Melusin Is a New Muscle-specific Interactor for β1 Integrin Cytoplasmic Domain*

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Here we describe the isolation and partial characterization of a new muscle-specific protein (Melusin) which interacts with the integrin cytoplasmic domain. The cDNA encoding Melusin was isolated in a two-hybrid screening of a rat neonatal heart library using β1A and β1D integrin cytoplasmic regions as baits. Melusin is a cysteine-rich cytoplasmic protein of 38 kDa, with a stretch of acidic amino acid residues at the extreme carboxyl-terminal end. In addition, putative binding sites for SH3 and SH2 domains are present in the amino-terminal half of the molecule. Chromosomic analysis showed that melusin gene maps at Xq12.1/13 in man and in the synthetic region X band D in mouse. Melusin is expressed in skeletal and cardiac muscles but not in smooth muscles or other tissues. Immunofluorescence analysis showed that Melusin is present in a costamere-like pattern consisting of two rows flanking α-actinin at Z line. Its expression is up-regulated during in vitro differentiation of the C2C12 murine myogenic cell line, and it is regulated during in vivo skeletal muscle development. A fragment corresponding to the tail region of Melusin interacted strongly and specifically with β1 integrin cytoplasmic domain in a two-hybrid test, but the full-length protein did not. Because the tail region of Melusin contains an acidic amino acid stretch resembling high capacity and low affinity calcium binding sites, we tested the possibility that Ca2+ regulates Melusin-integrin association. In vitro binding experiments demonstrated that interaction of full-length Melusin with detergent-solubilized integrin heterodimers occurred only in absence of cations, suggesting that it can be regulated by intracellular signals affecting Ca2+ concentration.

Integrins are heterodimeric αβ membrane receptors that link extracellular matrix proteins to cytoskeletal elements controlling adhesive and motile behavior of cells. They are also crucial in transfering signals that affect cell proliferation and differentiation. Both the ability to interact with cytoskeletal proteins and to generate intracellular signals depends on the integrin cytoplasmic domain that consists of short amino acid sequences, devoid of enzymatic activity. Mutational analyses have shown that the β1 subunit cytoplasmic domain is responsible for the localization of the integrin heterodimer in focal adhesions, the sites where actin filaments are connected to the plasma membrane (1, 2). β1 cytoplasmic domain interacts with several cytoskeletal and signaling molecules such as talin, filamin, α-actinin, paxillin, and p125*AK as shown by in vitro binding assays (3, 4). All these proteins are selectively concentrated at focal adhesions, and their association with integrins in vivo is likely to require the organization of supramolecular complexes. Using the two-hybrid system, new proteins such as the serine-threonine kinase ILK (Integrin Linked Kinase) (5), ICAP (Integrin Cytoplasmic Domain Associated Protein) (6, 7), and RACK1 (Receptor for Activated Protein Kinase C) (8) were shown to bind directly to the β1 integrin cytoplasmic domain. Analysis of the integrin cytoplasmic domain indicated the existence of four different splicing variants referred as β1A, B, C, and D (9). Whereas β1B and β1C are rare isoforms expressed at low level only in human species, the β1A is the most widely expressed isoform and β1D is selectively expressed in striated muscle tissues where it represents the only β1 integrin splice variant (10). We have previously shown that β1D cytoplasmic domain endows this isoform with higher binding affinity for both cytoskeletal and extracellular matrix proteins, indicative of the ability of β1D to form stable cytoskeleton/matrix connections (11).

