Journal of Cellular Biochemistry 83:463–472 (2001)

Elfin is Expressed During Early Heart Development

Masayo Kotaka,[1] Yee-man Lau,[1] Kwok-kuen Cheung,[2] Simon M.Y. Lee,[1] Hoi-yeung Li,[1] Wood-yee Chan,[2] Kwok-pui Fung,[1] Cheuk-yu Lee,[1] Mary M.Y. Waye,[1] and Stephen K.W. Tsui[1]*

[1]Department of Biochemistry, The Chinese University of Hong Kong, Hong Kong
[2]Department of Anatomy, The Chinese University of Hong Kong, Hong Kong

Abstract Elfin (previously named CLIM1) is a protein that possesses an N-terminal PDZ domain and a C-terminal LIM domain. It belongs to the family of Enigma proteins. Enigma proteins are a family of cytoplasmic proteins that contain an N-terminal PDZ domain and a series of C-terminal LIM domains. By virtue of these two protein interacting domains, Enigma proteins are capable of protein–protein interactions. It has been proposed that Enigma proteins may act as adapters between kinases and the cytoskeleton. We have previously shown that Elfin is most abundantly expressed in the heart and it colocalizes with α-actinin 2 at the Z-disks of the myocardium. In this report, Elfin was shown to localize at the actin stress fibers of myoblasts, as revealed by green fluorescent protein (GFP) tagging. In situ hybridization and immunostaining showed that Elfin expression begins at an early stage in mouse development and is present throughout the developing heart. Taken together, our experimental results suggest that Elfin may play an important role in myofibrillogenesis and heart development. J. Cell. Biochem. 83: 463–472, 2001.

Key words: CLIM1; CLP-36; Enigma protein; PDZ domain; LIM domain; heart muscle; embryonic development

During a human heart cDNA sequencing project, we have previously identified several LIM domain proteins [Chan et al., 1998; Lee et al., 1998, 1999]. One of which is a human 36 kDa protein (CLIM1), containing a N-terminal PDZ domain and a C-terminal LIM domain. This Enigma protein has been renamed Elfin to avoid confusion of this protein with LIM-domain binding protein LDB2, also known as CLIM1 [Ueki et al., 1999]. Elfin is a protein most abundantly expressed in heart [Kotaka et al., 1999]. It is associated through its LIM domain to the C-terminal calcium insensitive EF hand region of α-actinin 2. The interaction caused Elfin to colocalize with α-actinin 2 at the Z-disks of the human myocardium [Kotaka et al., 2000]. CLP-36 [Wang et al., 1995], the rat homolog of Elfin, was also found to interact with the spectrin-like repeats of α-actinin 1 and 4 through its PDZ domain [Vallenius et al., 2000].

Elfin belongs to the family of Enigma proteins, also known as the PDZ-LIM protein family. Enigma proteins are an emerging subclass of LIM proteins first identified by Xia et al. [1997]]. Enigma proteins are proteins that possess one to three LIM domains at the carboxyl terminal and a PDZ domain at the amino terminal. The PDZ domains of the Enigma family members are identified by the replacement of G-L in the G-L-G-F signature

Abbreviations used: ALP, actinin-associated LIM protein; CLIM1, 36 kDa carboxyl terminal LIM domain protein; CLP-36, carboxyl terminal LIM domain protein of 36 kDa; E, embryonic day; ENH, Enigma homologue; EST, expressed sequence tag; GFP, green fluorescent protein; MLP, muscle LIM protein; ORF, open reading frame; PBS, phosphate buffered saline; PBT, phosphate buffered saline with 0.1% Tween-20; RIL, reverse-induced LIM protein; TBS, Tris-buffered saline; TBST, 0.1% Tween 20 in TBS; ZASP, Z-band alternatively spliced PDZ-motif protein.

Drs. M. Kotaka and S.M.Y. Lee were supported by the postdoctoral fellowships of the Chinese University of Hong Kong.

Grant sponsor: Research Grant Council, Hong Kong SAR; Grant numbers: CUHK 205/96M, CUHK 4305/99M; Grant sponsor: Industry Department, Hong Kong SAR; Grant numbers: AF/47/98, AF/9/97.

Masayo Kotaka's present address is Department of Biochemistry, University of Hong Kong, Li Shu Fan Building, Sassoon Road, Hong Kong.

Hoi-yeung Li's present address is Howard Hughes Medical Institute, Carnegie Institution of Washington, 113 West University Parkway, Baltimore, MD 21210.

*Correspondence to: Dr. Stephen K.W. Tsui, Department of Biochemistry, Mong Man Wai Building, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong.
E-mail: kwtsui@cuhk.edu.hk

Received 20 February 2001; Accepted 18 May 2001

DOI 10.1002/jcb.1244

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]: Elfin /CLP-36 [Wang et al., 1995; Kotaka et al., 1999], reverse-induced LIM protein (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 protein Cypher [Zhou et al., 1999], Oracle [Passier et al., 2000] and Z-band alternatively spliced PDZ-motif protein (ZASP) [Faulkner et al., 1999] have three LIM domains.

Though the physiological functions of the Enigma family members are not fully understood, it has been proposed that they may act as adapters between kinases and the cytoskeleton [Nakagawa et al., 2000]. 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]. The assembly of such protein complexes mediated by Enigma proteins is thought to play a role in the intramolecular signal transduction to cytoskeletal proteins [Cuppen et al., 1998].

In this report, we demonstrate the localization of Elfin to the actin stress fibers in mouse myoblasts. In addition, we also report the expression of Elfin in developing mouse embryo begins as early as E7.5 to E8. When the developing heart was closely examined, it was observed that expression of Elfin is detected throughout the developing heart.

