LIM do-

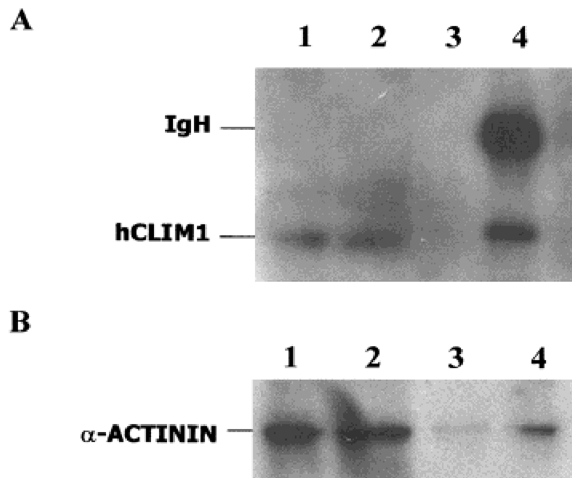


**Fig. 2.** Immunocoprecipitation of hCLIM1 and α-actinin. **A**: Immunoblot analysis of immunoprecipitation of hCLIM1 protein using anti-hCLIM1 antibody for detection. **Lane 1**: Total protein extract from rat heart; **Lane 2**: Total protein extract after preclearing with Protein A; **Lane 3**: Protein precipitated by Protein A alone; **Lane 4**: Protein precipitated with anti-hCLIM1 antibody. **B**: Immunoblot analysis of immunoprecipitation of α-actinin by anti-hCLIM1 antibody. α-actinin was detected using anti-α-actinin antibody. **Lane 1**: Total protein extract from rat heart; **Lane 2**: Total protein extract after preclearing with Protein A; **Lane 3**: Protein precipitated by Protein A alone; **Lane 4**: Protein precipitated with anti-hCLIM1 antibody.

main proteins. Members of the cysteine-rich protein (CRP) family are capable of interacting α-actinin [Louis et al., 1997]. Muscle LIM protein (MLP), a member of the CRP family was detected in the Z-disks of striated muscles. It was proposed to be involved in the early adaptation of cytoskeleton and myofibrillar structures to enhance contractility [Schneider et al., 1999]. Targeted disruption of MLP expression resulted in the disorganization of cardiomyocyte cytoarchitecture suggesting MLP plays an essential role for proper cardiomyocyte architectural organization [Arber et al., 1997].

α-actinin 2 is the skeletal muscle isoform of α-actinin and is found in both skeletal and cardiac muscles [Beggs et al., 1992]. α-actinin belongs to a superfamily of actin-binding and crosslinking proteins like spectrin and dystrophin [Hammonds, 1987]. Native α-actinin forms anti-parallel homodimers and they bundle F"2dactin into parallel arrays and anchor actin filaments at specific sites within the cell [Blanchard et al., 1989]. In skeletal, cardiac, and smooth muscles, α-actinin is localized at the Z-disks and analogous dense bodies where it is involved in constitutively anchoring the microfibrillar actin thin filaments [Endo and Masaki, 1984]. α-actinin has the capability to interact with different adhesion plaque and cytoskeletal proteins such as integrin, vinculin, and actin [Mimura and Asano, 1986; Pavalko et al., 199l, 1995].

The interaction of the LIM domain of hCLIM1 and α-actinin 2 caused the two proteins to colocalize at the Z-disks in the human myocardium, implying a biological significance of the interaction. Z-disks consist of sarcomeric α-actinin, the N-terminal of titin, the C-terminal of nebulin, and sarcomeric α-actin [Lin et al., 1998; Young et al., 1998]. Additional components such as PIP2 and several Enigma proteins are also present in the Z-disks [Xia et al., 1997; Zhou et al., 1999; Faulkner et al., 1999; Fukami et al., 1992]. The carboxyl terminal of α-actinin inhibits the distribution of α-actinin along the thin filaments of muscle, thereby maintaining a constant width of the periodic Z-disks [Lin et al., 1998]. Therefore, as hCLIM1 interacts with the carboxyl terminal region of α-actinin, it may play a role in maintaining the constant width of Z-disks in muscles.