In muscle tissue, the membrane-actin cytoskeleton interaction occurs at myotendinous junctions and costamers, two highly specialized junctional complexes. At myotendinous junctions, actomyosin filaments are anchored end-on to the plasma membrane, whereas at costamers they are joined laterally. Integrins are selectively enriched both in myotendinous junctions and costamers (10, 12, 13), suggesting an important role of these receptors in connecting the cytoskeleton to the extracellular matrix in muscles. Direct evidence of the role of integrins in muscle function and in actin organization also comes from gene knockout experiments. In Drosophila, lack of integrin β subunit expression causes muscle detachment from its attachment points when the first contraction occurs (14). Gene knockout experiments in mice indicated that β1 is not essential for myoblast fusion during in vitro myogenic differentiation (15), but an impaired sarcomere cytoarchitecture is observed in β1-null cardiomyocytes derived by in vitro differentiated embryonic stem cells (16). Moreover, mice lacking expression of α7, the major muscle integrin α subunit, develop muscular dystrophy postnatally and display major alterations in the muscle-tendon junction (17).

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Experiments directed to investigate whether integrins are involved in the formation of the sarcomeres or in their stabilization after formation indicate that localization of integrins to actin-membrane junctions occurs once the organization of actin in sarcomeres has already occurred (18, 19). These data strongly suggest that the localization of integrins at myotendinous junctions and costamers is driven by the organization of actin inside the cells. Their presence at these sites is crucial for the mechanical stabilization of these junctions as indicated by the gene knockout experiments (16, 17).

To better understand the mechanisms of integrin-cytoskeletal interactions, we searched for muscle proteins capable of interacting with the β1 integrin cytoplasmic domain. Using a two-hybrid screening, we isolated a new muscle-specific interactor capable of binding both β2A and β2D isoforms, but not other integrin β subunits.

**MATERIALS AND METHODS**

**Interaction Trap**—Screening for proteins that interact with cytoplasmic tails of β2A and β2D was performed as described (20). To construct bait plasmids encoding amino acids 722–788 and amino acids 752–801 of β2D were amplified by PCR1 using primers containing EcoRI and BamHI site on either ends (β2, 5′-GGAATTC-AAGCTTTAATGATAATT-3′ and 5′-CCGGAATCTCATTTCCTCTCATCATTCT-3′; β2D, 5′-GGAATTCAGGTGTTTATGATAATT-3′ and 5′-CCGGAATCTCAGGGACCGTCTTAC-3′) and cloned in pEG202 vector in frame with LexA coding sequence. Bait plasmids were unable to activate transcription when cotransformed in EGY48 yeast strain with in frame with LexA coding sequence. Both plasmids were unable to control baits such as bicoid, bFGF, a ciclyn A, and c-Myc (a gift from A. Z. V.). Other baits were produced to test the ability of Melusin to interact with other integrin cytoplasmic domain.

**Sequence and CDNA Cloning**—Positive clones were sequenced using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). D3–2 and D7–2 DNA fragments containing overlapping sequences of Melusin were used to isolate the full-length cdNA from a human skeletal muscle library in λgt10 (CLONTECH) and a mouse skeletal muscle library in λgt11 (CLONTECH). Human and mouse clones were subcloned in pBluescript II SK+ and sequenced. Sequenced with the BAC (Bacterial Artificial Chromosome) server at the National Center for Biotechnology Information. Our cdNA contains the complete Melusin cdNA sequence as indicated by the fact that mouse cdNA transfect COS cells show a band which co-migrates with the endogenous Melusin present in differentiated C2C12 myogenic cells. The first atg of the sequence was considered the putative start codon. Analysis of mouse genomic DNA sequences showed that two stop codons are present 18 and 30 nt upstream of this atg. This genomic region just upstream of the start codon is not present in dbEST (23) database that contains randomly expressed sequences.

**Northerm Blot**—RNA from C2C12 cells and from mouse embryo and neonatal skeletal muscle was extracted using RNeasy Mini Kit (Qiagen Inc.). Adult skeletal muscle RNA was extracted according to Chirgwin et al. (21). 20 μg of total RNA from each sample was run on 0.8% agarose-formaldehyde gels and transferred to N+ nylon membranes (Amersham Pharmacia Biotech). Poly(A)1 RNA isolated from human and mouse tissues and immobilized onto nitrocellulose filters after electrophoretic separation was obtained from CLONTECH (Multiple Tissue Northern blot). Filters were probed at 65 °C with D3–2 insert labeled with 32P using a random prime labeling system (Repligene II, Amersham Pharmacia Biotech) and were washed twice with 2× SSC, 1% SDS and twice with 0.4% SSC, 1% SDS at 65 °C, and exposed to x-ray film.

