Subcellular targeting of metabolic enzymes to titin in heart muscle may be mediated by DRAL/FHL-2

Stephan Lange[1,‡], Daniel Auerbach[1,*,‡], Patricia McLoughlin[2], Evelyne Perriard[1], Beat W. Schäfer[2], Jean-Claude Perriard[1,§] and Elisabeth Ehler[1]

[1]Institute of Cell Biology, ETH Hönggerberg, 8093 Zurich, Switzerland
[2]Division of Clinical Chemistry and Biochemistry, Department of Pediatrics, University of Zurich, 8032 Zurich, Switzerland
*Present address: Dualsystems Biotech AG, Winterthurerstrasse 190, 8057 Zürich, Switzerland
‡These authors contributed equally to this work
§Author for correspondence (e-mail: jcp@cell.biol.ethz.ch)

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Summary

During sarcomere contraction skeletal and cardiac muscle cells consume large amounts of energy. To satisfy this demand, metabolic enzymes are associated with distinct regions of the sarcomeres in the I-band and in the M-band, where they help to maintain high local concentrations of ATP. To date, the mechanism by which metabolic enzymes are coupled to the sarcomere has not been elucidated. Here, we show that the four and a half LIM-only protein DRAL/FHL-2 mediates targeting of the metabolic enzymes creatine kinase, adenylate kinase and phosphofructokinase by interaction with the elastic filament protein titin in cardiomyocytes. Using yeast two-hybrid assays, colocalisation experiments, co-immunoprecipitation and protein pull-down assays, we show that DRAL/FHL-2 is bound to two distinct sites on titin. One binding site is situated in the N2B region, a cardiac-specific insertion in the I-band part of titin, and the other is located in the is2 region of M-band titin. We also show that DRAL/FHL-2 binds to the metabolic enzymes creatine kinase, adenylate kinase and phosphofructokinase and might target these enzymes to the N2B and is2 regions in titin. We propose that DRAL/FHL-2 acts as a specific adaptor protein to couple metabolic enzymes to sites of high energy consumption in the cardiac sarcomere.

Key words: LIM domain, Sarcomere, Cardiac cytoarchitecture,

Introduction

Members of the expanding LIM domain protein family are characterised by the possession of one or several LIM-domains, consisting of double zinc finger structures that are thought to mediate protein-protein interactions (Schmeichel and Beckerle, 1994; Bach, 2000) and have been implicated in many aspects of vertebrate development (Lumsden, 1995; Dawid et al., 1998; Hobert and Westphal, 2000). They are divided into two general classes, namely those containing LIM domains in combination with other functional domain types, such as homeodomains or kinase domains and into LIM-only proteins (for a review, see Bach, 2000).

In skeletal muscles and in the heart several LIM domain proteins have been discovered that participate in muscle differentiation and play important roles in linking the myofibrils to the surrounding cytoskeleton (Arber et al., 1994; Luo et al., 1997; Pomies et al., 1999; Zhou et al., 2001). Mice deficient in the muscle LIM protein MLP display severe alterations in intercalated disc structure and ultimately develop dilated cardiomyopathy (Arber et al., 1997; Ehler et al., 2001). Mice lacking the α-actinin associated LIM protein Alp show a specific cardiomyopathy of the right ventricle (Pashmforoush et al., 2001). Ablation of cypher, a striated muscle specific LIM-PDZ domain protein, leads to congenital myopathy and ventricular dilation in mice (Zhou et al., 2001). Elevated expression levels of N-RAP, an intercalated disc associated LIM-domain protein, serve as one of the earliest indicators for dilated cardiomyopathy (Ehler et al., 2001). These findings suggest that LIM domain proteins act as adaptors in striated muscle in general and the heart especially and help to maintain the cytoarchitecture during contraction. In their absence, decreased power output results, often followed by dilated cardiomyopathy (reviewed by Chien, 1999; Chien, 2000).

DRAL/FHL-2 is a member of the four and a half LIM domain protein subfamily which possesses an N-terminal half LIM domain followed by four complete LIM domains. Members of this family include FHL-1, DRAL also called FHL-2, FHL-3, FHL-4 and ACT (Morgan and Madgwick, 1996; Genini et al., 1997; Fimia et al., 1999; Morgan and Madgwick, 1999a). A characteristic property of all FHL proteins is their tissue-specific expression pattern (Chu et al., 2000a). Only FHL-1 is expressed in a broad range of tissues, ranging from skeletal and heart muscle to kidney, lung and brain (Fimia et al., 2000). DRAL/FHL-2 was discovered in a screen for cancer-related genes that are downregulated in a rhabdomyosarcoma cell line (hence the name DRAL, for downregulated rhabdomyosarcoma LIM protein) and was subsequently shown to be highly expressed in the heart (Genini et al., 1997; Chan et al., 1998; Chu et al., 2000a; Müller et al., 2000; Scholl et al., 2000; Li et al., 2001a). FHL-3 is strongly expressed in skeletal muscle, where it may serve similar functions as DRAL/FHL-2 in the heart (Fimia et al., 2000; Morgan and Madgwick, 1999b). FHL-4 and ACT are different from the other members of the family since they are not

expressed in either heart or skeletal muscles, but in the testis (Fimia et al., 2000).

To date, several binding partners of DRAL/FHL-2 have been identified. The N-terminal fragment of the Alzheimer's disease-associated protein presenilin-2 interacts with DRAL/FHL-2 via its hydrophilic loop region (Tanahashi and Tabira, 2000). The cytoplasmic domains of several α- and β-integrins also interact with DRAL/FHL-2, implicating DRAL/FHL-2 as an adaptor/docking protein involved in integrin signalling (Wixler et al., 2000). DRAL/FHL-2 can form homodimers as well as heterodimers together with its family member FHL-3 (Wixler et al., 2000; Li et al., 2001b). DRAL/FHL-2 has been identified as a tissue-specific coactivator of the androgen receptor (Müller et al., 2000), and also interacts with insulin-like growth factor-binding protein 5 (Amaar et al., 2002), the DNA-binding nuclear protein hNP220 (Ng et al., 2002) and the transcription factor CREB (Fimia et al., 1999; Fimia et al., 2000). CREB is induced in cardiac cells after β-adrenergic stimulation (Goldspink and Russell, 1996) and mice bearing a dominant negative isoform of CREB have been shown to develop dilated cardiomyopathy (Fentzke et al., 1998). DRAL/FHL-2 knockout experiments also lend support to a role for DRAL/FHL-2 as an adaptor protein involved in cardiac stress management and signalling. Mice lacking DRAL/FHL-2 develop normally but show a hypertrophic response following β-adrenergic stimulation, indicating an involvement of DRAL/FHL-2 in the remodelling mechanisms employed by cardiomyocytes in response to stress (Kong et al., 2001).

In cardiomyocytes, DRAL/FHL-2 is associated with sarcomeres where it is found in a cross-striated pattern (Scholl et al., 2000). Sarcomeres are the contractile units of muscle and are composed of highly regular, interdigitating arrays of thick and thin filaments. Two transverse structures, the M-band and the Z-disc, serve as primary anchor points for the thick and thin filaments, respectively. Sarcomere assembly and integrity are controlled by the elastic filament system composed of the giant protein titin. Titin molecules form elastic scaffolding structures spanning more than 1 μm from a Z-disc to the M-band (Obermann et al., 1996; Fürst et al., 1988; Trinick and Tskhovrebova, 1999). The distinct localisation of DRAL/FHL-2 at the M-band and, more prominently, around the Z-disc region in cardiomyocytes suggests that it may link other proteins to the sarcomere. To investigate a potential function of DRAL/FHL-2 as a sarcomeric adaptor molecule in the heart we searched for interaction partners that mediate the binding of DRAL/FHL-2 to the sarcomere. We have identified two different regions in the sarcomeric ruler molecule titin that act as binding sites for DRAL/FHL-2. In addition, we show an interaction of DRAL/FHL-2 with the metabolic enzymes creatine kinase, phosphofructokinase and adenylate kinase. We propose that DRAL/FHL-2 plays a crucial role in the recruitment of metabolic enzymes to sites of high energy consumption in the cardiac sarcomere.