MATERIALS AND METHODS

Sequence Analysis of Mouse Elfin

By nucleotide sequence homology search using the BLAST alogrithm, several mouse expressed sequence tags (ESTs) (GenBank Accession Numbers: AA592176, AA265401, AA146393, AA620177, AA870784, and AI326107) were found to share high homology with the cDNA sequence of human Elfin. The sequences of these clones were aligned by the software DNASIS to generate a putative full-length sequence of mouse Elfin cDNA. The open reading frame (ORF) of Elfin was amplified by PCR with a pair of specific primers designed from the 5′ and 3′ untranslated region of the putative sequence (Forward primer: 5′ TAG GCC GAA TTC GAT TGT GCC TAG CGA TGA CCA CC 3′; Reverse primer: 5′ TAG GCC GTC GAC GCG CAT GAC TAA CAC TAA GTA AGC 3′) from Marathon-Ready Mouse Heart cDNA library (Clontech, Palo Alto, CA). An *Eco*RI site is present in the forward primer and a *Sal*I site in the reverse primer as underlined. The PCR product was cloned into pBlueScript SK-plasmid. The complete sequence of the cDNA of mouse Elfin was determined by primer walking using dideoxy termination cycle sequencing with a combination of vector and insert-specific primers and ThermoSequenase cycle sequencing kit (Amersham Pharmacia Biotech, Ohio) and with the use of the A.L.F. automated DNA sequencer (Amersham Pharmacia Biotech). The amino acid sequence of the putative protein was predicted using the software DNASIS (Hitachi).

Tissue Distribution of Elfin mRNA in Mouse

A pair of gene-specific PCR primers from the coding region of mouse Elfin (Forward: 5′ CTG CAG GAG ATC CTG GAG TCA GAT GGG 3′; Reverse: 5′ GCG CAT GAC TAA CAC TAA GTA AGC 3′) were used to amplify Mouse Multiple Tissue cDNA Panel I (Clontech), which is a set of normalized first-strand cDNA generated using poly-$(A)^+$ RNA from various mouse tissues (quality control data sheet, Clontech).

PCR was performed using *Taq* polymerase (Amersham Pharmacia Biotech) with hot start and cycling conditions as follows: 95°C for 1 min, 38 cycles of 94°C (30 s), 53°C (1 min), and 72°C (1 min 30 s). Equal aliquots (5 μl) of PCR product were withdrawn from the reaction mixtures every four cycles from the 30^{th} cycle of amplification and analyzed on 1% agarose gel and

visualized under ultraviolet (UV) light by staining with ethidium bromide.

Subcloning, Expression, and Localization of Elfin in Cultured Mouse C2C12 Cells

The full-length Elfin cDNA was amplified by PCR with a pair of specific primers (5′ TAG GGC GAG CTC AAA CCA CCC AGC AGC ACA TAG ACC TC 3′ and 5′ TAG GGC GTC GAC CTG GAG AAC AGT GGT CAC ATC 3′) from human Elfin-pBlueScript SK-plasmid. A *Sac*I site is present in the 5′ primer and a *Sal*I site in the 3′ primer as underlined. After restriction digestion, the PCR product was subcloned into the green fluorescent protein (GFP) fusion expression plasmid, pEGFP-Cl (Clontech).

The Elfin-pEGFP-Cl construct was then transfected to C2C12 mouse myoblasts (ATCC no: CRL-1772) grown on coverslips at 60–70% confluence using Lipofectamine Plus reagent (Life Technologies, Gaithersberg, MD) according to the manufacturer's procedures. The cells were then fixed in 3.7% formaldehyde and stained with rhodamine phalloidin (Molecular Probes, Eugene, OR), according to the manufacturer's procedures. The coverslips were then mounted on a slide cell-side down with phosphate buffered saline (PBS)/glycerol mixture (1:1). The cells were viewed in a Zeiss Axioskop fluorescence microscope with the corresponding filters for GFP and rhodamine. Images were captured by a photometric cooled CCD camera and artificially colored using IpLab spectrum software.

In Situ Hybridization of Elfin to Mouse Embryos

The cDNA of mouse Elfin was used to synthesize the sense and antisense probes for in situ hybridization using DIG RNA labeling kit (Boehringer Mannheim, Mannheim, Germany), T7 and T3 polymerases (Boehringer Mannheim). Mouse embryos at E7.5 to E9.5 were fixed in 4% paraformaldehyde. The noon of the plug day was E0.5. The embryos were rehydrated in graded methanol/phosphate buffered saline with 0.1% Tween-20 (PBT) (75, 50, and 25%) for 5 min each, then bleached in 6% H_2O_2 in PBT, and treated with 10 μg/ml Proteinase K in PBT at room temperature. The embryos were then washed twice with fresh glycine in PBT (2 mg/ml) and twice with PBT. The embryos were then postfixed in 0.2% glutaraldehyde in 4% paraformaldehyde in PBT for 20 min at room temperature. The postfixed embryos were washed with PBT, and then prehybridized in hybridization buffer (50% formamide, 5 × SSC, 1% SDS, 50 μg/ml heparin, and 50 μg/ml yeast tRNA) for 2 h at 70°C. Hybridization was performed by incubating the embryos with 1 μg/ml RNA probe in fresh hybridization buffer at 70°C overnight.

After hybridization, the embryos were washed sequentially with solution I (50% formamide, 5× SSC, 1% SDS) at 70°C, solution I/solution II mixture (1:1) at 70°C and in solution II (0.5 M NaCl, 10 mM Tris-HCl, 0.1% Tween-20, pH 7.5) at room temperature. The embryos were then treated with 100 μg/ml RNase A in solution II at 37°C. After RNase treatment, the embryos were washed with solution II, then in solution III (50% formamide, 2 × SSC) at room temperature. The embryos were then washed twice in solution III at 65°C.

