Unusual splicing events result in distinct Xin isoforms that associate differentially with filamin c and Mena/VASP

Peter F.M. van der Ven a,⁎, Elisabeth Ehler b, Padmanabhan Vakeel a, c, Stefan Eulitz a, Jörg A. Schenk d, Hendrik Milting e, Burkhard Micheel d, Dieter O. Fürst a

a Department of Molecular Cell Biology, University of Bonn, Ulrich-Haberland-Str. 61a, D-53121 Bonn, Germany
b The Randall Division of Cell and Molecular Biophysics and the Cardiovascular Division, King’s College London, UK
c Department of Cell Biology, University of Potsdam, Germany
d Department of Biotechnology, University of Potsdam, Germany
e Herz-und Diabeteszentrum NRW, Universitätsklinikum der Ruhr-Universität, Erich und Hanna Klessmann-Institut für Kardiovaskuläre Forschung und Entwicklung, Bad Oeynhausen, Germany

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ABSTRACT

Filamin c is the predominantly expressed filamin isoform in striated muscles. It is localized in myofibrillar Z-discs, where it binds FATZ and myotilin, and in myotendinous junctions and intercalated discs. Here, we identify Xin, the protein encoded by the human gene ‘cardiomyopathy associated 1’ (CMYA1) as filamin c binding partner at these specialized structures where the ends of myofibrils are attached to the sarcolemma. Xin directly binds the EVH1 domain proteins Mena and VASP. In the adult heart, Xin and Mena/VASP colocalize with filamin c in intercalated discs. In cultured cardiomyocytes, the proteins also localize in the nonstriated part of myofibrils, where sarcomeres are assembled and an extensive reorganization of the actin cytoskeleton occurs. Unusual intraexonic splicing events result in the existence of three Xin isoforms that associate differentially with its ligands. The identification of the complex filamin c–Xin–Mena/VASP provides a first glance on the role of Xin in the molecular mechanisms involved in developmental and adaptive remodeling of the actin cytoskeleton during cardiac morphogenesis and sarcomere assembly.

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Introduction

Myodifferentiation is associated with extensive remodeling of all classes of cytoskeletal filaments and culminates in the assembly of a highly specialized contractile machinery. This transition is accompanied by a spatially and temporally precisely regulated expression of muscle-specific proteins or muscle-specific isoforms of proteins [1,2]. In particular, a dramatic reorganization of the actin cytoskeleton is involved in this process: different actin isoforms are expressed during the maturation of developing myofibrils and are transformed from a stress-fiber-like arrangement to the thin myofilaments of the sarcomere with strictly defined length. In immature myofibrils, different actin isoforms have been detected at distinct localizations [3]. Whereas the expression of these isoforms is transcriptionally regulated, their precise localization and reorganization into I-Z-I stacks must be regulated by actin binding proteins. One of the actin binding proteins that was implied to play an important role in the assembly of the Z-disc is filamin c [4,5]. This protein belongs to a family of high...
molecular mass proteins (~280 kDa) whose members are characterized by an aminoterminal actin binding domain followed by 24 immunoglobulin-like (Ig) modules [6,7]. Only filamin c contains a unique insertion in Ig domain 20. The most carboxy-terminally situated Ig domain is required and sufficient for dimerization [8], which enables the filamins to cross-link actin filaments into a meshwork or into bundles.

In addition, all members of the filamin family have numerous further binding partners with highly diverse functions, such as ion channels, signaling proteins, and structural proteins [6,7,9]. This indicates that the organization of the actin cytoskeleton is only one of the many biological functions of the filamins. A complicated and essential role of the filamins in bone morphogenesis is exemplified by mutations in the FLNA and FLNB genes, which cause otopalatodigital and spondyloepiphyseal dysplasia. Further defects in FLNA lead to abnormalities in brain development [9]. Very recently, a mutation in the human FLNC gene was identified, which results in a novel form of the muscle disease myofibrillar myopathy [10]. Thus, all filamins appear to be involved in the regulation of tissue and organ development.

In mouse embryos, FLNC transcripts can be detected in the primitive heart tube at 8 days p.c., at the time the cardiac tube begins to contract. In skeletal muscle cells, expression already occurs in the myotomes at 10.5 days p.c., 1 day before the myocytes begin to fuse and form primary myotubes [11]. In cultured primary skeletal muscle cells, filamin c protein is detected during the earliest stages of skeletal muscle differentiation where it gradually replaces the a isoform. Filamin c is the only isoform expressed in mature myotubes and in adult muscle fibers, where it is mainly organized in Z-bodies and the myofibrillar Z-discs, respectively [4,5]. The suggested role for filamin c in the assembly of these structures is exemplified by its capacity to directly bind further Z-disc components such as myotilin [5] and FATZ/calsarcin/myozenin [12–14].

Interestingly, a small quantity of filamin c is associated with the sarcolemma of myofibers, where it binds γ- and δ-sarcoglycan [15]. Its association with the transmembrane dystrophin-associated glycoprotein complex, which is thought to mechanically stabilize the plasma membrane of muscle cells and serve a cell signaling role (for a recent review, see [16]), lead to speculations that filamin c not only remodels the actin cytoskeleton, but also functions in signaling processes between the sarcolemma and the myofibrillar Z-disc [5,15]. Interestingly, phosphorylation of filamin a by calcium/calmodulin-dependent kinase (CaMKII) has been reported to result in a relocalization of filamin from the submembranous meshwork to the actin cytoskeleton, whereas phosphorylation by PKA and PKC attenuates this translocation [17].