Materials and Methods

Preparation of muscle extracts and immunoblotting

Freshly dissected muscle tissue was homogenised by freeze slamming and solubilised in a modified version of SDS-sample buffer (Ehler et al., 1999). Samples were run on 12.5% SDS polyacrylamide gel minislabs and blotted overnight onto nitrocellulose (Amersham). Detection of the antigen of interest was carried out by chemiluminescence as described previously (Ehler et al., 2001).

Whole mount staining of rat papillary muscle

Papillary muscle was dissected from adult rat hearts, incubated for 30 minutes in relaxation buffer (7 mM EGTA, 20 mM Imidazol, 1 mM $MgCl_2$, 14.5 mM creatine phosphate, 4 mM MgATP, 100 mM KCl, pH7.0, 0.1% saponin), tied onto plastic strips at slightly extended length and fixed in 4% PFA/PBS for 1 hour. Permeabilisation with 0.2% Triton X-100 was carried out for 30 minutes, subsequently the muscle strips were cut into smaller pieces and stained as a whole mount as described previously for embryonic heart whole mount preparations (Ehler et al., 1999).

Eukaryotic expression constructs

The eukaryotic expression vector pMCS-HA was constructed by removal of GFP in pEGFP (Clontech) with *Bam*HI and *Not*I and ligation of a linker encoding the HA epitope followed by a stop codon into the *Bam*HI and *Not*I sites. The constructs NF-DRAL/FHL-2 and DRAL/FHL-2-CF, encoding N- and C-terminally FLAG-tagged DRAL/FHL-2 in the pcDNA3.1 vector (Invitrogen, Basel, Switzerland) have been described (Scholl et al., 2000). The plasmid pGFP-DRAL/FHL-2 was constructed by excising DRAL/FHL-2 from DRAL/FHL-2-CF using *Bam*HI and *Xho*I and subcloning into the *Bgl*II and *Sal*I sites of pEGFP. The constructs GFP-is2 and MM-CK-HA were made by subcloning is2 and MM-CK from the original yeast two-hybrid library plasmids into pEGFP and pMCS-HA using *Eco*RI and *Xho*I sites. To create GFP-N2B, the sequence encoding amino acids 3455 to 4427 in the primary sequence of human cardiac titin (Labeit and Kolmerer, 1995) was amplified from embryonic human cardiac RNA with the Qiagen one step RT-PCR kit (Qiagen, Basel, Switzerland) using the primers n2bfw (5′gaagatctatggaaggcactggcc-caattttcatcaaagaa3′) and n2brv (5′ccgctcgagcactgtcacagttagtgtggctg-tacagct3′). Reverse transcription was carried out using a three step protocol: 50°C for 30 minutes, 60°C for 30 minutes and 95°C for 15 minutes. This was followed by the amplification step: 35 cycles of 30 seconds at 94°C, 45 seconds at 65°C and 3 minutes at 72°C and a final incubation for 10 minutes at 72°C. The PCR product was subcloned into the *Bgl*II and *Sal*I sites of pEGFP. To construct GFP-tagged adenylate kinase and phosphofructokinase, the entire open reading frame was amplified using the primers akfw (5′gaagatctaccatggaaga-gaagctgaagaaggcc3′), akrv (5′ccgctcgagcttcagggagtcaagataggtgca3′), pfkfw (5′gaagatctaccatgacccatgaagagcatcatgc3′) and pfkrv (5′ccgctc-gaggacggcggcttctccagaccg3′) and subcloned into the Bgl II and Sal I sites in pEGFP. To construct GFP-tagged Smpx/Csl, the entire open reading frame was amplified by PCR using the primers cslfw (5′gaa-gatctaccatgtcgaagcagccaatttccaat3′) and cslrv (5′ccgctcgagctgttcac-ctttggggacaaattt3′) and subcloned into the *Bgl*II and *Sal*I sites of pEGFP. GFP-N2B deletion mutants were constructed by removing the fragments indicated in Fig. 5 using internal restriction sites. All constructs derived by PCR were fully sequenced on both strands.

Yeast two-hybrid assays

The entire coding region of human DRAL/FHL-2 was amplified by PCR and cloned into the *Eco*RI and *Not*I sites of pGilda (Gyuris et al., 1993), Clontech, Palo Alto, CA). Yeast two-hybrid screening procedures and β-galactosidase filter assays were carried out as described by the manufacturer. The plasmid was transferred into the yeast strain EGY48 (Estojak et al., 1995), which was then transformed with an adult human cardiac cDNA library (Clontech). 1×10^7 primary transformants were screened, resulting in 200 clones that grew on selection plates lacking the amino acids histidine, tryptophan and leucine and were positive in a β-galactosidase filter assay. Library

plasmids from positive clones were isolated and sequenced. To confirm the interaction between DRAL/FHL-2 and either is2 or MM-CK, bait and library inserts were switched using the *Eco*RI and *Xho*I sites in the bait and prey vectors, cotransformed into EGY48 and assayed for growth on selective plates and β-galactosidase activity. To assay the interaction between DRAL/FHL-2 and N2B, the coding sequence of DRAL/FHL-2 was subcloned into the *Eco*RI and *Xho*I sites of the pLexA bait vector (Stenmark et al., 1995). The sequence encoding N2B was excised from GFP-N2B by cutting with *Xho*I, filling of 5′ overhangs using Klenow enzyme and cutting with *Bam*HI and cloned into the *Bgl*II and *Sma*I sites of the vector pACT2 (Clontech). The two plasmids were cotransformed into the yeast strain L40 (Vojtek et al., 1993) and assayed for growth on selection plates lacking the amino acids tryptophan, leucine and histidine as well as for β-galactosidase activity.

Immunofluorescent staining of NRC and sections

Primary cultures of neonatal rat cardiomyocytes were isolated and cultured as previously described (Auerbach et al., 1999). Cells were cultured in Maintenance medium (20% medium M199, 75% DBSS-K, 4% horse serum, 4 mM glutamine, 1% penicillin/streptomycin, 0.1 mM phenylephrine; DBSS-K: 6.8 g/l NaCl, 0.14 mM NaH_2PO_4, 0.2 mM $CaCl_2$, 0.2 mM $MgSO_4$, 1 mM dextrose, 2.7 mM $NaHCO_3$) and transient transfections were carried out after 1 day in culture using the reagent Escort III (Sigma) according to the manufacturer. 24-48 hours after transfection, cells were fixed in 4% paraformaldehyde/PBS for 10 minutes and stained with different antibodies as previously described (Auerbach et al., 1999).

Confocal microscopy

The specimen were analysed using confocal microscopy on an inverted microscope DM IRB/E equipped with a true confocal scanner TCS NT, a PL APO 63×/1.32 oil and a PL APO 100×/1.40 immersion objective (Leica) as well as an argon-krypton mixed gas laser. Image processing was done on a Silicon Graphics workstation using Imaris® (Bitplane AG), a 3D multichannel image processing software specialised for confocal microscopy data sets (Messerli et al., 1993).

Preparation of COS-1 total lysates

COS-1 cells (Gluzman, 1981) were cultured in maintenance medium consisting of DMEM, 10% fetal calf serum and 4 mM glutamine (Gibco, Basel, Switzerland). For transfection, the cells were grown to 80% confluency in 10 cm dishes, 10^7 cells were pelleted, washed twice with PBS and resuspended in 800 μl PBS. 10 μg of plasmid were added to a 4 mm gap cuvette (BTX, Axon Lab, Baden, Switzerland) on ice, mixed with 800 μl of cells by tapping and incubated for 5 minutes on ice. Cells were electroporated in a BTX Electro cell manipulator 600 (BTX) using the following conditions: capacitance setting 125 μF, voltage 300 V, resistance 72 Ω. After electroporation, cells were left on ice for 5 minutes, transferred to 10 cm dishes with maintenance medium and incubated for 48-72 hours. Lysates were prepared by washing cells twice with ice-cold PBS, adding 1 ml of lysis buffer (10 mM HEPES pH 7.9, 100 mM KCl, 5 mM $MgSO_4$, 50 μM $ZnSO_4$, 1.5% Triton X-100, 1 mM DTT, protease inhibitors) and incubation for 10 minutes on ice. Cells were scraped off the plate, transferred to eppendorf tubes and incubated for 10 minutes on ice. Lysates were sonicated in an ice/water bath (ELMA sonicator, Transsonic Digitals, Singen, Germany) at maximum setting for 10 minutes and centrifuged for 5 minutes at 5000 ***g***. The supernatants were transferred to fresh tubes and stored on ice.