The embryos were washed with 2 mM levamisole (Sigma, St. Louis, MO.) in 0.1% Tween 20 in TBS (TBST) three times at room temperature for 5 min, then blocked with 10% heat inactivated sheep serum in TBST for 60 min at room temperature. After blocking, the embryos were incubated with anti-DIG antibody (Boehringer Mannheim), preadsorbed with embryo powder and 1% heat-inactivated sheep serum, in 2 mM levamisole in TBST at 4°C, overnight with rocking. After incubation, the embryos were washed with series of TBST, then with alkaline phosphatase buffer (100 mM NaCl, 100 mM Tris, 50 mM $MgCl_2$, 0.1% Tween-20, pH 9.5) with 2 mM levamisole. Color development was performed by incubating the embryos with 175 μg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Boehringer Mannheim) and 450 μg/ml nitroblue tetrazolium (NBT) (Boehringer Mannheim) in alkaline phosphatase buffer in the dark. Color reaction was terminated by the addition of 1 mM EDTA in PBT. The embryos were fixed in 4% paraformaldehyde in PBS at 4°C overnight. The embryos were washed with PBT and cleared sequentially with glycerol/PBT (1:1), 1 mM EDTA, then glycerol/PBT (4:1), 1 mM EDTA.

Immunostaining of Transverse Sections of Mouse Embryos With Polyclonal Anti-Elfin Antibody

The polyclonal anti-Elfin antibody was produced from rabbit as previously described [Kotaka et al., 2000]. Mouse embryos at E8.5, E10.5, and E12.5 were used. The noon of the plug day was E0.5. The wax mount tissue

sections were deparaffinized in xylene three times for 5 min each. The sections were then rehydrated with graded alcohol series (absolute, 95, 80, 75, and 70%) for 5 min each. They were rinsed in tap water for 5 min and then in PBS three times for 5 min each. The tissue sections were blocked with goat serum for 1 h at room temperature. Excess serum was drained off and the sections were probed with polyclonal anti-Elfin antibody (1:50) in 1% BSA in PBS and incubated at 4°C overnight. The sections were washed with PBS three times for 5 min each. Biotinylated goat anti-rabbit IgG antibodies (1:200) in 1% BSA in PBS (Vectastain ABC Kit, Standard) were added to the sections and the sections were incubated at room temperature for 1 h. After washing with PBS, the sections were incubated with peroxidase substrate (0.3% H_2O_2 in methanol) for 1 h to quench endogenous peroxidase activity. Vectastain ABC reagent was prepared by mixing equal volumes of reagent A and B in PBS (1:1:100). The ABC mixture was allowed to stand on ice for 30 min. After washing with PBS, the sections were incubated with ABC reagent for 30 min and then washed with TB buffer (50 mM Tris-HCl, pH 7.2). Six microliter of 30% H_2O_2 was added to 30 ml of 0.05% DAB (3′,3′-diaminobenzidine) in TB buffer and was immediately applied onto the sections for color development. The duration of incubation was dependent on the color intensity developed in the section. The sections were washed with TB buffer twice and tap water. Counterstaining was carried out by immersing the sections sequentially in hematoxylin, acid alcohol and then Scott's tap, with rinses with tap water between each solution. The sections were dehydrated with graded alcohol series (70, 80, and 95%, absolute) then in xylene for 5 min each. The sections were then mounted with Permount (BDH).

RESULTS AND DISCUSSION

Sequence Analysis of Mouse Elfin cDNA

Sequence determination of the cDNA revealed that it is composed of 1440 bp, encoding a 327 amino acid protein. The putative protein encoded by this cDNA was named Elfin. The nucleotide sequence data have been submitted to the GenBank/EMBL Data Libraries under the accession number AF053367. When the

```
mElfin          MTTQQIVLQGPGPWGFRLVG-KDFEQPLAISRVTPGSKAAIANLCIGDLITAIDGEDTSS 59
CLP36           MTTQQIVLQGPGPWGFRLVGGKDFEQPLAISRVTPGSKAAIANLCIGDLITAIDGEDTSS 60
hElfin          MTTQQIDLQGPGPWGFRLVGRKDFEQPLAISRVTPGSKAALANLCIGDVITAIDGENTSN 60
                ****** ************* ******************* ******* ******* **

mElfin          MTHLEAQNKIKGCADNMTLTVSRSEQKIWSPLVTEEGKRHPYKMNLASEPQEVLHIGSAH 119
CLP36           MTHLEAQNKIKGCVDNMTLTVSRSEQKIWSPLVTEEGKRHPYKMNLASEPQEVLHIGSAH 120
hElfin          MTHLEAQNRIKGCTDNLTLTVARSEHKVWSPLVTEEGKRHPYKMNLASEPQEVLHIGSAH 120
                ******** **** ** **** *** * ********************************

mElfin          NRSAMPFTASPAPST--RVITNQYNSPTGLYSSENISNFNNAVESKTSASGEEANSRPVV 177
CLP36           NRSAMPFTASPAPGT--RVITNQYNSPTGLYSSENISNFNNAVESKTSASGEEANSRPSA 178
hElfin          NRSAMPFTASPASSTTARVITNQYNNPAGLYSSENISNFNNALESKTAASGVEANSRPLD 180
                ************  *  ******** * ************** **** *** ******

mElfin          QPHPSGSLIIDKDSEVYKMLQEKQELNEPPKQSTSFLVLQEILESDGKGDPNKPSGFRSV 237
CLP36           QPHPSGGLIIDKESEVYKMLQEKQELNEPPKQSTSFLVLQEILESDGKGDPNKPSGFRSV 238
hElfin          HAQPPSSLVIDKESEVYKMLQEKQELNEPPKQSTSFLVLQEILESEEKGDPNKPSGFRSV 240
                   *   * *** ********************************  *************

mElfin          KAPVTKVAASVGNAQKLPICDKCGTGIVGVFVKLRDHHRHPECYVCTDCGINLKQKGHFF 297
CLP36           KAPVTKVAASVGNAQKLPICDKCGTGIVGVFVKLRDHHPHPECYVCTDCGINLKQKGHFF 298
hElfin          KAPVTKVAASIGNAQKLPMCDKCGTGIVGVFVKLRDRHRHPECYVCTDCGTNLKQKGHFF 300
                ********** ******* ***************** * *********** *********

mElfin          VEDQIYCEKHARERVTPPEGYDVVTVFRE 326
CLP36           VGDQIYCEKHARERVTPPEGYDVVTVFPK 327
hElfin          VEDQIYCEKHARERVTPPEGYEVVTVFPK 329
                * ******************* *****
```