**In Situ Hybridization**—Human metaphase spreads were obtained from PHA-stimulated peripheral lymphocytes of a normal donor by standard procedures. Mouse spreads were prepared from a mouse cell line expressing a large multiple were cultured overnight and prepared for in situ hybridization using an easy identification of the mouse chromosome (25). The cell line was a generous gift from Dr. H. Hameister (Ulm, Germany). Full-length human Melusin cdNA and 14.8-kilobase mouse genomic DNA fragment spanning the atg-containing exon and the three following ones were used as probes. Chromosome preparations were hybridized in situ with probes labeled with biotin nick translation, essentially as described by Lichter et al. (26), with minor modifications. Briefly, 500 ng of labeled probe were used for the FISH experiments; hybridization was performed at 37 °C in 2× SSC, 50%, 10/100 dextran sulfate, 5 μg COTI DNA (Roche Molecular Biochemicals), and 3 μg of sonicated salmon sperm DNA in a volume of 10 μl. Post-hybridization washing was performed at 42 °C in 2× SSC, 50% formamide (×3) followed by three washes in 0.1× SSC at 60 °C. Biotin-labeled DNA was detected with Cy3-conjugated avidin (Amersham Pharmacia Biotech). Chromosome identification was obtained by simultaneous DAPI staining, that produces a q-band pattern.

**Antibody Preparation**—GST-Melusin fusion protein was produced by expressing the entire sequence of the human Melusin cloned in pGEX 4T2 (Amersham Pharmacia Biotech) in *Escherichia coli* BL21 bacterial strain. Human Melusin was purified on glutathione-Sepharose 4B (Amersham Pharmacia Biotech), and elution was performed following the recommendations of the manufacturer. Rabbits were immunized by repeated intramuscular injections of the purified fusion protein (500 μg) suspended in Complete Freund Adjuvant. Specificity of the antibody was demonstrated in Western blots on protein extracts from wild type and Melusin cDNA-transfected COS cells. To affinity purify antibody, rabbit serum, Melusin was fused to maltose-binding protein (MBP) by cloning the cdNA into the pMALp2 vector (New England Biologial). The MBP-Melusin fusion protein was purified on an amylose column according to the instructions of the manufacturer and coupled to Sepharose. Antibodies were adsorbed on the MBP-Melusin-Sepharose column and eluted with 50 mM glycine-HCl buffer.

**Cell Culture and Western Blot**—C2C12 mouse skeletal muscle cell line was maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Cells were induced to differentiate into myotubes by switching to culture medium with 2% horse serum.

Western blots on cell and tissue extracts were performed as follows. Cells were washed twice with PBS and lysed in Tris-buffered saline (TBS) containing 0.5% Triton X-100 and the following protease inhibitors: 10 μg/ml leupeptin, 4 μg/ml pepstatin, and 0.1 TIU/ml aprotinin for 10 min at 4 °C. Extracts were centrifuged at 14,000 rpm for 10 min to remove insoluble material. Tissues were frozen and triturated in liquid nitrogen and extracted in lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 8, 5 mM EDTA, 1% Nonidet P-40, 10 μg/ml leupeptin, 4 μg/ml pepstatin, and 0.1 TIU/ml aprotinin. Tissue extracts were sonicated three times for 10 s and centrifuged at 14,000 rpm for 10 min to remove insoluble material. Protein concentration was determined using Bio-Rad assay. 60 μg of every protein extract were separated on polyacrylamide gel in presence of SDS and subsequently blotted to nitrocellulose membranes. Membranes were saturated with TBS, 5% BSA and incubated in TBS, 1% BSA containing primary antibody overnight at 4 °C. After washing, the filters were incubated with peroxidase-conjugated secondary antibody for 2 h at room temperature,
and detection was performed with chemiluminescent substrate ECL (Amer sham Pharmacia Biotech).