Pull-down assays

A plasmid encoding GST-DRAL/FHL-2 fusion protein (Genini et al., 1997) was transformed into *E. coli* BL21 Star cells (Invitrogen). The cells were grown at 37°C in LB supplemented with 50 μM $ZnSO_4$ to an OD_{600} of 1.0, induced with 0.2 mM IPTG and grown for another 30 minutes at 37°C. Cells were pelleted and resuspended in 1/25 volume of lysis buffer (10 mM HEPES pH 7.9, 100 mM KCl, 50 μM $ZnSO_4$, 1% Triton X-100, 1 mM DTT) supplemented with 0.5 mg/ml lysozyme (Sigma), incubated for 30 minutes on ice and sonicated 5 times 10 seconds (Branson Sonifier 250, output setting 6, 60% duty cycle). Cellular debris were removed by centrifugation for 20 minutes at 15,000 ***g*** and GST-fusion protein was absorbed on beads by incubation with Glutathione-Sepharose 4B beads (Pharmacia, Uppsala, Sweden) for 30 minutes on ice. The beads were washed four times in ST buffer and stored on ice. For pull-down assays, defined amounts of bead-coupled GST-DRAL/FHL-2 or GST alone were washed once in IP buffer (10 mM HEPES pH 7.9, 100 mM KCl, 5 mM $MgSO_4$, 0.5% NP-40, 50 μM $ZnSO_4$, 1 mM DTT) supplemented with a protease inhibitor cocktail (Roche Diagnostics, Rotkreuz, Switzerland) and incubated for 3 hours on ice with precleared lysates from COS-1 cells expressing the respective binding partners or controls. Following incubation, the complexes were pelleted by centrifugation, washed four times in IP buffer, resuspended in SDS-PAGE sample buffer, boiled and loaded on 12.5% SDS-PAGE gels. The proteins were transferred to nitrocellulose membranes according to standard procedures and visualised by immunoblotting as described above.

Co-immunoprecipitation

COS cells were co-transfected with GFP-N2BΔ5 and DRAL/FHL-2-FLAG and lysed as described above. After preclearing by incubation with Protein G Plus/Protein A agarose (Oncogene) diluted in IP buffer (see above), the lysates were incubated with the monoclonal mouse anti-FLAG antibody at 4°C overnight. Then Protein G Plus/Protein A agarose was added and incubated for 3 hours at 4°C. Following several washes with IP buffer, the beads were processed for an SDS sample and immunoblotting with the Horse radish peroxidase-conjugated polyclonal rabbit anti-GFP antibody (Clontech) was carried out as described above.

Antibodies

Monoclonal mouse antibodies against α-cardiac actin were obtained from Progen (Heidelberg, Germany), monoclonal mouse antibodies against FLAG M2 were from Sigma (Buchs, Switzerland), monoclonal mouse anti-GFP (clones 7.1 and 13.1) and monoclonal rat anti-HA tag (clone 3F10) antibodies were from Roche Diagnostics (Rotkreuz, Switzerland) and polyclonal Horse radish peroxidase-conjugated rabbit anti-GFP antibodies were from Clontech. Polyclonal rabbit antibodies against DRAL/FHL-2 were produced in the lab (Scholl et al., 2000), polyclonal rabbit antibodies against the titin m8 epitope and monoclonal mouse antibodies against the titin N2B epitope (clone I19) were generously donated by Mathias Gautel (King's College London, UK). Polyclonal chicken antibodies against N2A were a kind gift from Carol Gregorio (University of Arizona, Tucson, AZ) and monoclonal mouse anti-titin T12 epitope antibodies were generously provided by Dieter Fürst (University of Potsdam, Germany). The monoclonal mouse anti-titin 9D10 epitope antibody was obtained from the Developmental Studies Hybridoma Bank, University of Iowa, USA.

As secondary antibodies FITC-conjugated anti-rabbit and anti-mouse Igs were employed (Cappel, via ICN Germany). Cy3-conjugated anti-mouse Igs, Cy3-conjugated anti-rat Igs (mouse Ig adsorbed), Cy5-conjugated anti-rabbit and anti-mouse Igs, Cy5-conjugated anti-mouse Igs (rat Ig absorbed) were all purchased from Jackson Immunochemicals via Milan (La Roche, Switzerland). FITC anti-mouse IgM was from Sigma, FITC anti-chicken IgY was from The Binding Site Ltd (University of Birmingham, UK). Horse radish

peroxidase-conjugated anti-rabbit Igs were from Calbiochem (Luzern, Switzerland), horse radish peroxidase-conjugated anti-mouse and anti-rat Igs were from DAKO (Zug, Switzerland).

Results

Expression of DRAL/FHL-2 in the heart is developmentally regulated

Previous reports have demonstrated the tissue-specific expression of DRAL/FHL-2 in the heart at the mRNA level (Chan et al., 1998; Scholl et al., 2000). In order to investigate the expression of DRAL/FHL-2 at the protein level we performed immunoblot analysis of fast and slow twitch skeletal muscle and cardiac muscle samples using a polyclonal antibody directed against DRAL/FHL-2. No signal was detected in fast or slow twitch skeletal muscles, but a strong signal around 34 kDa was apparent in mouse cardiac muscle, corresponding well to the calculated molecular weight of DRAL/FHL-2 (Fig. 1A). We also analysed the expression pattern of DRAL/FHL-2 in cardiac muscle at different time points during development (Fig. 1B). Very weak expression of DRAL/FHL-2 was first detected at embryonic stage E12.5. Expression of DRAL/FHL-2 increased up to postnatal stage P0 (Fig. 1B, top). The same immunoblot was reprobed with an antibody specific for α-cardiac actin to demonstrate that the increase in DRAL/FHL-2 expression levels is not due to differences in the number of differentiated cardiomyocytes in the extracts (Fig. 1B, bottom). Whereas α-cardiac actin expression levels remained unchanged, DRAL/FHL-2 was found to be upregulated during late fetal development. Thus, we confirm earlier reports that DRAL/FHL-2 is expressed only in significant amounts in the heart and is absent from skeletal muscle. In addition, DRAL/FHL-2 is expressed comparatively late during embryonic heart development, at a time when contracting sarcomeres have already been formed, and is upregulated significantly during prenatal muscle development.

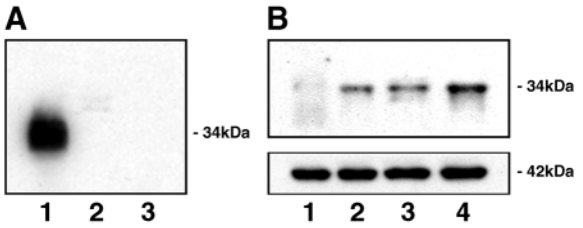

Fig. 1. Expression pattern of DRAL/FHL-2 in cardiac and skeletal muscle. (A) DRAL/FHL-2 is expressed only in cardiac muscle from adult mice. Immunoblots with a polyclonal rabbit anti-DRAL/FHL-2 antibody on whole muscle samples of adult mouse ventricle (lane 1), mouse tibialis anterior (fast twitch skeletal muscle, lane 2) and mouse soleus (slow twitch skeletal muscle, lane 3) show a band of the expected molecular weight of 34 kDa only in the heart. (B) DRAL/FHL-2 expression is upregulated in fetal mouse heart. Immunoblots with a polyclonal rabbit anti-DRAL/FHL-2 antibody on whole muscle samples of E12.5 (lane 1), E16.5 (lane 2), E18.5 (lane 3) and P0 (lane 4) heart show that DRAL/FHL-2 is expressed in cardiomyocytes during late embryonic development (upper panel). Equal amounts of heart tissue were loaded, as judged by the expression of α-cardiac actin (bottom panel).