Fig. 1. Comparison of the amino acid sequences of human mouse Elfin, and CLP36. Amino acids that are identical are shown in asterisk.

ORF of mouse Elfin was compared to that of CLP36 and human CLIM1, the nucleotide sequences showed 85% homology (data not shown), whilst the amino acid sequences showed 97 and 88% homology respectively (Fig. 1). When compared to RIL and ALP, the close relatives of Elfin, Elfin showed an overall homology of 44 and 54% to RIL and ALP respectively. The PDZ domain of Elfin showed 66 and 58% homology to the PDZ domains of RIL and ALP respectively, and the LIM domain of Elfin showed 61 and 69% homology to the LIM domain of RIL and ALP respectively (Fig. 2).

Tissue Distribution of Elfin mRNA in Mouse Tissues

Elfin mRNA expression level in various mouse tissues was determined by amplification of a 452 bp fragment using PCR primers designed from the coding region of Elfin from the mouse MTC cDNA panel I (Clontech). An aliquot of the PCR reaction was withdrawn from the reaction tubes at 30, 34, and 38 cycles of amplification and analyzed by gel electrophoresis (Fig. 3). In order to give an accurate comparison of the Elfin transcripts in different mouse tissues, the semiquantitative PCR was repeated twice. A 452 bp major PCR fragment corresponds to the expected size of Elfin transcript. The semiquantitative PCR result revealed that the expression pattern of Elfin in various mouse tissues is similar to that in human as previously described [Kotaka et al., 1999]. It is most abundantly expressed in heart, moderately expressed in lung, spleen, testis, and skeletal muscle. In mouse embryos, the expression of Elfin begins as early as E7 and continues throughout the development of the embryo until E17.

Subcellular Localization of Elfin in Myoblasts

The subcellular localization of Elfin in myoblasts was determined using the GFP fusion technique. The full-length cDNA of Elfin was

```
ALP             MPQTVILPGPAPWGFRLSGGIDFNQPLVITRITPGSKAAAANLCPGDVILAIDGFGTESM 60
RLI             MPHSVTLRGPSPWGFRLVGGRDFSAPLTISRVHAGSKAALAALCPGDLIQAINGESTELM 60
mElfin          MALTVDVAGPAPWGFRITGGRDFHTPIMVTKVAERGKAKDADLRPGDIIVAINGESAEGM 60
                *   *   ** *****  ** **  *          **  * * *** * ** *   * *

ALP             THADAQDRIKAAAHQLCLKIDRGETHLWSPQVSEDGKAHPFKINLESEPQDGNYFEHKHN 120
RLI             THLEAQNRIKGCHDHLTLSVSRPEGRSW-PSAPDDSKAQAHRIHIDPEIQDG-------- 111
mElfin          LHAEAQSKIRQSPSPLRLQLDRSQ-----ATSPGQNNGDSSLEVLATRFQGS-------- 107
                 *  **  *      * *   *                              *

ALP             IRPKPFVIPGRSSGCSTPSGIDCGSGRSTPSSVSTVSTICPGDLKVAAKLAPNIPLEMEL 180
RLI             ----------SPTTSRRPSGTGTGPEDGRPS------------LGSPYGQPPRFPVPHNG 149
mElfin          ------------VRTYTESQFSLRSSYSSPTS------------LSRGPAAPSHHHPLAA 143
                           *          *                    *

ALP             PGVKIVHAQFNTPMQLYSDDNIMETLQGQVSTALGETPLMNEPTASVPPESDVYRMLHDN 240
RLI             SSEATLPAQMST---------LHVSPPPSADPARG---LPRSRDCRVDLGSEVYRMLREP 197
mElfin          PSLERQPSAAGG-LGRAGDSAVLVLPPSPGPRSSRPSMDSEGGSLLLDEDSEVFKMLQEN 202
                                                          * *  **

ALP             RN-EPTQPRQSGSFRVLQGMVDDGSDD---RPAGTRSVRAPVTKVHGGSGGAQRMPLCDK 296
RLI             AEPVAAEPKQSGSFRYLQGMLEAGEGGDWPGPGGPRNLKPTASKLGAPLSGLQGLPECTR 257
mElfin          RE-GRAAPRQSSSFRLLQEALEAEERGGTPAFLPSSLSPQSSLPASRALATPPKLHTCEK 261
                       * ** *** **                                      *

ALP             CGSGIVGAVVKAR-DKYRHPECFVCADCNLNLKQKGYFFIEGELYCETHARARTKPPEGY 355
RLI             CGHGIVGTIVKAR-DKLYHPECFMCSDCGLNLKQRGYFFLDERLYCESHAKARVKPPEGY 316
mElfin          CSTSIANQAVRIQEGRYRHPGCYTCADCGLNLKMRGHFWVGDELYCEKHARQRYSAPATL 321
                *   *    *         ** *  * ** ****  * *     **** **  *   *

ALP             DTVTLYPKA----- 364
RLI             DVVAVYPNAKVELV 330
mElfin          SSRA---------- 325
```

Fig. 2. Comparison of the amino acid sequences of mouse Elfin, ALP and RIL. Amino acids that are identical are shown in asterisk.