In our study, hCLIM1 was also found to colocalize with vinculin at the intercalated disks of the human myocardium. The intercalated disks are the specialized portions of the plasma membrane that connect the cardiac muscle cells. Vinculin is localized in the fascia adherens part of the intercalated disks [Kostin et al., 1998b]. Vinculin is a protein that serves as an anchor for actin and actin-binding proteins in the cell membrane. The interaction between vinculin and α-actinin is thought to be important to the pathway linking adhesion receptors to filamentous actin [McGregor et al., 1994]. Colocalization of hCLIM1 and vinculin is believed to be mediated via the interactions of the two proteins to α-actinin.

As it has been proposed that Enigma proteins act as adapters that bring together different molecules in dynamic microenvironments at specialized submembranous or cytoskeletal structures [Cuppen et al., 1998] and that the general function of the Enigma proteins may be to target protein kinases to actin filaments [Guy et al., 1999], the localization of hCLIM1 to the Z-disks and the intercalated disks might cause PDZ-binding proteins to be recruited to these regions via interaction with hCLIM1. Further studies of the interaction of the PDZ domain of hCLIM1 will determine whether hCLIM1 act as an adapter to target signaling molecules to the Z-disks and intercalated disks.

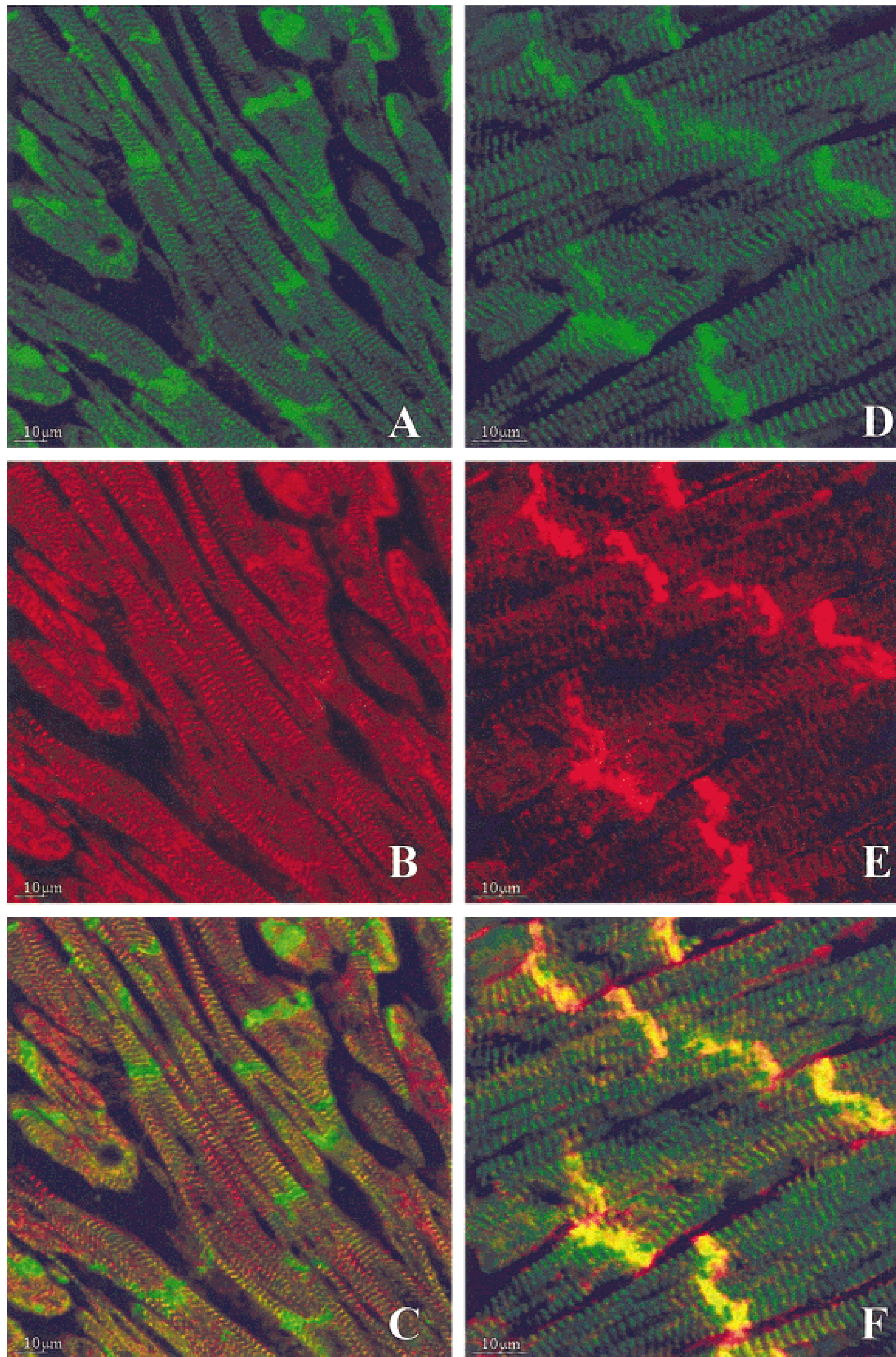


**Fig. 3.** Immunofluorescent confocal laser micrographs showing subcellular distribution of hCLIM1 in cardiac muscle. Longitudinal sections of human myocardium were dual-labeled with rabbit anti-hCLIM1 and mouse anti-α-actinin or anti-vinculin. The immunofluorescence of hCLIM1 (green, **A,C,D,F**) codistributes with α-actinin (red, **B**,C) at the Z-disks or with vinculin (red, **E**,F) at the intercalated disk. The labeling images of hCLIM1 are superimposed onto the same fields of α-actinin (C) or vinculin labeling (F), respectively.

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## REFERENCES