DRAL/FHL-2 localises to specific regions in the cardiac sarcomere

Initial studies have shown that ectopically expressed DRAL/FHL-2 as well as endogenous DRAL/FHL-2 is localised in a cross-striated pattern at two distinct sites in sarcomeres of cultured neonatal rat cardiomyocytes. DRAL/FHL-2 is found in broad striations at the Z-disc and in fainter striations at the M-band region (Scholl et al., 2000). In order to precisely identify the region near the Z-disc where DRAL/FHL-2 is bound, double labeling experiments with antibodies directed against DRAL/FHL-2 together with antibodies directed against different epitopes of the sarcomeric ruler titin were performed (Fig. 2). We employed antibodies against the T12 epitope, which is located at the outer edge of the Z-disc region of titin, against the I-band epitopes N2B and N2A and against the 9D10 epitope located in the extensible PEVK region (Fig. 2A). The signal for DRAL/FHL-2 was compared with the signals of the different titin epitopes obtained with myofibrils in different states of contraction (Fig. 2B). In relaxed sarcomeres, DRAL/FHL-2 was consistently found as a narrow doublet flanking the Z-disc and in very faint striations at the M-band (Fig. 2Bc′). In contrast, the T12 epitope, which denotes the border of the Z-disc, could not be resolved into a doublet. This finding suggests that DRAL/FHL-2 is located outside of the Z-disc. Comparison of the other two

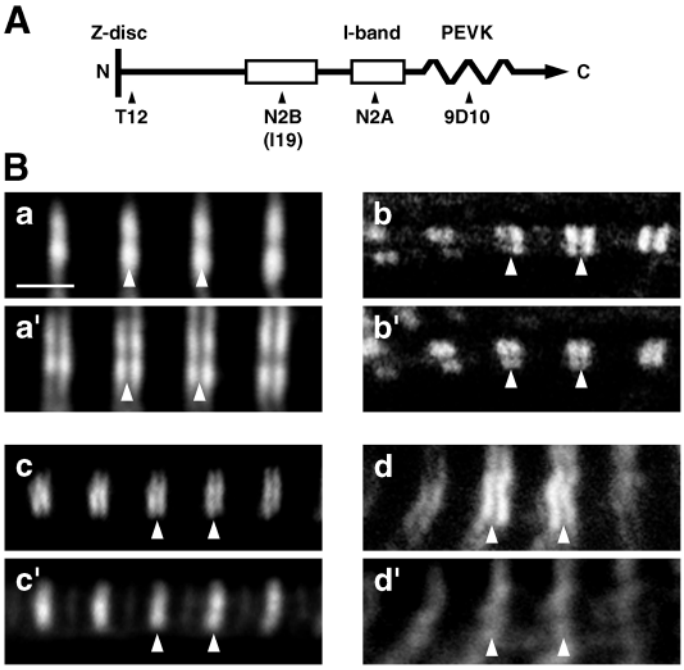

Fig. 2. DRAL/FHL-2 colocalises with the N2B region of titin. (A) Schematic representation of antibody epitopes on titin. (B) Mapping of the exact position of DRAL/FHL-2 in the I-band by costaining with antibodies against different titin epitopes. Cardiac myofibrils from papillary muscle (a,b,c) or neonatal rat cardiomyocytes (d) were stained with an antibody directed against DRAL/FHL-2 (a′, b′, c′, d′), together with antibodies directed against the N-terminus of titin (T12 epitope, a), the N2B region (I19 epitope, b), the N2A region (c) and against the PEVK region (9D10 epitope, d). DRAL/FHL-2 colocalises with the N2B region, but not with the other regions of titin. Arrowheads indicate the center of the Z-disc. Bar, 2 μm.

titin epitopes with DRAL/FHL-2 showed that DRAL/FHL-2 colocalised with the N2B epitope independently of the contraction state, whereas the N2A and PEVK epitopes were always located further towards the M-band than DRAL/FHL-2 (Fig. 2B). These results suggest that DRAL/FHL-2 is bound at or very near the N2B region of cardiac titin.

Identification of DRAL/FHL-2 binding partners by yeast two hybrid assay

The sarcomeric localisation of DRAL/FHL-2 at the Z-disc and at the M-band cannot be explained by any of its interaction partners that have been identified so far. For this reason, a yeast two-hybrid screen was carried out to identify sarcomeric proteins that bind to DRAL/FHL-2 and that may serve to anchor it to the sarcomere. Full-length DRAL/FHL-2 was used to screen an adult human cardiac cDNA library. Out of 1×10^7 primary transformants, 200 clones were isolated that survived selection and were positive in a β-galactosidase assay. Of these, one clone encoded a region of titin termed is2 (Labeit and Kolmerer, 1995) and five clones encoded the entire open reading frame of the muscle isoform of creatine kinase (MM-CK) (Ordahl et al., 1984; Wallimann et al., 1992). The interaction between DRAL/FHL-2 and either is2 or MM-CK was reproducible regardless of whether DRAL/FHL-2 or its interacting partners were fused to the DNA-binding domain or to the activation domain (data not shown). The is2 region is situated in the M-band region of titin (Labeit and Kolmerer, 1995) and MM-CK, despite being a cytosolic enzyme, is found at distinct sites near the M-band and near the Z-disc (Wallimann et al., 1992). For this reason, is2 and MM-CK represent potential binding partners for DRAL/FHL-2.

No clones were isolated in our yeast two-hybrid screen that would explain the localisation of DRAL/FHL-2 in the Z-disc region. Since DRAL/FHL-2 colocalises exactly with the N2B titin epitope, we reasoned that it may be bound to this region of titin. The direct interaction of DRAL/FHL-2 and N2B was investigated in a yeast two-hybrid assay. As shown in Fig. 3, the entire N2B region of titin interacts strongly and specifically with DRAL/FHL-2. N2B does not bind the LexA DNA-binding domain alone, and it does not interact with an unrelated bait (Fig. 3). In summary, the yeast two-hybrid assays demonstrate an interaction of DRAL/FHL-2 with the is2 and N2B regions of titin, and with the metabolic enzyme MM-CK. The interaction with two distinct regions of titin may thus serve to anchor DRAL/FHL-2 to the sarcomere.

Confirmation of the interactions by colocalisation and pull-down assays

Next, we sought to confirm the interaction of DRAL/FHL-2 with titin in an in vivo environment. Primary cultures of neonatal rat cardiomyocytes were cotransfected with constructs encoding FLAG-tagged DRAL/FHL-2 in combination with GFP-tagged is2 or GFP-tagged N2B segments of titin. In cotransfected cardiomyocytes, DRAL/FHL-2 was found in a doublet flanking the Z-disc and in a faint striation in the M-band (Fig. 4A), colocalising exactly with transfected N2B. An identical pattern was observed in cells cotransfected with DRAL/FHL-2 and is2: both proteins were found in a doublet flanking the Z-disc and in a weaker striation at the M-band (Fig. 4A). The finding