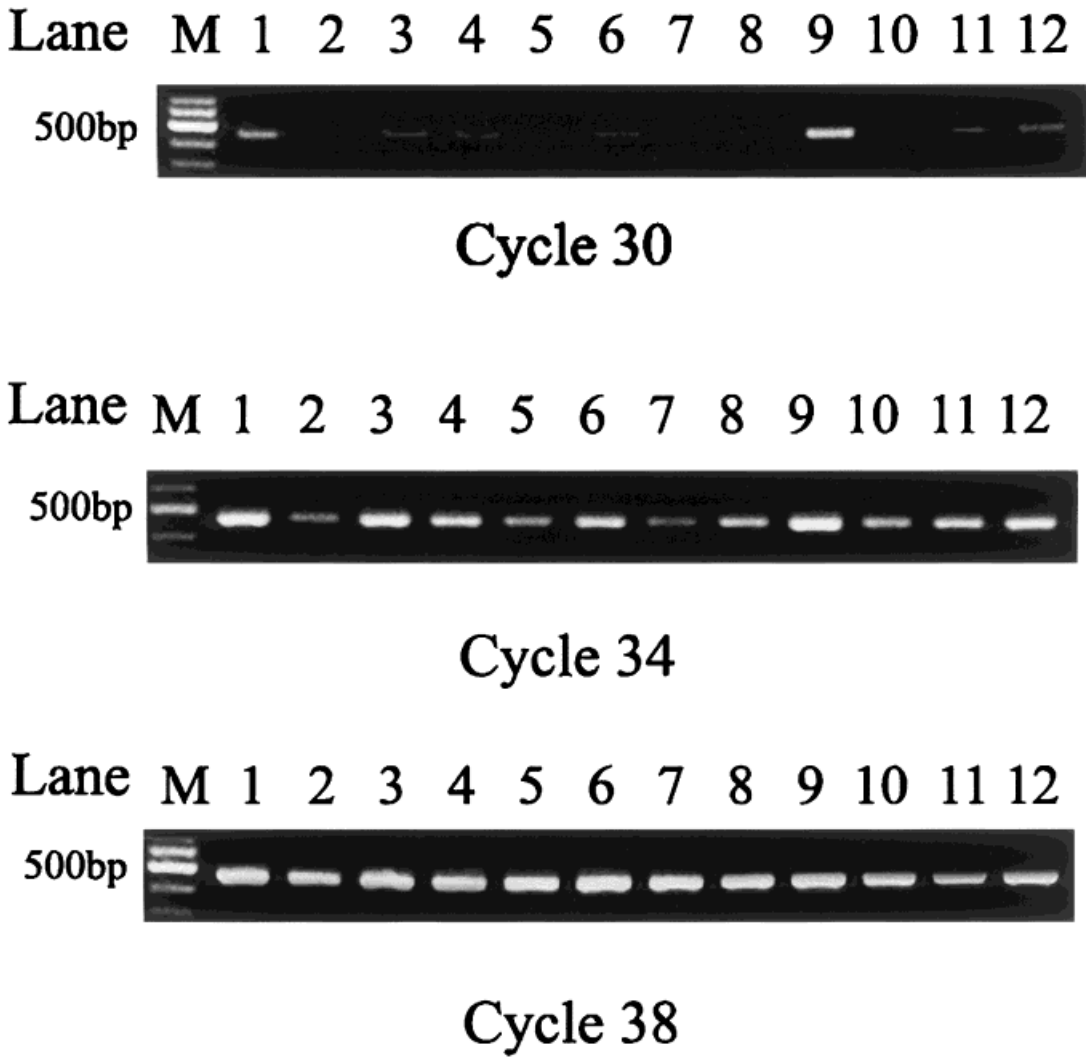

Fig. 3. Elfin mRNA expression in various mouse tissues. Key: **Lane 1**, Heart; **Lane 2**, Brain; **Lane 3**, Spleen; **Lane 4**, Lung; **Lane 5**, Liver; **Lane 6**, Skeletal Muscle; **Lane 7**, Kidney; **Lane 8**, Testis; **Lane 9**, Embryo at E7; **Lane 10**, Embryo at E11; **Lane 11**, Embryo at E15; **Lane 12**, Embryo at E17.

subcloned into the GFP expression plasmid and Elfin was expressed as a GFP fusion protein with the GFP tagged to the amino terminal of Elfin. After transfection to the mouse myoblast cell line C2C12, the Elfin-GFP fusion protein was transiently expressed. When viewed under the fluorescent microscope, the fusion proteins were found to colocalize with the phalloidin stained actin stress fibers (Fig. 4). α-actinin is also concentrated at the dense bodies of actin stress fibers in cell [Dabiri et al., 1997]. In our previous study, we have shown that Elfin interacts with α-actinin and it colocalizes at the Z-disks with α-actinin in the mature myofibrils of the myocardium [Kotaka et al., 2000]. During myofibrillogenesis, premyofibrils containing α-actinin-rich Z-bodies are formed de novo at the spreading edges of the cardiomyocytes and in time the premyofibrils would fuse and form mature myofibrils with α-actinin-rich Z-disks [Dabiri et al., 1997]. As members of the Enigma family protein such as Enigma [Wu et al., 1996], ENH [Kuroda et al., 1996], RIL [Cuppen et al., 1998], ALP [Xia et al., 1997], ZASP [Faulkner et al., 1999], and Cypher [Xhou et al., 1999], were shown to act as adapters that between kinases and the cytoskeleton, Elfin may act as an adapter that recruits other proteins to α-actinin during the process of myofibrillogenesis.