**Muscle Regeneration**—Adult CBA male mice were anesthetized with Avertin (17 μl of 2.5% Avertin per gram of body weight). Tibialis anterior muscles were exposed, and degeneration was induced by deep freezing with liquid nitrogen-cooled steel rod according to Toyota et al. (27). Muscles were surgically removed 3, 6, 9, and 12 days after freezing, and protein extracts were obtained as described above. 100 μg of every total extract were separated on polyacrylamide gel, and equal loading was verified by Ponceau red staining. Untreated tibialis anterior muscle was used as control.

**Inclusion Interactor**—1-week old mouse limb muscles and soleus muscle from 6-months old mice were collected and fixed in PBS, 4% paraformaldehyde for 2 h at room temperature. After washing in PBS and PBS, 15% sucrose for cryoprotection, muscle fragments were frozen in liquid nitrogen in Embedding Medium Compound (Bio-Optica S.p.a.). 10-μm cryosections were collected on polylysin-subbed slides. Sections were saturated with goat serum 1:100 in PBS, 1% BSA and incubated overnight at room temperature with primary antibody in PBS, 1% BSA, followed by 2-h incubation with fluorochrome-conjugated secondary antibody. The following primary antibodies were used: 5 μg/ml affinity purified rabbit anti-Melusin and 5 μg/ml monoclonal antibody EA-53 to sarcomeric α-actinin (Sigma). Secondary antibodies specific for rat or mouse IgG were labeled with fluorescein, while antibodies specific for rabbit IgG were labeled with Texas red (Molecular Probes). The species specificity of the secondary antibodies was cross-tested. Negative controls, rabbit and mouse IgG were used as controls and resulted in negative staining. Samples were observed under Olympus fluorescence microscope, and pictures were taken with an Olympus DP10 digital photomicrography system. Confocal images were obtained with Olympus IX70 inverted confocal laser scanning microscope equipped with a krypton-argon ion laser (488/568 nm).

**Results**

**Isolation of Melusin Full-length cDNA**—Using D3–2 cDNA fragment as probe we isolated human and mouse full-length Melusin cDNAs from human and mouse skeletal muscle libraries. The human clone is 1235 nt in length and conceptual translation of this sequence revealed the presence of an open reading frame of 347 amino acids (GenBankTM AF140690). The mouse cDNA was 1420 nt in length, with an open reading frame coding for 350 amino acids (GenBankTM AF140691), with 92% identity with the human amino acid sequence. The D3–2 and D7–2 cDNA fragments isolated by the two-hybrid screening were found to code for amino acid residues 211–350 and 164–350 respectively.

Sequence analysis by BLAST homology search (22) revealed no evident homology with any other known protein. Inspection of the sequence indicated the presence at the extreme carboxy-terminal portion of the molecule of a region highly enriched in aspartic and glutamic acid residues. Analysis by FTHOM domain homology search (22, 28, 29) indicated that this acidic sequence of Melusin closely resembles calreticulin and calsequestrin C-domain, known to bind calcium at high capacity and low affinity (30, 31). At the amino-terminal end, Melusin contains two cysteine rich repeats spaced by an intervening sequence of approximately 90 amino acid residues. The cysteine residues contained in the cysteine repeats are characteristically spaced with a pattern that was not found in other proteins (Fig. 1B). Moreover, four distinct PXXP motifs, representing the minimal consensus sequence recognized by SH3 domains (32, 33) and two XXXI/P sequences, putative binding sites for SH2 domains (34), are scattered in the amino-terminal half of the molecule.

The chromosomal localization of the Melusin gene was also investigated, both in man and in mouse. Fish analysis clearly showed that the gene is localized on the X chromosome in both species, respectively at Xq12.1–13 and at X band D.