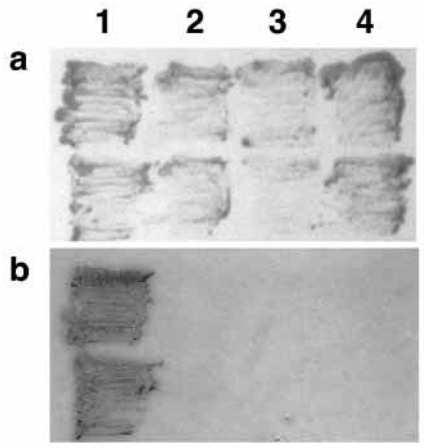

Fig. 3. DRAL/FHL-2 interacts with the N2B region of titin in a yeast two-hybrid assay. Yeast strain L40 was cotransformed with LexA-DRAL/FHL-2 and Gal4AD-N2B (lane 1), LexA-lamin C and Gal4AD-N2B (lane 2), LexA and Gal4AD-N2B (lane 3) and LexA-DRAL/FHL-2 and Gal4AD (lane 4). Yeast transformants were streaked out on selective plates (a) and β-galactosidase activity was detected using a filter assay (b). Results of two independent colonies per yeast transformation are shown. Only LexA-DRAL/FHL-2 in combination with Gal4AD-N2B yields any measurable β-galactosidase activity (lane 1). N2B does not interact with an unrelated bait (LexA-lamin C, lane 2) or with LexA alone (lane 3) and LexA-DRAL/FHL-2 does not interact with Gal4AD alone (lane 4).

that exogenously expressed N2B was also localised at the M-band, whereas is2 was also found in a doublet flanking the Z-disc strongly suggests that these fragments are targeted to the sites by means of their interaction with DRAL/FHL-2.

The direct interaction of DRAL/FHL-2 with the is2 and N2B regions of titin was further confirmed by an in vitro binding assay (Fig. 4B). Recombinant GST-DRAL/FHL-2 was incubated with total lysates from COS-1 cells that had been transfected with eukaryotic expression constructs encoding GFP-tagged is2 or N2B, respectively. As a control, GST alone was incubated in parallel with the same lysates. Both is2 and N2B were found in the GST-DRAL/FHL-2 fraction, whereas they were absent from the GST fraction. This result confirms the observed colocalisation in the transient transfection experiments and suggests that DRAL/FHL-2 is targeted to these sites in the sarcomere by a direct interaction with the N2B and the is2 regions of titin, respectively.

The N2B region of titin contains an N-terminal and a C-terminal block of two Ig-like domains interrupted by a large stretch of unique sequence (Fig. 5A) (Labeit and Kolmerer, 1995). In order to determine whether DRAL/FHL-2 binds to the blocks of the Ig-like domains or to the unique sequence that connects them, we constructed several deletion mutants of N2B and assayed these for interaction with GST-DRAL/FHL-2 in pull-down assays (Fig. 5B). GFP-N2BΔ1, which lacks the two C-terminal Ig-like domains, was still capable of interacting with DRAL/FHL-2. Additional deletion of the C-terminal half of the unique sequence in GFP-N2BΔ2 did not affect its binding to DRAL/FHL-2, either. However, deletion of another 270 amino acids from the central part of the unique sequence in GFP-N2BΔ4 completely abolished the interaction of

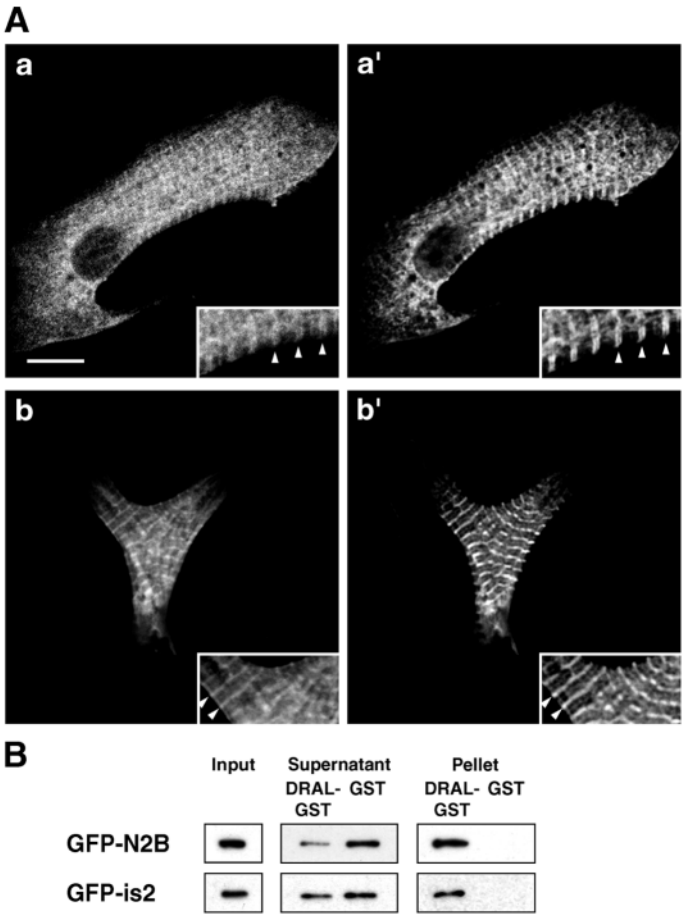

Fig. 4. Confirmation of the interaction between DRAL/FHL-2 and the N2B and is2 regions of titin by colocalisation and pull-down experiments. (A) Transient transfection assays. GFP-N2B (a) and DRAL/FHL-2-FLAG (a′) are localised in a similar pattern in transiently transfected NRC, with a broad doublet flanking the Z-disc (arrowheads) and a weaker striation at the M-band. A similar colocalisation can be observed in neonatal rat cardiomyocytes transiently transfected with GFP-is2 (b) and DRAL/FHL-2-FLAG (b′). (B) Pull-down assays: GFP-N2B and GFP-is2 interact with DRAL/FHL-2-GST but not with GST alone. Proteins in the supernatant and pellet fractions were detected by immunoblotting using a GFP antibody.

DRAL/FHL-2 and N2B. Likewise, GFP-N2BΔ3, which contains the C-terminal end of the unique sequence followed by the two C-terminal Ig-like domains, did not retain any affinity for DRAL/FHL-2. These results suggest that the interaction with DRAL/FHL-2 is mediated by a binding site located within the central 270 amino acids of the N2B region. To investigate this putative binding site more closely, we created an additional construct, GFP-N2BΔ5, which comprises just this sequence. This minimal binding domain shows targeting that is indistinguishable from the full-length titin N2B segment (Fig. 5C). Additional proof for an interaction between DRAL/FHL-2 and this region in titin N2B was provided by assaying COS cells that were transiently transfected with DRAL/FHL-2-FLAG and GFP-N2BΔ5. Following immunoprecipitation with an anti-FLAG antibody, GFP-N2BΔ5 could be visualised on a immunoblot with anti-