Filamin c is also highly concentrated at myotendinous junctions (MTJ) of skeletal muscles and intercalated discs (ICD) of the heart [4], both structures at the end of the myocytes that transduce force from the myofibrils via the cell membrane to the extracellular matrix and neighboring cells, respectively. In MTJs, the ends of the myofibrils are attached to the membrane, and via transmembrane proteins such as integrins, cadherins, and the dystrophin/utrophin-associated protein complex to the extracellular matrix of the tendon [18–20]. In cardiomyocytes, the structures functionally corresponding to MTJs in terms of myofibril attachment to the membrane are the adherens junctions of the ICD. Furthermore, the ICD interconnect individual cardiomyocytes and play a role in intercellular signaling [21].

Myotilin [5] and members of the FATZ/myozenin/calsarcin family [12–14] were shown to be binding partners of filamin c within the Z-disc and γ- and δ-sarcoglycan [15] within MTJ and costameres (lateral myofibril attachment sites to the sarcolemma), respectively. Other binding partners include the KY protein [22] and some of the β1 integrins [23–25]. Thus far, no direct binding partners within ICD were identified.

Here, we introduce Xin as a binding partner of filamin c in MTJ and ICD and show that Xin binds the unique insertion-containing Ig domain 20. Xin in turn directly binds F-actin [26] and the EVH1 domain containing proteins Mena and VASP. The coding region of Xin is encoded by a single large exon. Intragenic splicing leads to the expression of at least three different isoforms, each containing a different combination of the binding sites for actin, filamin c, and Mena/VASP. Together with the observation that experimental down-regulation of Xin expression in chicken embryos leads to abnormal development of the heart [27], our results indicate that these proteins are part of a complex that plays an important role in the morphogenesis of the heart, early myofibrillogenesis, and the maintenance of striated muscle integrity.

Materials and methods

Yeast two-hybrid assays

Human skeletal muscle and cardiac muscle cDNA libraries (Matchmaker cDNA libraries HL4047AH and HL4042AH, respectively; Clontech, Heidelberg, Germany) were screened with the filamin c cDNA comprising Ig domains 19–21 as described before [5]. For submapping the interaction between filamin and Xin, constructs encoding distinct portions of filamin c were cloned into the pLexA vector and cotransformed with the coding region of the smallest interacting Xin prey construct into L40 yeast cells. Growth on SD-LWH agar plates and activity of β-galactosidase were assayed as described [5].

To investigate whether the proline-rich regions of Xin interact with EVH1 domains, cDNA fragments encoding residues 1–54 and 1120–1416 were cloned in the pLexA vector. The constructs were cotransformed with fragments derived from human Mena, VASP, and EVL cDNAs that were cloned in pACT2 [28]. The latter constructs were a generous gift of Dr. J. Wehland, Braunschweig, Germany.

RT PCR and 5′ RACE

cDNA fragments covering different parts of human Xin (see Figs. 2 and 4) obtained by RT-PCR using total RNA purified from cultured human skeletal muscle cells as a template using Expand reverse transcriptase and the ‘Expand long template PCR system’ (both from Roche Diagnostics, Mannheim, Germany) were cloned into the pGEM-T vector (Promega, Mannheim, Germany) and sequenced (AGOWA, Berlin, Germany). 5′ RACE was performed using the 5′ RACE System (Invitrogen). cDNA was synthesized using a primer corresponding to nucleotides 413–433 of the Xin cDNA (AJ626900; see legend to...
Construction of eukaryotic expression constructs

A DNA fragment covering the entire ORF of human XinA was amplified by PCR using human genomic DNA and the 'Expand long template PCR system' (Roche Diagnostics, Mannheim, Germany), cloned into the pMYP vector, and verified by sequencing. For the construction of pMYP, the multiple cloning site of pMYP [29] was replaced by a novel cloning cassette and the sequence encoding EGFP, which was amplified from pEGFPC2 (Clontech). This vector was used to amplify and subclone truncated variants of Xin in the same vector, using Pfu polymerase. Constructs were transformed to and propagated in Escherichia coli JM109 bacteria. DNAs were purified using the Qiagen Plasmid Midi Kit (Qiagen, Hilden, Germany).

Construction of bacterial expression constructs and purification of recombinant proteins

cDNA fragments covering different parts of Xin, filamins, and VASP were cloned in pET23-EEF [5], pET23-T7 [30], or pGEX6P3 (Amersham Biosciences). Constructs were transformed to the E. coli strain BL21-CodonPlus(DE3)-RP (Stratagene). Expression and purification were carried out essentially as described before [30]. Protein concentrations were determined as described [31].

Peptide scans and Western blot overlay assays

Peptide scans were kindly provided by Dr. Ronald Frank (GBF, Braunschweig, Germany). Spot synthesis was performed as described with an Abimed ASP 222 Automated SPOT Robot [32]. The strip was incubated with recombinant protein diluted to 0.2 mg/ml in blocking solution (TBST + 1% milk powder) for 90 min. After three washes with TBST, bound protein was immunodetected using an anti-GST antibody and HRP-conjugated goat anti-mouse IgG, which were diluted in blocking buffer.

