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Melusin Is a New Muscle-specific Interactor for β_1 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 β_1A and β_1D 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 synthenic 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 β_1 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 domains, we tested the possibility that Ca^{2+} 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 Ca^{2+} concentration.

Integrins are heterodimeric $\alpha\beta$ membrane receptors that link extracellular matrix proteins to cytoskeletal elements controlling adhesive and motile behavior of cells. They are also crucial in transferring 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 β_1 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). β_1 cytoplasmic domain interacts with several cytoskeletal and signaling molecules such as talin, filamin, α-actinin, paxillin, and $p125^{FAK}$ 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 β_1 integrin cytoplasmic domain. Analysis of the integrin cytoplasmic domain indicated the existence of four different splicing variants referred as β_1A, -B, -C, and -D (9). Whereas β_1B and β_1C are rare isoforms expressed at low level only in human species, the β_1A is the most widely expressed isoform and β_1D is selectively expressed in striated muscle tissues where it represents the only β_1 integrin splice variant (10). We have previously shown that β_1D cytoplasmic domain endows this isoform with higher binding affinity for both cytoskeletal and extracellular matrix proteins, indicative of the ability of β_1D 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 β_1 is not essential for myoblast fusion during *in vitro* myogenic differentiation (15), but an impaired sarcomere cytoarchitecture is observed in β_1-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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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF140690 and AF140691.

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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 β_1 integrin cytoplasmic domain. Using a two-hybrid screening, we isolated a new muscle-specific interactor capable of binding both β_1A and β_1D isoforms, but not other integrin β subunits.

MATERIALS AND METHODS

Interaction Trap—Screening for proteins that interact with cytoplasmic tails of β_1A and β_1D was performed as described (20). To construct bait plasmids, sequences encoding amino acids 752–798 of β_1A and amino acids 752–801 of β_1D were amplified by PCR[1] using primers containing *Eco*RI and *Bam*HI site on either ends (β_1A, 5′-GGAATTC-AAGCTTTTAATGATAATT-3′and 5′-CGGGATCCTCATTTTCCCTCA-TACTT-3′; β_1D, 5′-GGAATTCAAGCTTTTAATGATAATT-3′and 5′-CG-GGATCCTCAGAGACCAGCTTTACG-3′) and cloned in pEG202 vector in frame with LexA coding sequence. Both plasmids were unable to activate transcription when cotransformed in EGY48 yeast strain with pSH18–34 reporter plasmid (data not shown). We confirmed the expression of these fusion proteins in yeast total protein extracts by Western blot analysis using an anti-LexA antibody (a gift from A. Zervos). A cDNA library from heart neonatal rat fused to a galactose-inducible activation domain (a gift from A. Zervos) was transformed in yeast strain EGY48 that already contained pSH18–34 reporter plasmid and pEG202-β_1A or -β_1D using a LiAc high efficiency transformation protocol (21). Primary transformants (2×10^6) were screened by plating 10 million colonies on selectable medium lacking Ura, His, Trp, and Leu. Several positive clones for β_1A and β_1D were isolated, and their specific binding to integrins was tested by assaying the interaction with control baits such as bicoid, bFGF, a ciclyn A, and c-Myc (a gift from A. Zervos).

Other baits were produced to test the ability of Melusin to interact with other integrin cytoplasmic tails: sequences encoding amino acids 724–769 of β_2 (5′-CATGCCATGGAAGGCTCTGATCCACCTG-3′ and 5′CCGCTCGAGCTAACTCTCAGCAAACTT-3′), 716–762 of human β_3 (5′-GCATGCCATGGAAACTCCTCATCACCATC-3′ and 5′-CCGCTCG-AGTTAAGTGCCCCGGTACGT-3′), 752–789 of β_1B (5′-GGAATTCAA-GCTTTTAATGATAATT-3 and 5′-CGGGATCCTTATAAGCCACTTTG-CTT-3′), 752–777 of β_1 common region (5′-GGAATTCAAGCTTTTAAT-GATAATT-3 and 5′-GGATCCTCACGTGTCCCATTTGGC-3′), and 1022–1049 of α_5 (5′-GGAATTCAAGCTTGGATTCTTCAAA-3′ and 5′-C-GGGATCCTCAGGCATCAGAGGTGGC-3′) were amplified by PCR and cloned in frame with LexA coding sequence in pEG202.

To map the integrin binding site in Melusin, an additional construct was created. The Melusin cDNA fragment coding for amino acids 211–320 (D3–2Δ) was amplified by PCR (5′-CGGAATTCTGGGCAAAGCA-GCTGCCA-3′ and 5′-CCCTCGAGTTATAGTAAAACCCCTGCCCT-3′) and cloned in frame with the B42 transactivation domain in pJG4–5.