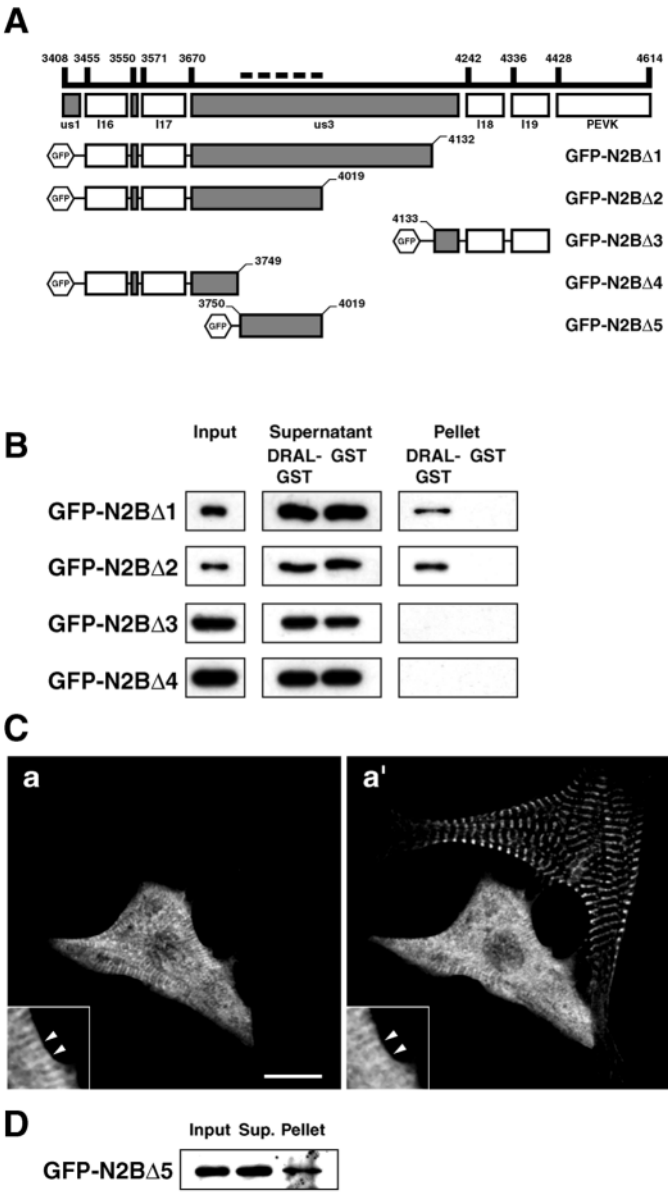

Fig. 5. Mapping of the DRAL/FHL-2-binding site in the N2B region of titin. Five different GFP-tagged deletion constructs of the N2B region were assayed for their interaction with DRAL/FHL-2 in pull-down assays. (A) Schematic representation of the employed constructs. (B) Investigation of the interaction between deletion constructs of the N2B region and DRAL by a GST pull-down assay. Only constructs that contain the residues between 3749 and 4019 of the unique sequence 3 are able to bind to DRAL/FHL-2-GST. The putative DRAL/FHL-2-binding site is indicated by a dotted line in the schematic drawing of the titin N2B region. (C) The putative DRAL/FHL-2-binding site alone shows proper targeting to the sarcomere, as shown in NRC double-transfected with GFP-N2BΔ5 (a) and DRAL/FHL-2-FLAG (a′). Arrowheads in the insets point at the doublet flanking the Z-disc. (D) Coimmunoprecipitation of GFP-N2BΔ5 and DRAL/FHL-2-FLAG. COS cells were cotransfected with GFP-N2BΔ5 and DRAL/FHL-2-FLAG, immunprecipitation was carried out with a monoclonal anti-FLAG antibody followed by immunoblotting with a peroxidase-conjugated polyclonal rabbit anti-GFP antibody. The domain nomenclature and amino acid numbering correspond to that of the human cardiac titin sequence [X90568 (Labeit and Kolmerer, 1995)]. Bar, 10 μm.

GFP antibodies, confirming the stabile interaction of these protein domains in vivo (Fig. 5D).

In summary, the colocalisation and pull-down experiments confirm the interaction between DRAL/FHL-2 and two distinct regions in the giant protein titin and strongly suggest that DRAL/FHL-2 is targeted to two sarcomeric sites in cardiomyocytes by virtue of those interactions. Furthermore, the DRAL/FHL-2-binding site on N2B is located in the central 270 amino acids of the unique insertion connecting the Ig-like domains.

Interaction of DRAL/FHL-2 with the enzymes creatine kinase, adenylate kinase and phosphofructokinase

The metabolic enzyme MM-CK (EC 2.7.3.2) was isolated five times in our yeast two-hybrid screen. MM-CK is a predominantly cytosolic enzyme, but a minor fraction is located at two distinct sites in the sarcomere near the M-band and flanking the Z-disc (Schäfer and Perriard, 1988; Wallimann et al., 1992; Wegmann et al., 1992). In order to investigate whether exogenous DRAL/FHL-2 and MM-CK colocalise in the sarcomere, we cotransfected primary cultures of rat cardiomyocytes with constructs encoding FLAG-tagged DRAL/FHL-2 and GFP-tagged MM-CK. Both DRAL/FHL-2 and MM-CK were found in a doublet pattern flanking the Z-disc and in a single striation at the M-band (Fig. 6A). However, while there was less DRAL/FHL-2 bound to the M-band than to the Z-disc region, the signals for MM-CK flanking the Z-disc and the M-band appeared equally intense (compare insets in Fig. 6Aa and a′), indicating that MM-CK may target to the M-band by interaction with other proteins apart from DRAL/FHL-2 (Hornemann et al., 2000).

Two other metabolic enzymes, adenylate kinase (EC 2.7.4.3) and phosphofructokinase (EC 2.7.1.11), are also found in the M-band and, depending on the buffer conditions used for fixation, in the I-band as well (Dolken et al., 1975; Kraft et al., 2000). We therefore asked whether those proteins may achieve their localisation in the sarcomere via an interaction with DRAL/FHL-2, too. First, we compared the sarcomeric localisation of FLAG-tagged DRAL/FHL-2 and either adenylate kinase or phosphofructokinase tagged with GFP by cotransfection into rat cardiomyocytes (Fig. 6A). Adenylate kinase and phosphofructokinase were found in a doublet flanking the Z-disc and in a single striation at the M-band (Fig. 6A).

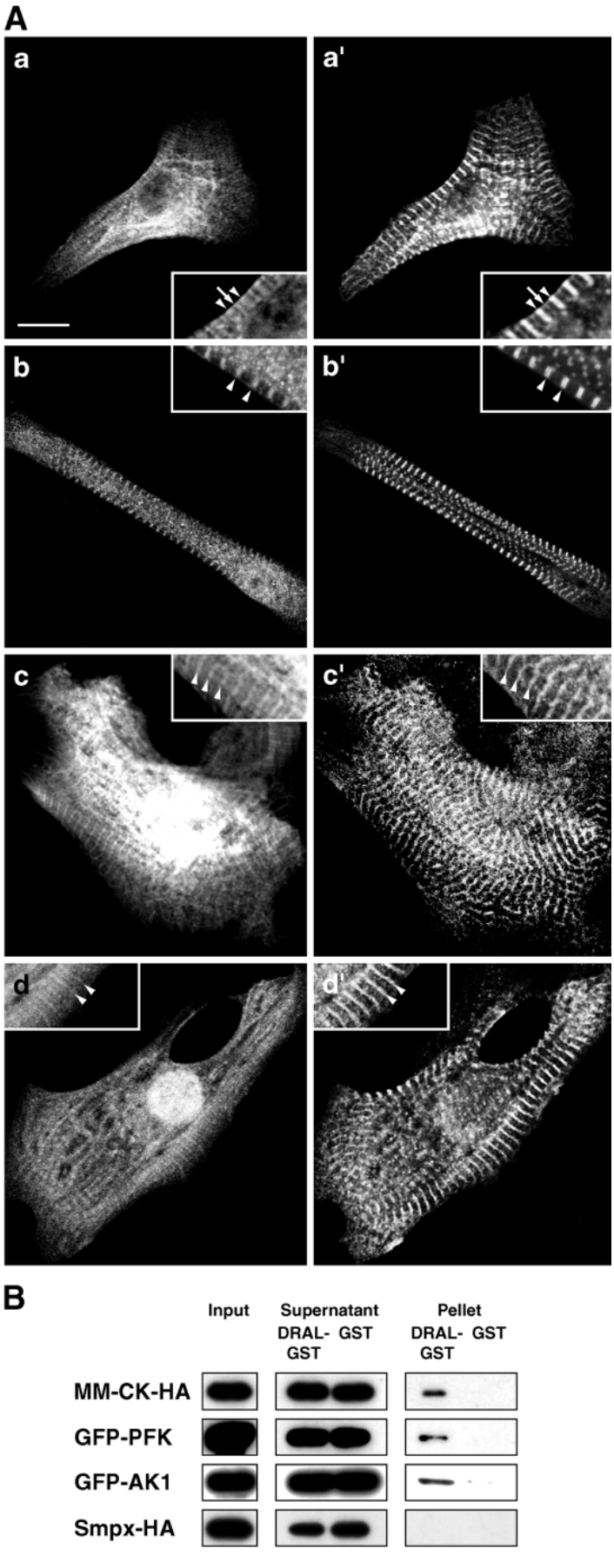

Fig. 6. DRAL/FHL-2 interacts with the metabolic enzymes MM-CK, adenylate kinase and phosphofructokinase. (A) Transient transfection assays. GFP-tagged MM-CK (a), phosphofructokinase (b), and adenylate kinase (c) colocalise with DRAL/FHL-2-FLAG (a′, b′, c′, d′) in neonatal rat cardiomyocytes, with a strong signal around the Z-disc (arrowheads) and a weaker signal in the M-band (arrows). GFP-tagged Smpx/Csl (d) is also colocalised with DRAL/FHL-2. (B) Interaction of the metabolic enzymes with DRAL/FHL-2 in pull-down assays. MM-CK, adenlyate kinase and phosphofructokinase interact with DRAL/FHL-2-GST, but not with GST alone. Despite its similar localisation pattern in cardiomyocytes, Smpx/Csl does not interact with DRAL/FHL-2 in the pull-down assay. PFK, phosphofructokinase; AK1, adenylate kinase. Bar, 10 μm.