**Regulation of Melusin Expression during Myogenic Differentiation**—To investigate the expression of this new gene, poly(A)+ RNA from human and mouse tissues was analyzed by Northern blotting with a Melusin probe. A single transcript of 1.4 kilobases was detected in human skeletal and cardiac muscles, whereas no hybridization occurred in all other tested tissues (Fig. 2A). Identical expression pattern was detected in mouse tissues (not shown). Analysis of the Melusin protein by Western blotting with polyclonal antibodies raised against a GST-Melusin fusion protein confirmed the specific expression in striated muscles (Fig. 2B).

To evaluate if Melusin expression was regulated during muscle differentiation, we analyzed the C2C12 myogenic cell line that can be induced to differentiate to form myotubes by serum starvation. Melusin expression was tested both by Western and Northern blotting. As shown in Fig. 3A, Melusin was absent in undifferentiated myoblasts, and its expression was turned on in differentiated myotubes after 6 days of serum starvation.

Melusin expression was also examined during mouse embryonic development in vivo. Melusin protein and mRNA became detectable in embryo limbs at day 15 (E15), reached a maximum in newborn mice, and declined in adult limb muscles (Fig. 3B). In adult muscles a doublet of protein bands was detected by Western blotting, suggesting possible posttranslational

**Muscle-specific Interactor for β1 Integrin** (Amer sham Pharmacia Biotech).
modifications. During heart development, on the other hand, Melusin level remains steady with no major changes in expression from embryonic day 15 to adult stage.

To investigate if Melusin expression is regulated in regenerating adult muscle, we induced regeneration of mouse tibialis anterior following freeze injury. 3, 6, 9, and 12 days after freeze trauma, muscles were collected and Melusin expression was investigated by Western blot analysis on total protein extracts using normal muscle as control. As shown in Fig. 3C, Melusin is up-regulated from day 6 on during muscle regeneration, consistent with a role of this molecule in myogenetic processes.

Subcellular Localization of Melusin by Immunofluorescence—Using affinity purified Melusin antibody, we performed immunofluorescence analysis on newborn and adult mouse muscle cryosections. Fluorescence was localized only on skeletal muscle and not in the surrounding tissues, confirming the muscle-specific expression. Longitudinal sections of posterior leg muscles from 1-week old mice clearly revealed a striated pattern of two Melusin rows flanking the α-actinin band in the Z disc (Fig. 4). In adult soleus muscle, the striated localization of Melusin was still detectable although to a lower intensity compared with newborn muscles (Fig. 4).

Mapping of the β1 Integrin-Melusin Interaction Sites with the Two-hybrid Test—The integrin β subunit cytoplasmic domains are highly homologous to each other (3). Using the two-hybrid test, we analyzed whether Melusin was capable of interacting with β subunits other than β1. Using the protein fragment coded by plasmid D3–2, corresponding to the carboxyl-terminal of Melusin (tail domain) as a bait, interaction occurred with β1 but not with β2 and β3 cytoplasmic domains (Table I). Moreover, no interaction occurred with the integrin α5 cytoplasmic domain used as control (Table I). The β1 cytoplasmic domain consists of a 26-amino acid long membrane proximal subdomain common to all known isoforms and of variable carboxyl-terminal subdomains specific for each splicing variant (35). As shown in Table I, the interaction occurred with all three tested isoforms (β1A, β1B, and β1D) suggesting that the interaction involved the common subdomain. To further test this hypoth-

FIG. 1. Amino acid sequence of Melusin. A, the amino acid sequence was deduced from the full-length human and mouse cDNAs. Identical residues are indicated by vertical bars and conservative substitutions by double dots. The two cysteine-rich domains are underlined, and the acidic amino acid stretch at the extreme carboxy-terminal is double underlined. PXXP sequences, putative SH3 domain binding motifs, are boxed. YXXY/P sequences, putative SH2 binding sites are shown in dashed line boxes. B, the spacing of cysteine residues in the two cysteine-rich motifs in mouse and human Melusin are indicated.