Arber S, Hunter JJ, Ross JJ, Hongo M, Sansig G, Borg J, Perriard JC, Chien KR, Caroni P. 1997. MLP-deficient mice exhibit a disruption of cardiac cytoarchitectural organization, dilated cardiomyopathy, and heart failure. Cell 88:393–403.

Beggs AH, Byers TJ, Knoll JH, Boyce FM, Bruns GA, Kunkel LM. 1992. Cloning and characterization of two human skeletal muscle alpha-actinin genes located on chromosomes 1 and 11. J Biol Chem 267:9281–9288.

Blanchard A, Ohanian V, Critchley D. 1989. The structure and function of alpha-actinin. J Muscle Res Cell Motil 10:280–289.

Cuppen E, Gerrits H, Pepers B, Wieringa B, Hendriks W. 1998. PDZ motifs in PTP-BL and RIL bind to internal protein segments in the LIM domain protein RIL. Mol Biol Cell 9:671–683.

Dawid IB, Breen JJ, Toyama R. 1998. LIM domains: Multiple roles as adapters and functional modifiers in protein interactions. Trends Genet 14:156–162.

Dawid IB, Toyama R, Taira M. 1995. LIM domain proteins. CR Acad Sci III 318:295–306.

Endo T, Masaki T. 1984. Differential expression and distribution of chicken skeletal- and smooth-muscle-type alpha-actinins during myogenesis in culture. J Cell Biol 99:2322–2332.

Faulkner G, Pallavicini A, Formentin E, Cornelli A, Ievolella C, Trevisan S, Bortoletto G, Scannapieco P, Salamon M, Mouly V, Valle G, Lanfranchi G. 1999. ZASP: A new Z-band alternatively spliced PDZ-motif protein. J Cell Biol 146:465–475.

Fukami K, Kuruhasi K, Inaki M, Endo T, Hatano S, Takenawa T. 1992. Requirement of phosphatidylinositol 4,5-bisphosphate for α-actinin function. Nature 359:150–152.

Gill GN. 1995. The enigma of LIM domains. Structure 3:1285–1289.

Gill GN, Wu RY. 1994. LIM domain recognition of a tyrosine-containing tight turn. J Biol Chem 269:25085–25090.

Guy PM, Kenny DA, Gill GN. 1999. The PDZ domain of the LIM protein Enigma binds to β-tropomyosin. Mol Biol Cell 10:1973–1984.

Hammonds RGJ. 1987. Protein sequence of DMD gene is related to actin-binding domain of alpha-actinin [letter]. Cell 51:1.

Hein S, Fujitani N, Rennollet H, Brand T, Friedl A, Schaper J. 1994. Altered expression of titin and contractile proteins in failing human myocardium. J Mol Cell Cardiol 26:1291–1306.