Superimpositions showed that both proteins colocalised exactly with DRAL/FHL-2. Thus, MM-CK, adenylate kinase and phosphofructokinase are all located at the same sarcomeric sites flanking the Z-disc and the M-band together with DRAL/FHL-2.

To investigate wether the colocalisation of the three metabolic enzymes is due to their direct interaction with DRAL/FHL-2, we performed in vitro pull-down assays. GST-DRAL/FHL-2 was incubated with lysates of COS-1 cells expressing MM-CK, adenylate kinase or phosphofructokinase. As a control for non-specific binding, GST alone was used. As shown in Fig. 6B, all three enzymes interacted with GST-DRAL/FHL-2 but not with GST alone. A recently identified muscle protein, Smpx/Csl (Kemp et al., 2001; Palmer et al., 2001), which shows a localisation pattern similar to that of DRAL/FHL-2 (Fig. 6A), was also evaluated for its interaction with DRAL/FHL-2. Smpx/Csl interacted neither with DRAL/FHL-2-GST nor with GST alone, confirming the specificity of the pull-down assay. These results strongly suggest that in cardiomyocytes the three metabolic enzymes MM-CK, adenylate kinase and phosphofructokinase are bound to two distinct sarcomeric sites near the Z-disc and the M-band by their interaction with DRAL/FHL-2. In contrast to the interactions between DRAL/FHL-2 and titin, attempts to co-immunoprecipitate the metabolic enzymes with DRAL/FHL-2 were not successful (data not shown). The failure to detect the interaction of DRAL/FHL2 and the metabolic enzymes may indicate that their association is transient in nature and may serve as a means for dynamic compartmentalisation of these enzymes, rather than a strict immobilisation to the sarcomere.

DRAL/FHL-2 interacts with FHL-1 but not with the LIM domain protein MLP

Recently, it has been shown that DRAL/FHL-2 and FHL-3 associate to form heterodimers (Li et al., 2001b). We wanted to investigate whether DRAL/FHL-2 also forms dimers with the other four and a half LIM-only family members. We focused on FHL-1, since it is the only other family member apart from DRAL/FHL-2 that is also expressed in the heart (Fimia et al., 2000). First, we cotransfected rat cardiomyocytes with constructs encoding GFP-tagged DRAL/FHL-2 and FLAG-tagged FHL-1 to compare the localisation of the two proteins. DRAL/FHL-2 and FHL-1 colocalised exactly in two striations flanking the Z-disc and in a single striation in the M-band (data not shown). Next, we tested whether the colocalisation of DRAL/FHL-2 and FHL-1 in the sarcomere is due to a direct interaction using a pull-down assay. As shown in Fig. 7, FHL-1 bound to GST-DRAL/FHL-2 but not to GST alone. The interaction was abolished by the addition of 10 mM EDTA to the incubation buffer. EDTA complexes and removes the two Zn^{2+} ions that are bound to histidine and cysteine residues of a zinc finger, thereby unfolding it. Thus, the proper conformation of the LIM domain is needed for the interaction of DRAL/FHL-2 with FHL-1.

To exclude non-specific interactions between LIM domains, we also tested an unrelated LIM protein, MLP, for binding to DRAL/FHL-2. MLP is expressed in the heart but shows a different subcellular localisation than DRAL/FHL-2 (Arber et al., 1997), making a direct interaction of the two proteins extremely unlikely. For this reason, MLP serves as a good control to assess non-specific interactions between LIM domains. As expected, MLP interacted neither with GST-DRAL/FHL-2 nor with GST alone (Fig. 7). The fact that DRAL/FHL-2 does not bind to MLP in our pull-down assays suggests that no non-specific interaction between LIM domains occurs under our assay conditions. Furthermore, the loss of interaction between DRAL/FHL-2 and FHL-1 in the presence of EDTA shows that the binding between the two proteins is dependent on the proper folding of the LIM domains and is not mediated by the non-specific sticking of LIM domains to each other.

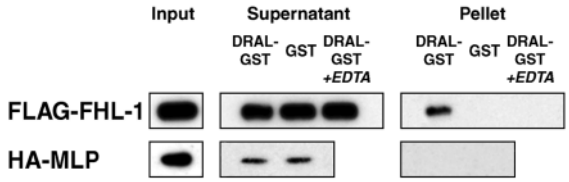

Fig. 7. DRAL/FHL-2 interacts with the four and a half LIM domain protein FHL-1 but not with the LIM-only protein MLP. Pull-down assays: FLAG-tagged FHL-1 interacts with DRAL/FHL-2-GST but not with GST alone. In contrast, HA-tagged MLP shows no interaction with either GST-DRAL/FHL-2 or GST alone. The interaction of FHL-1 with DRAL/FHL-2 can be abolished by the addition of 10 mM EDTA, which unfolds LIM domains through the removal of the coordinated metal ions.

Discussion

Sarcomeric association of DRAL/FHL-2

DRAL/FHL-2 is a member of the four and a half LIM-only protein family that is specifically expressed in cardiac muscle, where it is found near the Z-disc and in the M-band of the sarcomere. We have shown that DRAL/FHL-2 is targeted to these sites by an interaction with the N2B and is2 regions of titin, respectively, and that it serves as an adaptor protein by linking several metabolic enzymes to titin (Fig. 8). Because titin spans an entire half-sarcomere from the Z-disc to the M-band and because of its modular composition, titin is perfectly suited as an organiser of the sarcomere (Fürst and Gautel, 1995; Squire, 1997; Trinick and Tskhovrebova, 1999). A multitude of proteins bind to titin and in most cases the interaction with titin is essential for their subcellular targeting. In the Z-disc region the most prominent titin-binding protein is α-actinin (Young et al., 1998), but several other proteins that interact with N-terminal titin-like telethonin/T-cap (Gregorio et al., 1998; Mues et al., 1998) or obscurin (Young et al., 2001) have been identified. In the region of the A-band and the M-band, titin binding has been demonstrated for MyBP-C (Fürst et al., 1992; Freiburg and Gautel, 1996), myosin (Houmeida et al., 1995), myomesin (Obermann et al., 1995) and M-protein (Nave et al., 1989). The muscle-specific protein calpain3 (p94) is a titin interacting protein that shows a localisation pattern similar to DRAL/FHL-2. It binds to titin in the I-band region and in the M-band domain is-7 (Kinbara et al., 1997). Expression of this protein seems to be specific for skeletal muscle, since no calpain3 protein could be detected in heart (Fougerousse et al., 2000). Interactions with titin in general either occur via binding to modular sequences such as Ig-like