FIG. 2. Expression of Melusin is restricted to striated muscle tissue. A, poly(A)+ RNA from different human tissues was hybridized with D3–2 rat probe isolated with the two-hybrid screening as described under "Materials and Methods." A single band of approximately 1.4 kilobases was present only in heart and skeletal muscles. B, protein extracts from newborn mouse tissues were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filter. The filter was probed with polyclonal antibody raised against a GST-Melusin fusion protein as described under "Materials and Methods." A band of 38 kDa was detected in skeletal muscle and heart.

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In this work we describe a new muscle-specific protein, Melusin, interacting with the cytoplasmic domain of the β₁ integrin subunit. Melusin is expressed in striated skeletal and cardiac muscles, both at mRNA and protein level, but it is undetectable in all other tested tissues including gut smooth muscle, brain, placenta, lung, liver, kidney, and pancreas. Its expression appears to be regulated during myogenesis both in vitro and in vivo. In fact, Melusin was undetectable in cultured proliferating myoblasts, but it is highly expressed in differentiated myotubes. During in vivo skeletal muscle myogenesis, Melusin starts to be detectable in 15-day old embryos, and its level peaks in newborn mice. In adult skeletal muscle tissue...
Binding of integrin subunit cytoplasmic domains to melusin and its tail domain

Baits consisting of different β integrin cytoplasmic domains were used in the two-hybrid system to measure interaction with the D3–2 melusin fragment (coding for the tail domain, amino acid residues 201–350) and the full-length melusin protein. β1A, β1B, and β1D are different splicing variants of β1 integrin. β1Com is a mutant containing the cytoplasmic subdomain common to all splicing variants (35). α3 integrin subunit cytoplasmic domain was used as control.

| Baits | Cytoplasmic domain sequences | D3–2 | Melusin |
|-------|-------------------------------|------|--------|
| β1A   | KLLMIHDRREFAKFEKEMNAKWDTVSRYKSWQSGL | + + + | - |
| β1B   | KLLMIHDRREFAKFEKEMNAKWDTVSRYKSWQSGL | + + + | - |
| β1D   | KLLMIHDRREFAKFEKEMNAKWDTVSRYKSWQSGL | + + + | - |
| β1Com | KLLMIHDRREFAKFEKEMNAKWDTVSRYKSWQSGL | + + + | - |
| α3    | KLGFKRSFLPYGTAEMEQAQLKPATSDA | - | - |

Interaction of different melusin constructs with β1 integrin

Different melusin constructs used are schematized. The ability of the constructs to bind the β1 integrin cytoplasmic domain in the two-hybrid test or the intact integrin complexes from COS cell extracts are indicated. nt, not tested.

| CONSTRUCTS | RESIDUES | TWO HYBRID | IN VITRO BINDING |
|------------|----------|------------|------------------|
| Melusin full-length | 1-350 | - | + |
| D3-2 | 164-350 | + | nt |
| D3-2A | 211-320 | + | nt |
| D3-2A | 211-320 | + | nt |

![Fig. 5. Interaction of Melusin with integrins is Ca²⁺ dependent.](image)

The amino acid sequence of Melusin revealed four domains. The protein consists of 347 and 350 amino acid residues in man and mouse, respectively, with a 92% identity (96% considering conservative substitutions). A 55-amino acid long domain, containing a unique cysteine-rich motif, is repeated twice in the molecule. These repeats share 42% identity among each other, while the cysteine pattern is conserved. Interestingly, in human Melusin, the first repeat contains an extra cysteine residue immediately adjacent to cysteine 3. This is not a sequence polymorphism or a mutation in our clones but is conserved. The second cysteine-rich domain is repeated twice in the molecule. These repeats share 42% identity among each other, while the cysteine pattern is conserved. This pattern of localization suggests that Melusin is a component of the actin-integrin junctional complex in muscle.