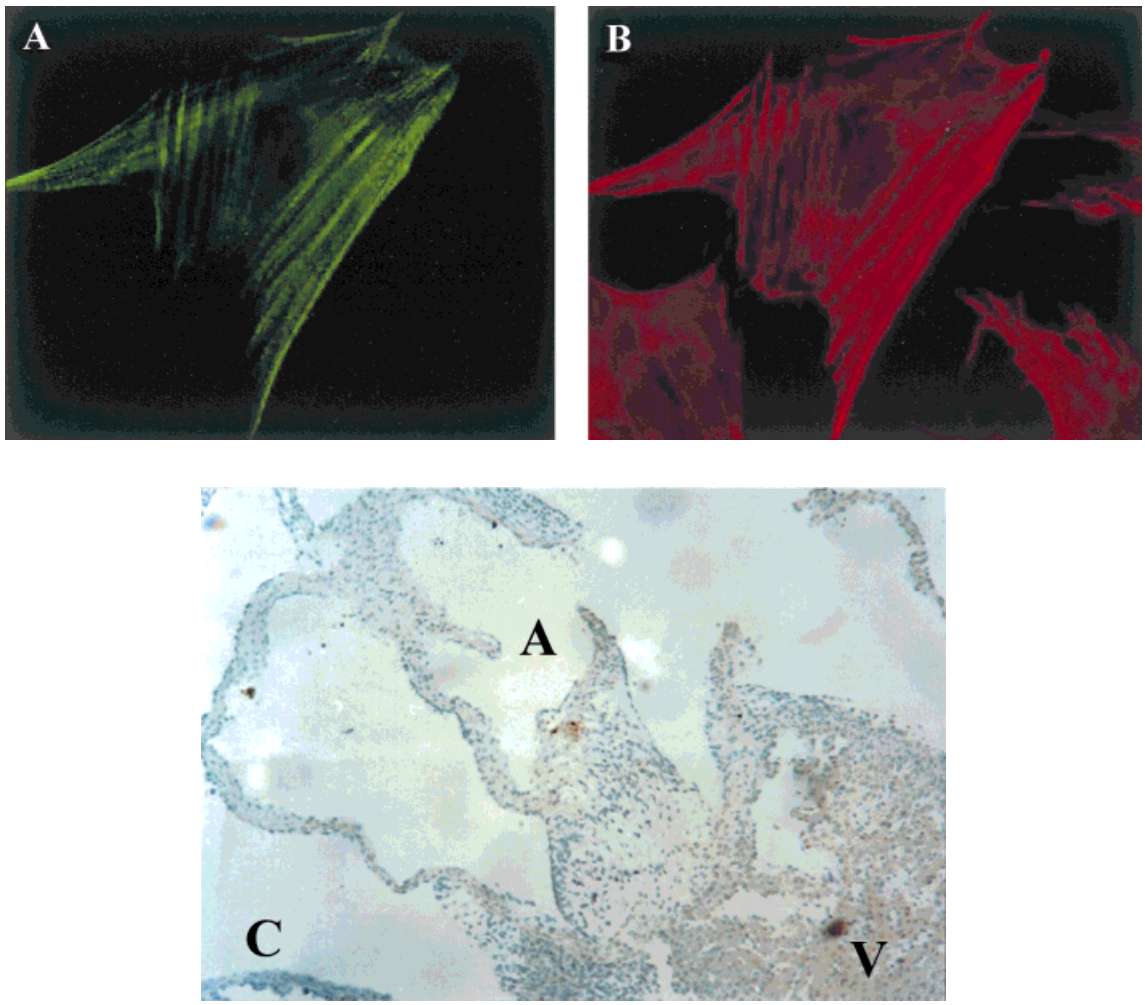

Fig. 4. Subcellular localization of Elfin in C2C12 myoblast cells. **Panel A**: Subcellular localization of Elfin as revealed by the green fluorescent signal emitted by the GFP tag. **Panel B**: Visualization of actin stress fibers by phalloidin staining.

Elfin Expression During Mouse Embryogenesis

To understand the potential function of Elfin in mouse embryogenesis, we have examined its expression pattern in whole mount mouse embryos. At E7.5–E8.0, the mouse is in the early head-fold, pre-somite stage. At this stage, the mesothelial lining of the ventral aspect of the intraembryonic coelom starts to thicken to form the future cardiogenic plate [Kaufman, 1992]. Whole mount in situ hybridization of Elfin mRNA at E7.5–E8.0 revealed that the expression of Elfin is detected when compared to the sense control (Fig. 5A), and this corresponds to the expression pattern seen from the semi-quantitative RT-PCR. At E8.5, a heart tube begins to form on the ventral side of the primitive thorax and starts to beat [Kaufman, 1992]. Our results indicated that Elfin expression was detected throughout the developing heart (Fig. 5B). The expression of Elfin remained high in the heart in E9.5 embryo (Fig. 5C). At this stage of development, expression of Elfin is also observed in other parts of the developing embryo, such as the limbs where skeletal muscles have began to develop. At E10.5–E11, the expression of Elfin in the heart maintained (Fig. 5D), while expression in other parts of the embryo is also observed (data not shown), which corresponds to the results of a previous study showing that CLP-36, the rat homolog of Elfin, is expressed in various parts of the embryo at E11 [Vallenius et al., 2000].