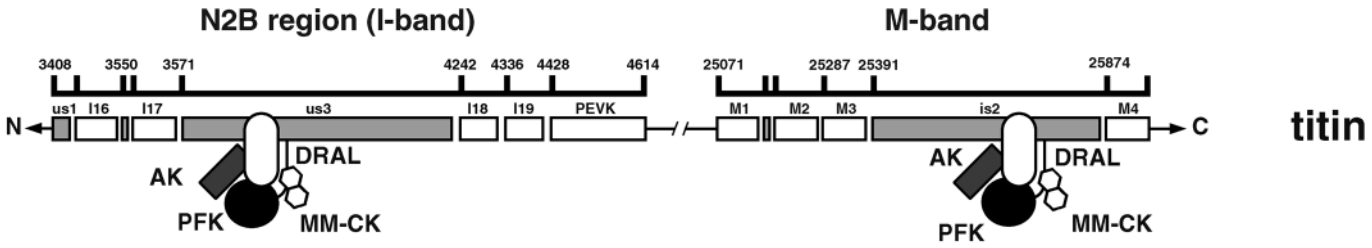

Fig. 8. Schematic drawing of the N2B and is2 regions of titin and their associated proteins. DRAL/FHL-2 interacts directly with the N2B and is2 regions and serves as an adaptor molecule to couple the metabolic enzymes MM-CK, adenylate kinase and phosphofructokinase to titin. In addition, MM-CK may be bound to the M-band region of the sarcomere via its association with additional M-band proteins (not shown here). The domain nomenclature and amino acid numbering correspond to that of the human cardiac titin sequence [X90568 (Labeit and Kolmerer, 1995)].

domains (Obermann et al., 1995; Freiburg and Gautel, 1996; Mues et al., 1998; Centner et al., 2001; Young et al., 2001), or by association with unique sequence insertions in titin [(Young et al., 1998) and this study]. To our knowledge, the interaction between DRAL/FHL-2 and titin is the first reported case of an interaction between a LIM domain protein and titin, and involves unique sequence insertions in the titin molecule such as the unique sequence 3 in the N2B region and the insertion sequence 2 in the M-band part. So far, interactions between LIM domain proteins such as ALP, cypher1/ZASP and MLP (Pomies et al., 1999; Zhou et al., 1999; Flick and Konieczny, 2000; Faulkner et al., 2001) with the sarcomere have been restricted to the Z-disk protein α-actinin. LIM domains have been suggested to act as protein-protein interaction domains and to mediate structural links (Schmeichel and Beckerle, 1994; Bach, 2000; Flick and Konieczny, 2000). The absence of LIM domain proteins in striated muscle has drastic consequences such as myopathies (Arber et al., 1997; Pashmforoush et al., 2001; Zhou et al., 2001), suggesting a role not in myofibril assembly per se but in the maintenance of cytoarchitecture and the ability to cope with stress. In most cases the interaction takes place between two different LIM-domains to form either homo- or heterodimers (Sadler et al., 1992; Wixler et al., 2000; Li et al., 2001a) but the binding can also involve regions outside the LIM domain such as in the interaction between the LIM domain proteins MLP and N-RAP (Ehler et al., 2001). However, the possession of a LIM domain and the expression in the same cell type do not automatically result in an interaction between different LIM-domain proteins as demonstrated in the case of MLP and DRAL/FHL-2 (this study).

DRAL/FHL-2 localises to the N2B region of titin, which is present only in cardiac-specific isoforms of titin (Gautel et al., 1996), with DRAL/FHL-2 being at the same time predominantly expressed in cardiac muscle. The importance of the N2B region for cardiomyocyte structure was demonstrated by the observation that overexpression of N2B fragments in cardiomyocytes can lead to a disruption of thin filaments and it has been suggested that the N2B region might be important for the binding of several sarcomeric components (Linke et al., 1999). In our transfection assays no deleterious effects of expression of N2B could be observed; however, we analysed the cardiomyocytes already 1-2 days after transfection, when the disruption of thin filament structures is probably not yet visible and the cross-striated pattern of ectopically expressed N2B in I-band and M-band can still be detected. A possible explanation of why ectopically expressed N2B fragments target to the I-band in transiently transfected NRC could be that DRAL/FHL-2 exerts its function as a homodimer, which has been strongly suggested by in vitro observations (Wixler et al., 2000). In contrast to the PEVK region, which is situated further towards the C-terminus of titin, no direct binding of N2B to actin could be found (Gutierrez-Cruz et al., 2001; Kulke et al., 2001; Yamasaki et al., 2001).

DRAL/FHL-2 interaction with metabolic enzymes in the heart

Targeting of metabolic enzymes to the I-band has been shown previously (Arnold and Pette, 1970; Dolken et al., 1975; Wegmann et al., 1992; Kraft et al., 2000) and is thought to be necessary for energy provision during contraction (Wallimann and Eppenberger, 1985). The I-band association seems to be dependent on the buffer conditions used for the sample preparation, since it is primarily observed if bivalent cation chelators such as EDTA are omitted (Wegmann et al., 1992). Since our experiments indicated that the presence of EDTA abolished the in vitro interaction between DRAL/FHL-2 and FHL-1, it seems very likely that the association of metabolic enzymes is also dependent on a conserved conformation of LIM domains in DRAL/FHL-2. DRAL/FHL-2 expression is upregulated in the heart only rather late during embryonic development. This is similar to results obtained for MM-CK expression levels in developing muscle and might explain the delay in sarcomeric compartmentalisation of this enzyme that is observed during development (Carlsson et al., 1982; Carlsson et al., 1990; Ventura-Clapier et al., 1998). Animals that are homozygous null for DRAL/FHL-2 seem normal and display no obvious abnormalities in myofibrils (Chu et al., 2000b). However, they seem to be more sensitive to stress situations such as β-adrenergic stimulation (Kong et al., 2001), which might suggest an impaired ability to cope with abnormal requirements on the cardiac energy metabolism.

DRAL/FHL-2 interactions with non-sarcomeric proteins

Previous investigations in non-cardiac cells have identified hCDC47, presenilin-2, the androgen receptor and several α- and β-integrins as potential interaction partners of DRAL/FHL-2 (Chan et al., 2000; Tanahashi and Tabira, 2000;

Müller et al., 2000; Wixler et al., 2000); however, the biological significance of these interactions in the heart is still unclear. It has been suggested that the androgen receptor might be involved in the development of hypertrophy in the heart (Marsh et al., 1998) and a decrease in DRAL/FHL-2 expression seems to occur in patients with dilated cardiomyopathy (Müller et al., 2000), again underlining the importance of LIM-domain-containing proteins for the maintenance of cardiac function (Arber et al., 1997; Pashmforoush et al., 2001). Despite the reported binding of DRAL/FHL-2 to integrins we were unable to see the typical costameric localisation pattern as expected for an integrin-associated protein in cardiomyocytes. Several other LIM-domain-containing proteins target to focal adhesions, such as paxillin (Brown et al., 1996) and zyxin (Sadler et al., 1992), or bind to integrin in vitro, such as N-RAP (Luo et al., 1999). DRAL/FHL-2 is indeed localised at focal adhesions in non-muscle cells (Scholl et al., 2000) and also in not yet differentiated muscle cells (Li et al., 2001b). However, as soon as myofibrils are formed, the preferential association of DRAL/FHL-2 seems to occur in the region of the I-band [(Li et al., 2001b) and this study] and no costameric association can be observed any longer. This might suggest that in cardiomyocytes the binding affinity of DRAL/FHL-2 for titin is so strong that it competes completely for the interaction with other potential binding partners. The localisation pattern of DRAL/FHL-2 or other LIM-domain-containing proteins could therefore be dependent on the molecular expression profile in different cell types.

In summary, subcellular localisation experiments and in vitro binding assays suggest that the localisation of DRAL/FHL-2 at two defined sites in the cardiac sarcomere is mediated by its interaction with the N2B and is2 regions of titin. In turn, DRAL/FHL-2 binds to the metabolic enzymes MM-CK, adenylate kinase and phosphofructokinase, thus serving as an adaptor to couple them to the N2B and is2 regions in titin. The results presented here point towards a crucial role for DRAL/FHL-2 in the compartmentalisation of metabolic enzymes in the heart. The participation of DRAL/FHL-2 in signalling and response to extracellular stimuli such as growth factors and mechanical stress remains to be established.

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