The level of expression slightly declines, and in Western blotting a doublet of bands becomes visible, suggesting that the molecule undergoes post-translational modifications. The doublet of bands could also be indicative of alternatively spliced isoforms of the protein, but reverse transcription PCR analysis of adult and neonatal muscle with primers covering the entire length of the molecule did not reveal the existence of alternatively spliced forms. This conclusion is also supported by the presence of a single band in Northern blot analysis from both newborn and adult mice (see Fig. 3B). The highest expression level of Melusin in skeletal muscle coincides with secondary myogenesis, a process in which a distinct myoblast population line up using primary myotubes as scaffold and fuse to each other forming secondary myotubes that will give rise to the muscle fibers of adult tissue. High level of Melusin expression was also observed in regenerating adult tibialis anterior muscle, further suggesting that Melusin might play a crucial role during maturation and/or organization of muscle cells. A possible role in myoblast fusion seems unlikely because Melusin is also expressed in heart where cardiomyocytes do not undergo cell fusion. The two-hybrid test showed that the tail domain of Melusin binds equally well to the cytoplasmic domain of both β1A and β1D integrin isoforms. These two isoforms are differentially expressed during muscle development (36). β1A is expressed in muscles during embryonic development and is down-regulated after birth. On the other hand, the β1D isoform starts to appear in skeletal muscle in 17-day embryos and becomes the only β1 isoform in adult muscles. The ability to bind β1A and β1D integrin isoforms allows Melusin to interact with integrins both in developing and in adult muscles. Immunofluorescence analysis showed that Melusin is localized in rows flanking the Z line containing α-actinin. Similar pattern has been described for vinculin (37) and β1 integrin (12, 13, 10) and is thought to correspond to sites of lateral interaction of actin with the plasma membrane known as costameres (37, 38). This pattern of localization suggests that Melusin is a component of the actin-integrin junctional complex in muscle.
residues and contains a stretch of 18/20 negatively charged amino acids at the extreme carboxyl-terminal. Similar acidic carboxy-terminal sequences are present in calsequestrin (31) and calreticulin and are shown to bind Ca\(^{2+}\) ions with high capacity and low affinity (30). As detected in the two-hybrid screening, the tail domain was sufficient to bind \(\beta_1\) cytoplasmic region, and deletion experiments allowed to exclude a role of the acidic amino acid stretch in this process (see Table II). Interestingly, the full-length Melusin protein was unable to interact with \(\beta_1\) cytoplasmic domain in the two-hybrid system (Tables I and II). In vitro binding experiments showed that the interaction of Melusin with integrins is regulated by divalent cations, and it occurs only in the absence of Ca\(^{2+}\) (Table II). It is possible that Ca\(^{2+}\) directly competes for binding to integrins. However, this is not the case, in fact, the presence of Ca\(^{2+}\) ions did not prevent binding of the truncated Melusin C-term fragment (see Table II). In addition, the acidic amino acid stretch of Melusin, that it is likely to bind Ca\(^{2+}\) ions, is not required for integrin binding (see Table II). Thus the most likely explanation is that Ca\(^{2+}\) modulates the conformation of Melusin exposing the integrin binding site located in the tail domain. In this model the amino-terminal region of Melusin masks the integrin binding site present in the tail domain of the molecule, and removal of Ca\(^{2+}\) releases this inhibition. These data suggest that Melusin-integrin interaction depends on Ca\(^{2+}\) concentration and can thus be regulated by intracellular alteration of Ca\(^{2+}\) level in response to extracellular stimuli.

 Whereas Melusin tail domain is responsible for the interaction with \(\beta_1\) integrin, the amino-terminal portion of the molecule can possibly bind to SH3- and SH2-containing proteins, as suggested by the presence of multiple proline-rich motifs and tyrosine phosphorylation sites. These properties suggest that Melusin could be an important molecular link between integrin receptors and cytoskeletal or transducing proteins in muscle cells.

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