Pellets of bacterial cells expressing recombinant polypeptides were boiled in SDS sample buffer. SDS-PAGE was performed using 14% polyacrylamide gels, and separated proteins were transferred to nitrocellulose membranes using a semidry blotting apparatus (BioRad). Nitrocellulose strips were blocked with 4% (w/v) low fat milk powder in TBST, and either incubated with a tag-specific antibody and a HRP-conjugated secondary antibody to detect the expressed polypeptide or overlaid with recombinant polypeptide diluted to 30 µg/ml in blocking solution for 90 min. After three washes with TBST, bound protein was immunodetected using a tag antibody, HRP-conjugated secondary antibodies (Jackson Immuno Research Laboratories, USA), and ECL using 'Super-Signal West Pico Chemiluminescent Substrate' (Pierce, Rockford, IL, USA) and Kodak XAR-351 film.

Tissues, preparation of tissue extracts, Western blotting, and immunodetection

Human myocardial tissue was obtained from the septum of nonfailing donor hearts, which could not be transplanted for technical reasons. The study was approved by the ethics committees of the University of Potsdam and the Heart and Diabetescenter NRW in Bad Oeynhausen. Approximately 5 mg frozen tissue was grinded to powder in liquid nitrogen, suspended in 2× SDS-sample buffer, incubated at 65°C for 15 min, and sonicated to shear the DNA. Proteins were separated on 6% polyacrylamide gels and transferred to nitrocellulose membranes using a Trans-Blot Plus Cell tank transfer system (BioRad). Membranes were incubated as described above.

Production and characterization of monoclonal Xin antibodies

Monoclonal antibodies specific for the repeat region and the carboxy-terminus of XinA were obtained from female Balb/c mice that had been immunized by a standard immunization scheme using purified bacterially expressed polypeptides XinA aa 83–285 and aa 1686–1843, respectively (see also Fig. 3A). Following fusion according to a protocol described before [33], hybridoma supernatants were tested for specific antibodies in a solid-phase enzyme immunoassay as described [34]. To document their specific reaction with the different Xin isoforms, their reactivity was analyzed by Western blotting on total human cardiac muscle extract as described above.

Further antibodies used in this study

RR90, a mouse mAb recognizing filamin a and filamin c [4]; mouse mAb B4 specific for myomesin [35]; mouse mAb EA53 (Sigma) and rabbit polyclonal antiserum (pAb) RaA653 [4], both specific for sarcomeric α-actinin; m8 a rabbit anti-M-band titin pAb [36]; M4, a rabbit pAb for VASP (immunoGlobe, Himmelstadt, Germany); YL1/2, a rat mAb raised against the carboxy-terminus of tyrosinated tubulin and recognizing the EEF tag [37], which was a kind gift of Dr. J. Wehland, Braunschweig, Germany; T7-tag antibody, a mouse mAb, specific for the T7-immunotag (Novagen, Madison, WI, USA); anti-VASP mouse mAb IE273 [38]; mouse mAb 49C2 specific for Mena [39]. The latter two antibodies were kind gifts of Dr. T. Stradal, Braunschweig, Germany.

Isotype-specific secondary antibodies conjugated with FITC, Cy3, Texas Red, or Cy5 were applied according to the recommendations of the manufacturers (Southern Biotechnology Associates, USA; Jackson Immunoresearch Laboratories, USA; Sigma). Alexa633-conjugated phallolidin (Molecular Probes) was used to stain F-actin.
Indirect immunofluorescence microscopy of cryosections of normal human heart specimens or cultured cardiomyocytes was performed as described previously [4,35]. All specimens were examined, and pictures were digitally acquired using a Zeiss Axiophot microscope (Carl Zeiss, Germany) equipped with a cooled CCD camera. Images of single confocal sections were taken on a Leica SP1 or a Zeiss LSM 510 confocal microscope equipped with argon and helium-neon lasers using the sequential scanning mode and a 63× oil immersion lens. Data sets were either processed using Imaris (Bitplane, Switzerland) or Adobe Photoshop.

Culture and transfection of neonatal rat cardiomyocytes

Primary neonatal rat cardiomyocytes were isolated, cultured, and transfected as described previously [35]. 24–48 h after transfection, cells were fixed in 4% paraformaldehyde/PBS for 10 min or washed with PBS and incubated with cold methanol for 5 min at –20°C. Cells were stained and analyzed by confocal microscopy as described [40].

Results

Filamin c binds the carboxy-terminal region of human Xin

In an effort to find novel binding partners of the filamin c Ig domain that harbors a unique insertion of 78 residues, the region encompassing domains 19–21 was used as a bait in yeast two-hybrid screens. A human skeletal muscle and a human cardiac muscle library were screened and plasmid DNA was rescued from 20 and 15 HIS3 and β-galactosidase-positive clones, respectively. While most of the clones encoded common false-positives such as hsp27, elongation and translation initiation factors (see: http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html), one of the skeletal muscle and three of the cardiac muscle clones contained sequences from a gene that was previously denominated cardiomypathy associated 1 (CMYA1; GenBank AW755250), the human homologue of mouse Xin [27]. The smallest clone encoded an ORF of only 158 amino acids (Fig. 1A), indicating that filamin c binds the extreme carboxy-terminal portion of Xin.

To establish which Ig domain of filamin c was responsible for binding Xin, subfragments of filamin c were tested for their ability to interact. In contrast to the binding capacities of filamin c to myotilin [5], fragments d19–20 and d20–21 both interacted with Xin (Fig. 1B). This indicates that the unique insert-containing filamin c domain 20 must be responsible and sufficient for binding Xin (Fig. 1E).