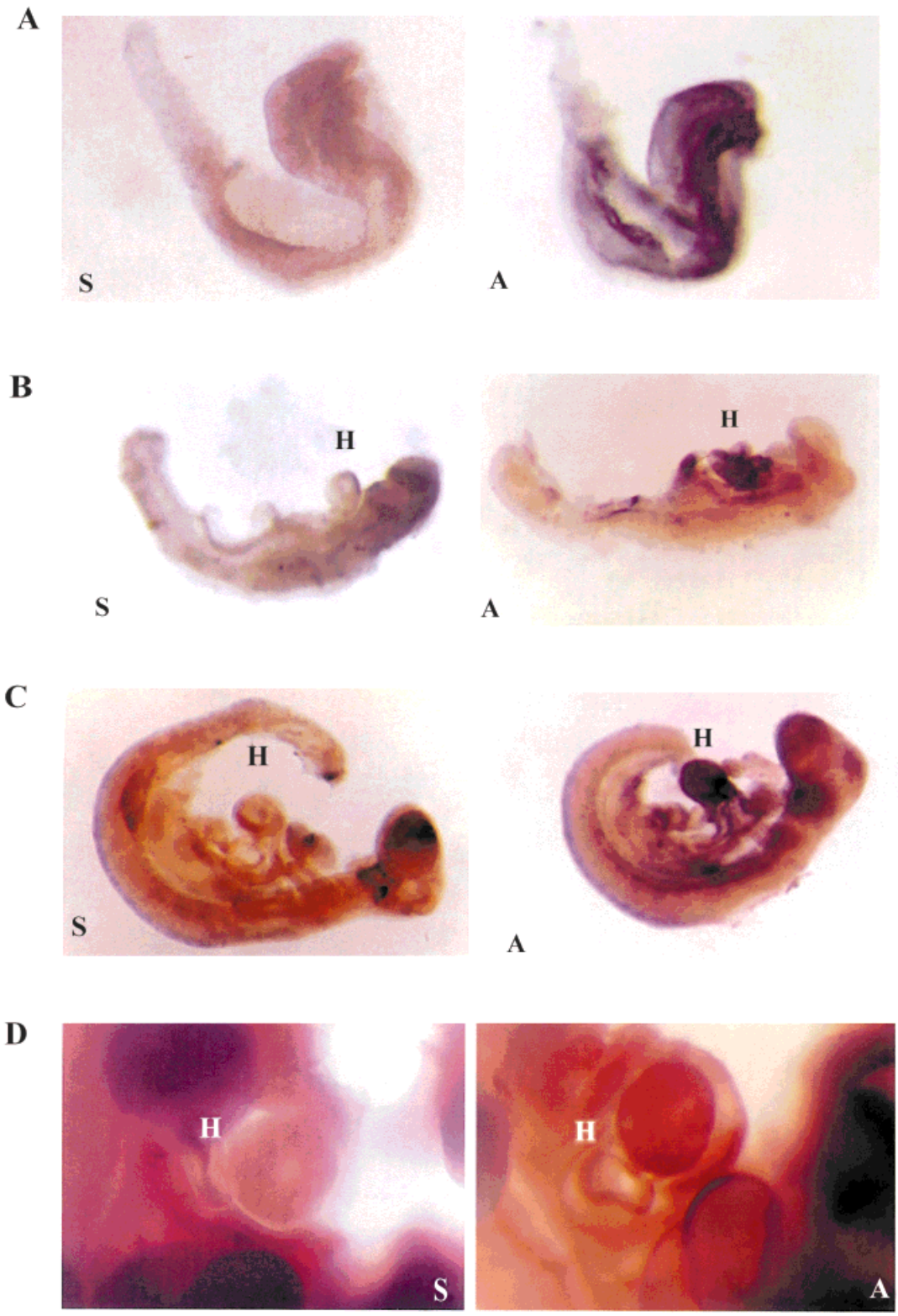

Fig. 5. Expression of Elfin mRNA in mouse embryos. **Panel A**: Whole mount in situ hybridization of Elfin at E7.5–E8. The sense control was shown on the left. **Panels B**, **C**, and **D**: Whole mount in situ hybridization of E8.5, E9.5, and E10.5 mouse embryos respectively, showing expression of Elfin in the developing heart (H).

Expression of Elfin in the Developing Heart

Our research group is particularly interested in the role of LIM proteins in cardiac development. Therefore, to have a better understanding of the potential function of Elfin in cardiovascular development and the localization of Elfin expression during cardiac development, immunostaining of Elfin in transverse sections through the cardiac region of mouse embryos of various stages was conducted. Our results indicated that Elfin expression occurs throughout the developing heart at E8.5 (Fig. 6A). At E10.5, the expression of Elfin remained high in the heart. Expression was high in the atria and developing ventricles (Fig. 6B). This expression pattern of Elfin was maintained in the heart at E12.5 and is localized in the myocardium (Fig. 6C). From our results, it is shown that embryonic expression of Elfin shares significant similarity to other Enigma proteins. The expression of ALP is heart and skeletal muscle specific in embryos and adult [Xia et al., 1997]. Oracle, Cypher, and ENH are detected at the early stage of cardiac development and are expressed in a myocardium specific manner [Zhou et al., 1999; Nakagawa et al., 2000; Passier et al., 2000]. Similarly, Elfin is expressed at the early stage of embryonic development and is expressed in the atrial and