Kiess M, Scharm B, Aguzzi A, Hajnal A, Klemenz R, Schwarte-Waldhoff I, Schafer R. 1995. Expression of ril, a novel LIM domain gene, is down-regulated in Hras-transformed cells and restored in phenotypic revertants. Oncogene 10:61–68.

Kostin S, Scholz D, Shimada T, Maeno Y, Mollnau H, Hein S, Schaper J. 1998a. The internal and external protein scaffold of the T-tubular system in cardiomyocytes. Cell Tissue Res 294:449–460.

Kostin S, Helling A, Hein S, Scholz D, Klovekorn WP, Schaper J. 1998b. The protein composition of the normal and diseased cardiac myocyte. Heart Failure Rev 2:245–260.

Kotaka M, Garcia-Barcelo M, Ngai SM, Tsui SKW, Fung KP, Lee CY, Waye MMY. 1999. Characterization of human 36kDa carboxyl terminal LIM domain protein. J Cell Biochem 72:279–285.

Kuroda S, Tokunaga C, Kiyohara Y, Higuchi O, Konishi H, Mizuno K, Gill GN, Kikkawa U. 1996. Protein-protein interaction of zinc finger LIM domains with protein kinase C. J Biol Chem 271:31029–31032.

Lin Z, Hijikata T, Zhang Z, Choi J, Holtzer S, Sweeney HL, Holtzer H. 1998. Dispensability of the actin-binding site and spectrin repeats for targeting sarcomeric α-actinin into maturing Z bands in vivo: Implications for in vitro binding studies. Dev Biol 199:291–308.

Louis HA, Pino JD, Schmeichel KL, Pomies P, Beckerle MC. 1997. Comparison of three members of the cysteine-rich protein family reveals functional conservation and divergent patterns of gene expression. J Biol Chem 272: 27484–27491.

McGregor A, Blanchard AD, Rowe AJ, Critchley DR. 1994. Identification of the vinculin-binding site in the cytoskeletal protein alpha-actinin. Biochem J 301:225–233.

Mimura N, Asano A. 1986. Isolation and characterization of a conserved actin-binding domain from rat hepatic actinogelin, rat skeletal muscle, and chicken gizzard alpha-actinins. J Biol Chem 261:10680–10687.

Pavalko FM, Otey CA, Simon KO, Burridge K. 1991. Alpha-actinin: A direct link between actin and integrins. Biochem Soc Trans 19:1065–1069.

Pavalko FM, Schneider G, Burridge K, Lim SS. 1995. Immunodetection of alpha-actinin in focal adhesions is limited by antibody inaccessibility. Exp Cell Res 217:534–540.

Sanchez-Garcia I, Rabbitts TH. 1994. The LIM domain: A new structural motif found in zinc-finger-like proteins. Trends Genet 10:315–320.

Schneider AG, Sultan KR, Pette D. 1999. Muscle LIM protein: Expressed in slow muscle and induced in fast muscle by enhanced contractile activity. Am J Physiol 276:C900–C906

Taira M, Evrard JL, Steinmetz A, Dawid IB. 1995. Classification of LIM proteins [letter]. Trends Genet 11:431–432.

Wang H, Harrison-Shotak DC, Lemasters JJ, Herman B. 1995. Cloning of a rat cDNA encoding a novel LIM domain protein with high homology to rat RIL. Gene 165: 267–271.

Wu R, Durick K, Songyang Z, Cantley LC, Taylor SS, Gill GN. 1996. Specificity of LIM domain interactions with receptor tyrosine kinases. J Biol Chem 271:15934–15941.

Xia H, Winokur ST, Kuo WL, Altherr MR, Bredt DS. 1997. Actinin-associated LIM protein: Identification of a domain interaction between PDZ and spectrin-like repeat motifs. J Cell Biol 139:507–515.

Young P, Ferguson C, Banuelos S, Gautel M. 1998. Molecular structure of the sarcomeric Z-disk: Two types of titin interactions leads to an asymetrical sorting of α-actinin. EMBO J 17:1614–1624.

Zhou Q, Ruiz-Lozano P, Martone ME, Chen J. 1999. Cypher, a striated muscle-restricted PDZ and LIM domain-containing protein, binds to α-actinin 2 and protein kinase C. J Biol Chem 274:19807–19813.