Western blot overlay assays confirm the association of Xin with filamin c d20

The results obtained with the yeast two-hybrid screen were confirmed by a biochemical assay. Subfragments of filamin a, filamin b, and filamin c were expressed with a carboxy-terminal EEF tag. They were separated on polyacrylamide gels and blotted. The nitrocellulose filters were overlaid with a T7-tagged Xin fragment corresponding to the smallest expressed polypeptide found to interact with filamin c in yeast cells. Xin showed binding to recombinant fragments that contained at least Ig domain 20 of filamin c, whereas filamin c d23–24 and the filamin b and filamin a constructs were not bound (Fig. 1C). Furthermore, a T7-tagged nonrelated polypeptide did not show any binding (not shown). This assay demonstrated specific binding of Xin to filamin c.

The filamin c-binding Xin fragment colocalizes with filamin c upon transient expression in neonatal rat cardiomyocytes

The Xin fragment that bound filamin c was transiently expressed in neonatal rat cardiomyocytes. Obvious targeting of the Xin polypeptide to all structures known to contain filamin c ([5], see also Fig. 6), including the nonstriated parts of myofibrils (NSMF), myofibrillar Z-discs, and ICD, was observed, as demonstrated by staining of the transfected cells with an antibody specific for myomesin, and Alexa633-phalloidin (Fig. 1D).

The human CMYA1 gene encodes Xin, a protein of 1843 amino acid

With the longest Xin clone found in the yeast two-hybrid screens as a starting point, the complete human Xin cDNA sequence was obtained using a combined RT-PCR and 5’RACE approach. This allowed us to establish the complete coding region of human Xin (AJ626900). Thus, Xin is predicted to be a protein with a length of 1843 amino acids and an Mr of 199 × 10^3 (Fig. 2). For reasons mentioned below, we named this protein XinA. 5’RACE was performed to identify the 5’ end of the Xin mRNA including its 5’ UTR. This yielded three distinct sequences. In all cases, the identical ATG triplet was predicted to be the start codon using Netstart (http://www.cbs.dtu.dk/services/NetStart/) and ATGpr (http://www.hri.co.jp/atgpr/). Variant one contains 229 nucleotides as the predicted 5’ UTR. The sequence of the second variant lacked an internal portion of the UTR of 76 nucleotides. A third variant contained an entirely different sequence, except the 4 nucleotides directly upstream from the predicted ATG start codon (Fig. 2A). These findings prompted us to investigate the structure of the human CMYA1 gene in detail.

The human CMYA1 gene gives rise to several splice variants by intraexonic splicing

A search with the Xin mRNA sequence for genomic sequences using NCBI BLAST (http://www.ncbi.nlm.nih.gov) identified human genomic clones mapping exclusively to chromosome 3p22.2, indicating that CMYA1 is a single copy gene. Only the repeat region showed similarity to a second gene, CMYA3, encoding a protein we had previously designated Xin repeat protein 2 (XIRP2 [26]). The structure of CMYA1 has several highly unusual features: The entire coding region and the 3’ UTR are contained within a single exon comprising 6310 nucleotides (exon 2). Part of the 5’ UTR is contained in a leading 149 bp exon located 3 kb upstream from exon 2. This simple gene structure sharply contrasts the multiple isoforms that were revealed by EST searches and our expression analysis and implies that this variability arises by a
mechanism distinct from the simple alternative splicing of individual coding exons. In the case of Xin, this is achieved by usage of intrarexonic splice sites. Different 5′ UTRs are generated by joining either exon 1 to exon 2a, or to exon 2b, that starts 76 nucleotides downstream of exon 2a. In a third variant, the first exon is not used and the mRNA starts with a 5′ extended second exon (Fig. 2A). In the latter case, the complete Xin mRNA sequence is encoded by a single exon. The longest cDNA starts at position −216 from the ATG start codon, but we cannot exclude that the transcription start site is further upstream. In agreement with the assumption that the intron between exons 1 and 2 contains a second, alternative promoter, we have identified a wealth of transcription factor binding sites upstream of both alternative starting exons (our unpublished observation). Notably, the intron between exons 1 and 2 contains a region that is highly conserved among mammals and contains putative binding sites for MEF2 and MyoD. Similarly, the region directly upstream from exon 1 contains conserved putative binding sites for, for example, MyoD and nrx-2.

Additional to these splicing events, a pair of splice donor and acceptor sites is used in exon 2. These sites flank a portion of 427 bp and removal of this fragment (exon 2c) gives rise to a correspondingly shorter mRNA (AJ626899). The resulting