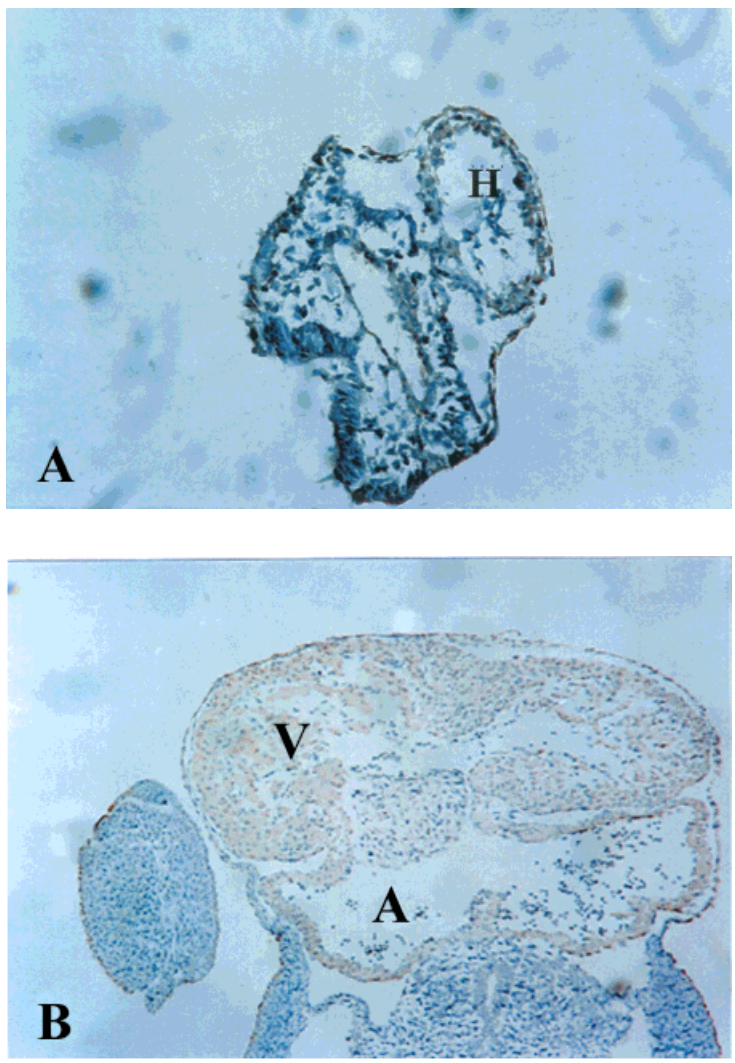

Fig. 6. Expression of Elfin protein in transverse sections of developing mouse heart. The expression of Elfin is visualized by the brown cells as compared to the blue cells that are stained by hematoxylin. **Panel A**: The expression of Elfin protein at E8.5. **Panels B** and **C**: Expression of Elfin at E10.5 and E12.5 resepectively. The developing ventricles were indicated by V and the atria were indicated by A.

ventricular myocardial cells. The expression of Elfin in the myocardium in the developing heart may be important for the maintenance of the cytoarchitecture of the developing heart. Another LIM domain protein, muscle LIM protein (MLP), was detected also at the Z disks of striated muscles and is found to be an essential regulator of myogenic differentiation [Arber et al., 1994]. Targeted disruption of MLP in mice resulted in the disorganization of cardiomyocyte cytoarchitecture [Arber et al., 1997]. From a previous study, it was shown that the expression level of Elfin remains unchanged in dilated cardiomyopathic hearts [Zimmermann et al., 1999], thus to determine the role of Elfin in heart development and diseased cardiomyocytes, it would be interesting to investigate the effect of the disruption of Elfin expression.

REFERENCES

Arber S, Halder G, Caroni P. 1994. Muscle LIM protein, a novel essential regulator of myogenesis, promotes myogenic differentiation. Cell 79:221–231.

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.

Chan KK, Tsui SKW, Lee SMY, Luk SCW, Liew CC, Fung KP, Waye MMY, Lee CY. 1998. Molecular cloning and characterization of FHL2, a novel LIM domain protein preferentially expressed in human heart. Gene 210:345–350.

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.

Dabiri GA, Turnacioglu KK, Sanger JM, Sanger JW. 1997. Myofibrillogenesis visualized in living embryonic cardiomyocytes. Proc Natl Acad Sci USA 94:9493–9498.

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.

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.

Kaufman MH. 1992. The atlas of mouse development. Cambridge: Academic Press.

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.

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

Kotaka M, Kostin S, Ngai SM, Chan KK, Lau YM, Lee SMY, Li HY, Ng EKO, Schaper J, Tsui SKW, Fung KP, Lee CY, Waye MMY. 2000. Interaction of hCLIM1, an Enigma family protein, with α-actinin 2. J Cell Biochem 78:558–565.

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.

Lee SMY, Tsui SKW, Chan KK, Garcia-Barcelo M, Waye MMY, Fung KP, Liew CC, Lee CY. 1998. Chromosomal mapping, tissue distribution and cDNA sequence of four-and-a-half LIM domain protein 1 (FHL1). Gene 216:163–170.

Lee SMY, Tsui SKW, Chan KK, Kotaka M, Li HY, Chim SSC, Waye MMY, Fung KP, Lee CY. 1999. Chromosomal mapping of a skeletal muscle specific LIM-only protein FHL3 to the distal end of the short arm of human chromosome 1. Som Cell Mol Genet 24:197–202.

Nakagawa N, Hoshijima M, Oyasu M, Saito N, Tanizawa K, Kuroda S. 2000. ENH, containing PDZ and LIM domains, heart/skeletal muscle-specific protein, associates with cytoskeletal proteins through the PDZ domain. Biochem Biophys Res Commun 272:505–512.

Passier R, Richardson JA, Olson EN. 2000. Oracle, a novel PDZ-LIM domain protein expressed in heart and skeletal muscle. Mech Dev 92:277–284.

Ueki N, Seki N, Yano K, Ohira M, Saito T, Masuho Y, Muramatsu M. 1999. Isolation of chromosomal assignment of human genes encoding cofactor of LIM homeodomain proteins, CLIM1 and CLIM2. J Hum Genet 44:112–115.

Vallenius T, Luukko K, Makela TP. 2000. CLP-36 PDZ-LIM protein associates with nonmuscle alpha-actinin-1 and alpha-actinin-4. J Biol Chem 275:11100–11105.

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.

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.

Zimmermann R, Kostin S, Kotaka M, Waye MMY, Tsui SKW, Lee SMY, Chan KK, Lee CY, Fung KP, Klsverkorn WP, Schaper J. 1999. A possible role for novel LIM domain proteins in dilated cardiomyopathy. Circulation (Suppl. 1) 100:565.