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**Fig. 1** - Characterization of the interaction of filamin c with Xin. (A) The Xin clones that were obtained in two yeast two-hybrid screens using a human skeletal or cardiac muscle library and filamin c d19–21 as a bait. (B) The results of an assay with different filamin c constructs as a bait, and the smallest interacting Xin clone or full-length myotilin, as a prey. (+) indicates growth on selective plates and restored β-galactosidase activity. (−) indicates no interaction. (C) Binding properties of a recombinant Xin fragment in Western blot overlay experiments. Different parts of filamin c, filamin b, or filamin a were separated by SDS-PAGE and transferred to nitrocellulose. Nitrocellulose strips were either stained with an antibody specific for the immunotag of the filamin fragments (lanes 1) or overlaid with the filamin c-binding Xin fragment. Binding was detected with an antibody specific for the immunotag of the Xin fragment (lanes 2). Xin binds all constructs containing filamin c domain 20, but not the corresponding domains of filamin a or filamin b. (D) Transient expression of the filamin c-binding part of Xin fused to EGFP in neonatal rat cardiomyocytes. The fragment binds nonstriated myofibrils (arrowheads), myofibrillar Z-discs, and ICD (arrows). These structures were identified upon staining the cells with an antibody specific for myomesin (c) and phalloidin (d). The merged triple stain is shown in panel A. (E) Schematic representation of filamin c’s molecular interactions. The domain at the N terminus is an actin-binding domain, whereas the 24 repeats that follow the ABD are immunoglobulin-like modules. Presented are a selection of proteins that interact with filamin c. The domains involved in each interaction are depicted by red bars.
frameshift generates a stop codon immediately after the splice site. ESTs (e.g., AK096421) were also identified, in which exon 1 is joined to exon 2d. The resulting mRNA encodes only the 3′ half of the XinA ORF (Fig. 2B).

In summary, these splicing events predict three Xin variants: (1) XinA, the ‘full-length’ protein (see above); (2) a carboxy-terminally truncated variant, XinB (1121 amino acids; 122.1 kDa), and (3) an aminoterminally truncated variant XinC (526 amino acids; 56.3 kDa). Database entries (BX116988, A1703241) point to the existence of a further splice variant that results in the deletion of amino acids 41–85 of the XinA sequence. This region is highly conserved between both human Xin repeat proteins and Xin repeat proteins from other species and was predicted to be a putative nuclear localization signal[27]. We could not confirm the expression of this isoform in striated muscle cells. Therefore, this potential Xin variant was not further studied.

Expression of Xin variants in human cardiac muscle

To analyze Xin isoform expression in the human heart at the protein level, monoclonal antibodies specific for the aminoterminus and carboxy-terminus of Xin were developed (Fig. 3A). In agreement with the expectation that antibodies specific for the aminoterminus portion of Xin (repeats 1–6, XR) detect XinA and XinB (see above), three different monoclonal antibodies specifically stained two bands of approximately 205 and 130 kDa in Western blots of human cardiac muscle extracts. In contrast, four antibodies raised against the carboxy-terminus, only stained the long 205 kDa Xin variant (Fig. 3B). Our antibodies did not detect a protein with the predicted size of the aminoterminally truncated XinC variant.

To investigate the expression of Xin at the mRNA level, and to exclude that the 130 kDa variant is a proteolytic product of the full-length protein, the expression of Xin variants in the human heart was examined by RT-PCR using isoform-specific primers. Although we were unable to detect XinC on Western blots of normal human heart protein extracts, at least two independent primer pairs for each isoform (Fig. 3A, see Supplementary Material) demonstrated the simultaneous expression of all three predicted variants in the normal human heart (Fig. 3C).

The proline-rich region in the aminoterminus of Xin interacts with EVH1 domains

The Xin cDNA sequence revealed several proline rich regions indicative of potential protein-protein interaction sites: aminoterminally from the repeats (amino acids 20–40, PR1; see Fig. 2A), at the carboxy-terminus of XinB (aa 1111–1121, PR2), in the part of XinA that is specific for this isoform (amino acids 1253–1270, PR3), and at the aminoterminus of XinC (between amino acids 1318 and 1350 in XinA, PR4). All sequences are highly conserved between human, mouse (AF051945), and rat (XM236702) Xin. PR1 (EDLPLPPPALEDLPLPPKE) seemed particularly interesting.
because it is highly homologous to the consensus sequence of class I EVH1 ligands (F/W/Y/L)PPPPX(D/E)(D/E)(D/E)Φ [41].

Using the yeast two-hybrid system, we could confirm that all constructs containing the EVH1 domain of either VASP, Mena, or EVL were found to interact with this part of Xin, whereas no interaction was detected with a truncated VASP protein that lacked the EVH1 domain (Fig. 4). No interaction of EVH1 domains was detected with PR3 and PR4, or with the empty pAct2 vector. This result was confirmed by biochemical assays. The N-terminal 54 residues of Xin (PR1) were expressed with the EEF-tag, and full-length VASP, the VASP EVH1 domain and VASP without EVH1 domain (VASP-ΔEVH1) with the T7-tag. PR1 showed specific binding to full-length VASP and its EVH1 domain, but not to VASP-ΔEVH1 (Fig. 4A). These results could be confirmed under more native conditions using dot-blot overlays (Fig. 5B). Thus, the specificity of the association of Xin with EVH1 domains was verified.

**Peptide scans identify the Xin sequence that associates with EVH1 domains**

A set of immobilized overlapping synthetic peptides covering the sequence of the aminoterminal 54 amino acids of Xin was used to map precisely the binding site for EVH1 domains. The cDNA encoding the EVH1 domain of VASP was cloned in pGEX-6P3, which allowed expression and purification of this domain fused to GST. Immobilized peptides were overlaid with GST-EVH1, and association of the fusion protein with the peptides was analyzed using a GST-antibody. The peptide that bound the EVH1 domain of VASP most strongly includes the sequence EDLPLPPPPALED, whereas deletion of the carboxy-terminal or aminoterminal acidic residues significantly reduced (peptide 8) or even completely abolished (peptide 6) binding, respectively (Fig. 5C). GST alone showed no binding.

**Xin colocalizes with filamin c, Mena, and VASP in the intercalated discs and nonstriated myofibrils of mammalian cardiac muscle cells**

In the mammalian heart, filamin c is mainly localized in ICD. In contrast to skeletal muscles, only minor amounts of the protein are associated with myofibrillar Z-discs [4]. Immunofluorescence microscopy of cryosections of normal human heart specimens revealed a strict colocalization of Xin with filamin c in ICD with both the XR and XC antibodies (Fig. 6A). Xin did not colocalize with filamin c in myofibrillar Z-discs. Double staining with an antiserum specific for VASP demonstrated Xin colocalization with VASP in ICD (Fig. 6B).

To investigate the localization of Xin during early developmental stages, we stained cultured neonatal rat cardiomyocytes...
with different combinations of antibodies specific for Xin, Mena, filamin, and titin. Whereas the titin antibodies exclusively stained myofibrils, Xin colocalized with filamin c, and filamin c with Mena in immature NSMF and ICD, which were identified using β-catenin antibodies, indicating that all four proteins colocalize in these cells (Figs. 6C–E). Only filamin c was also found in myofibrillar Z-discs, whereas both Xin and Mena were hardly detectable in mature myofibrils. Xin is an actin-binding and stabilizing protein, and its repeats are responsible for the association with F-actin [26]. In the developing and adult mammalian heart, Xin is exclusively found associated with the membrane, and not with the actin filaments of mature myofibrils ([27,42]; this report), indicating a strict regulation of binding of Xin to a specific subpopulation of actin filaments.

To study the localization of Xin isoforms and truncated variants upon transient expression in cultured NRCS, we cloned the full-length ORF of Xin and both truncated variants in a modified EGFP vector. All Xin constructs were incorporated into the NSMF (Figs. 7A–F). Transfection of XinA and XinB, which both contain the actin-binding Xin repeats, resulted in more prominent and elongated NSMF (Figs. 7A, B, and E). This is compatible with the property of Xin repeats to stabilize actin filaments [26]. Furthermore, an obvious targeting of XinA and XinC to ICD was observed (Figs. 7A, C, D, and F), whereas the carboxy-terminally truncated variant XinB that does not contain the filamin c binding site was excluded from these structures (Figs. 7B and E). Interestingly, XinC, which lacks the amino-terminal repeats, was the only construct that was strongly targeted to myofibrillar Z-discs (Fig. 7C). XinA and XinB were excluded from these structures and only localized to the nonstriated parts of the myofibrils. Furthermore, cells

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**Fig. 4** – Characterization of the interaction of the aminoterminus of Xin with EVH1 domains. Yeast cells pretransformed with a pLex vector containing Xin cDNA encoding the aminoterminial 54 amino acids (PR1) were cotransformed with constructs containing fragments of either VASP, Mena, or EVL, as indicated. Cotransformed cells were grown on selective LWH plates and tested for β-galactosidase activity. Note that all constructs with at least the EVH1 domain of either VASP, Mena, or EVL interact (+) with PR1, whereas a truncated VASP protein that lacked the EVH1 domain does not (−). No interaction of EVH1 domains was detected with PR3 + 4, another part of Xin that contains conserved proline-rich sequences.

**Fig. 5** – Biochemical characterization of the interaction of Xin with EVH1 domains. (A) The first three lanes show recombinant VASP fragments detected with an antibody against their T7 immunotag to show that they are intact. The identity of the polypeptides is indicated above each lane. The second set of three lanes shows the identical VASP fragments overlaid with PR1, and binding of the Xin fragment was detected with a monoclonal antibody to its EEF tag. Note that the Xin fragment only binds both VASP constructs containing the EVH1 domain. (B) Purified native Xin PR1, BSA, and Xin PR3 + 4 were immobilized on nitrocellulose and overlaid with T7-tagged VASP fragments that were purified under native conditions. Both VASP fragments containing the EVH1 domain bind PR1, but not BSA or PR3 + 4. (C) Peptides corresponding to the Xin aminoterminus were synthesized on a derivatized cellulose membrane. Each peptide comprised 15 amino acids with sequences overlapping for 12 amino acids starting with amino acids 1–15 of XinA at position 1 and ending with amino acids 40–54 at position 14. Peptide sequences are given with the sequence of the binding peptide in bold. The membrane was incubated with GST-VASP-EVH1 fusion protein and bound protein was detected with a GST antibody.
transfected with XinC remained significantly smaller than cells transfected with XinA or XinB and nontransfected cells.

Discussion

The morphogenesis of the contractile apparatus of cross-striated muscle cells is an impressive example of self-assembly into a higher order biological structure. A hallmark of this process is the complete rebuilding of the actin filament system from stress fiber-like filament bundles to stacks of thin myofilaments characterized by their uniform length and orientation. Obviously, this dramatic reorganization requires tight control by several accessory proteins. A role in this process was implied for filamin c, the muscle isoform of the filamins [43], by its expression pattern in developing human skeletal muscle cells [4,5]. However, the molecular mechanisms by which this important function might be exerted have remained elusive. Here, we describe novel protein interactions of filamin c and Xin, both of which are multiadapter proteins involved in actin filament reorganization during myofibril formation and remodeling.

Initially, we searched for muscle proteins that interact with filamin c by yeast two-hybrid analyses. The first protein

Fig. 6 – Immunolocalization of Xin in the human heart and cultured neonatal rat cardiomyocytes. Cryostat sections of normal human heart specimens were double stained with antibodies specific for (A) Xin (XR1, red) and filamin (green) or (B) Xin (green) and VASP (red). Note that all three proteins precisely colocalize in ICD. Neonatal rat cardiomyocytes were triple stained with antibodies specific for filamin c (green), Mena (red), and M-band titin (blue) (C); with antibodies specific for filamin c (green), Xin (red), and titin (D); or with antibodies specific for filamin c (green), Xin (red), and β-catenin (blue) (E). Note that Mena and filamin c (C) and filamin c, Xin, and β-catenin colocalize in ICD (D, E, arrows) and in NSMF (arrowheads), and that filamin c and Xin staining extends somewhat more into the cytoplasm when compared to β-catenin. Bar, 10 μm.
Mutations in \textit{TTID}, the gene encoding myotilin, cause limb girdle muscular dystrophy 1A \cite{44,45}. The late expression during muscle development implied a function for myotilin mainly in the context of Z-disc maintenance and stabilization rather than early development \cite{29}.

Subsequently, we identified Xin as a novel binding partner for the unique Ig domain 20 of filamin c. Because the (apart from the 80 amino acids long insertion) highly homologous corresponding regions of the nonmuscle filamin isoforms did not bind, we conclude that this insertion represents the actual interacting region. Thus, we identified Xin isoforms in neonatal rat cardiomyocytes. Neonatal rat cardiomyocytes transiently transfected with constructs encoding EGFP fusion proteins of XinA, XinB, or XinC were fixed and stained with an antibody specific for myomesin (A, B) or sarcomeric \(\alpha\)-actinin (C) to identify myofibrils, or with an antibody specific for \(\beta\)-catenin (D–F) to identify ICD, respectively. All Xin isoforms are targeted to immature, nonstriated myofibrils. Whereas XinA and XinC are localized in ICD (arrows in panels A, C, D, F), XinB is excluded from these structures (arrows in panels B, E). Transfection with XinA and XinB, which contain the Xin repeats, results in the stabilization of NSMF that are longer and more prominent than in nontransfected cells. XinC also colocalizes with \(\alpha\)-actinin in Z-discs. Cells transfected with XinC have normal myofibrils, but they often contain aggregates of the polypeptide and most cells do not spread to reach their normal size. Scale bar, 10 \(\mu\)m.
Filamin c interacts with the C-terminal ~160 amino acids of Xin that, except the homologous proteins from other species, lack significant homology to other sequences in databases. Consequently, information about the potential domain structure of this region is unavailable. Nonetheless, transient transfections implied that this domain is sufficient to direct Xin to sites where filamin c is located (Fig. 1D).

The comparative analysis of the Xin cDNA and the CMYA1 gene turned out to be quite surprising. On the one hand, the entire coding region of the ~200 kDa polypeptide is contained in a single large exon; on the other hand, intronic splice sites are used to generate distinct variants of the protein, both being rather unique properties, which are in part shared by the related gene CMYA3 encoding a protein that we had termed XIRP2 [26]. By this unique splicing pathway, at least three functionally very diverse isoforms of Xin arise (Fig. 2) that might be expressed under the control of two different promoters.

Closer inspection of Xin revealed several proline-rich clusters, including a conserved N-terminal motif highly reminiscent of EVH1 domain binding sites. Indeed, yeast two-hybrid experiments and biochemical assays confirmed this predicted interaction (Figs. 4 and 5). Furthermore, immunofluorescence microscopy of cardiac tissue and cultured cardiomyocytes demonstrated colocalization of Xin with filamin c, Mena, and VASP in ICD (Fig. 6). They consist of structurally and functionally divergent parts: while longitudinal portions contain gap junctions, transverse regions contain desmosomes intermingled with adherens junctions, where the ends of myofibrils are anchored to the sarcolemma and force is transmitted to neighboring cells [21]. The major proteins involved in establishing transmembrane contacts in striated muscles are β1-integrins and N-cadherin. Interestingly, previous reports have shown that the filamins are binding partners of β1-integrins [23–25], and that filamin c directly binds γ- and δ-sarcoglycan [15], two transmembrane proteins of the dystrophin-associated protein complex that link dystrophin to the extracellular matrix. A physical association between the dystrophin complex and the integrin adhesion system was already shown [46], and filamin c was suggested to function as a linker [15]. Because Xin was demonstrated to interact with the N-cadherin transmembrane complex [42], it appears that Xin and its binding partners establish a molecular link between the three different transmembrane systems (Fig. 8).

The early expression of Xin during cardiac development and its localization at myofibril-forming areas argues for a role in the actin filament remodeling that occurs during myofibril formation. In line with this, our data show direct binding of Xin to F-actin [26] as well as to filamin c and EVH1 domain-containing proteins like Mena and VASP (this work). Its colocalization with EVH1 proteins is in perfect agreement with the proposed function of Mena and VASP as ‘anti-capping’ proteins that antagonize capping of the barbed end of actin filaments by CapZ [47,48].

Freshly isolated cardiomyocytes contain in the body of the cell fully assembled myofibrils, and the NSMF gradually mature [49,50]. Although we could not detect Xin, Mena, and VASP together with filamin c in the myofibrillar Z-discs, all these proteins colocalized both in submembranous regions that were identified as ICD and in the extended nonstriated myofibril terminals (Figs. 6C and D). These peripheral parts of the

Fig. 8 – Schematic scheme showing the interactions of filamin c, Xin, and Mena/VASP in intercalated discs and myotendinous junctions. The complex is part of intricate subsarcolemmal structures that indirectly link the actin filaments that extend from the terminal Z-disc of the myofibrils to the sarcolemma and via three different types of transmembrane proteins to the
neighboring cells or the extracellular matrix (not depicted).
myofibrils are laterally attached to the sarcolemma [51] and contain several myofibrillar proteins that are not yet organized into sarcomeres [49,52]. This indicates that Xin and filamin c act as adapter proteins involved in both, controlling the dramatic reshaping of the actin filament system concomitant with myocyte differentiation, and the attachment of myofibrils to specialized membrane regions. A similar function was proposed for N-RAP [53,54]. On the other hand, the localization of N-RAP in the adult heart and cultured cardiomyocytes resembles that of Xin. On the other hand, the expression of Xin was already detected in the mouse heart at embryonic day 8.0 [42], whereas N-RAP was not detected before E10 [55], indicating that Xin is already functional during very early stages of in vivo muscle development. Analogous to Xin, N-RAP contains repeats (in the case of N-RAP nebulin-like repeats) that bind F-actin and is a ligand of several ICD proteins (filamin c, talin, and vinculin) [54,55]. N-RAP [56] and Xin (our unpublished observations) are tightly associated with the cytoskeleton and essentially insoluble, which eliminates the possibility to study in vivo protein interactions by co-immunoprecipitation.

Transient expression of XinA in cardiomyocytes showed that it is targeted to structures that were also stained with the Xin antibodies. XinB, which lacks the filamin c-binding site, binds NSMF, but seems to be excluded from ICD. Transfection of XinC, which lacks the actin-binding Xin repeats but contains the filamin c-binding site, results in an obvious targeting not only to NSMF and ICD, but also to Z-discs. These results indicate that binding to filamin c is essential for targeting to ICD. Furthermore, the presence of the Xin repeats seems to be sufficient to target a protein to NSMFs but not to Z-discs. Overexpression of XinC often leads to abnormal spreading of cardiomyocytes (Figs. 7C and F). These data indicate that the three different Xin isoforms have discriminative functions in the assembly of the contractile apparatus and its attachment to the membrane.

During adulthood, the complex of filamin c–Xin–Mena/VASP could be involved in mechanosensory processes, capable of transforming, for example, increased mechanical load into building additional sarcomeres. In this respect, it is interesting to note that the addition of extra sarcomeres, observed to occur in skeletal muscles after eccentric contractions, is accompanied by increased levels of Xin mRNA [57,58]. Furthermore, observations made in a genome-scale expression analysis lead to naming the gene coding for Xin CMYA1—‘cardiomyopathy associated 1’ [59]; see also GenBank AW755250). The interesting possibility that the expression of one or more of the Xin variants is indeed altered in human cardiomyopathies clearly awaits the characterization of Xin isoform expression patterns in myopathic heart specimens.

Both Mena and VASP were shown to be localized in the fascia adherens junctions of cultured epithelial cells, and it was suggested that these proteins play a role in the reorganization of the actin cytoskeleton necessary for epithelial sheet formation [60]. Interestingly, upon transient expression of Xin in epithelial cells [26], the protein is targeted to structures that are highly homologous to the adhesion zippers described by these authors, indicating that the protein complex cadherin–catenin–Xin–Mena/VASP–filamin c in the adherens junctions of the heart is also involved in developing the force necessary to efficiently assemble these structures. In the early heart, stable cell–cell contacts are indispensable for cardiac looping, and it is precisely this process that is disturbed in chick embryos treated with Xin antisense oligonucleotides [27].

Recently we have identified a mutation within the FLNC gene that is causative for filaminopathy, a novel form of myofibrillar myopathy [10]. The mutated filamin c cannot dimerize, and consequently skeletal muscle fibers contain large aggregates of filamin c and several other proteins. Surprisingly, affected individuals do not have any cardiac disease. Similarly, mice with Mena [61] or VASP [62,63] null mutations do not seem to have an apparent cardiac phenotype. However, experimental displacement of Mena and VASP from ICD resulted in dilated cardiomyopathy associated with cardiomyocyte hypertrophy, bradycardia, and early postnatal death [64]. A null mutation of N-cadherin, a further component of the Xin–complex [42], leads to dramatic defects within the developing heart tube [65], and an induced deletion of the N-cadherin gene in the adult heart results in defective anchorage of myofibrils to the sarcolemma and disintegration of the ICD [66].

In summary, the identification of the interactions of filamin c–Xin–Mena/VASP provides a first glance on the molecular events that control developmental and adaptive remodeling of the actin cytoskeleton in cross-striated muscle cells. Together with the observation that the lack of Mena and VASP in ICD leads to severe heart failure, these findings reinforce the idea that the genes encoding the proteins from this complex are candidate genes for cardiac diseases, in particular of cardiomyopathies associated with developmental defects or hypertrophy. Thus, it will be interesting to determine if such patients have disease-causing mutations in the genes coding for filamin c and Xin.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.yexcr.2006.03.015.

REFERENCES