Sequencing 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 cDNA 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). Positive human and mouse clones were subcloned in pBluescript II SK$^-$ and sequenced. Sequences were analyzed with the BLAST (22) server at the National Center for Biotechnology Information. Our cDNA contains the complete Melusin coding sequence as indicated by the fact that mouse cDNA transfected 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 putative atg. This genomic region just upstream of the start codon is not an intronic sequence because it is present in dbEST (23) data base that contains randomly expressed sequences.

Northern 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 Chomczynsky and Sacchi (24). 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)$^+$ 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 ^{32}P using a random prime labeling system (Rediprime 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 containing multiple well characterized Robertsonian translocations allowing an easy identification of the mouse chromosomes (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% (v/v) formamide, 10% (w/v) dextran sulfate, 5 μg COT1 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-banding 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. GST-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 antiserum was demonstrated in Western blots on protein extracts from wild type and Melusin cDNA-transfected COS cells. To affinity purify antibodies from rabbit serum, Melusin was fused to maltose-binding protein (MBP) by cloning the cDNA into the pMALp2 vector (New England Biolabs). 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 pH 3 glycin-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,

[1] The abbreviations used are: PCR, polymerase chain reaction; dbEST, data base of expressed sequence tags; GST, glutathione *S*-transferase; MBP, maltose binding protein; bFGF, bovine fibroblast growth factor; nt, nucleotide(s); DAPI, 4,6-diamidino-2-phenylindole; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; TIU, trypsin inhibitory units; BSA, bovine serum albumin; Cterm, carboxyl-terminal.

and detection was performed with chemiluminescent substrate ECL (Amersham 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.

Immunofluorescence—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 polylysine-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 specie specificity of the secondary antibodies was cross-tested. Non-immune 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 1X70 inverted confocal laser scanning microscope equipped with a krypton-argon ion laser (488/568 nm).

Melusin-Integrin in Vitro Binding Assay—GST fusion proteins were prepared by cloning the full-length human Melusin cDNA and a cDNA fragment coding for amino acid residues 149–350 (Cterm) in pGEX vectors (Amersham Pharmacia Biotech). GST and GST fusion proteins were expressed in *E. coli* BL21 bacterial strain and purified on glutathione-Sepharose 4B. COS cells, used as source of β_1 integrin heterodimers, were washed twice with cold PBS and lysed in TBS (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM $NaVO_4$, 10 mM NaF, 10 μg/ml leupeptin, 4 μg/ml pepstatin and 0.1 TIU/ml aprotinin) 0.5% Nonidet P-40 + 1 mM Ca^{2+}, or with TBS, 0.5% Nonidet P-40 + 5 mM EDTA. Cell extracts were precleared for 1 h at 4 °C with 10 μg of GST-Sepharose. 2 mg of every protein extract were incubated overnight at 4 °C with 10 μg of GST-Melusin, GST-Cterm and GST alone (as control). Sepharose beads were then washed, boiled in Laemmli buffer and proteins were separated on 6% polyacrylamide gel in nonreducing conditions. The gel was blotted to a nitrocellulose filter that was then saturated with TBS, 5% BSA and incubated over night at 4 °C in TBS 1% BSA containing 10 μg/ml TS2/16 monoclonal antibody to β_1 integrin. After washing, the filter was incubated with peroxidase conjugated anti-mouse antibody for 2 h at room temperature and detection was performed with chemiluminescent substrate ECL (Amersham).

RESULTS

Isolation of β_1 Integrin Cytoplasmic Domain Interactors—To identify muscle proteins able to interact with the β_1 integrin subunit cytoplasmic domain, we carried out an interaction trap approach (20). We screened a heart neonatal rat library using the entire cytoplasmic region of β_1A, the ubiquitously expressed isoform, and β_1D, the muscle-specific isoform, as baits. We obtained 3 different specific interactors for β_1A and β_1D. One of the β_1A interactors was identified as the rat homologues of ICAP-1α (Integrin Cytoplasmic Domain-Associated Protein), also called bodenin, and recently described as a β_1 interacting protein (6, 7). Another protein that we found to weakly interact with β_1D was RACK-1 (Receptor for Activated Protein Kinase C), a molecule recently described as interactor for β_1, β2 and β5 integrin cytoplasmic domains (8, 7).

The most abundantly retrieved cDNA corresponded to an unknown sequence and was isolated once in the β_1A and 8 times in the β_1D screening, where it was obtained as two different overlapping cDNA fragments with an open reading frame of 423 nt (D3–2) and 561 nt (D7–2). To verify the specificity of this interaction we tested the ability of the proteins coded by the D3–2 and D7–2 cDNA fragments to bind β_1A, β_1D and other unrelated baits (cyclin A, bFGF, c-Myc and bicoid) in a yeast interaction test. Our results showed that D3–2 and D7–2 protein fragments bind specifically and strongly to β_1A and β_1D cytoplasmic domains and not to the unrelated baits. We consequently focused our study on this new interactor that we called Melusin.

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 (GenBank™ AF140690). The mouse cDNA was 1420 nt in length, with an open reading frame coding for 350 amino acids (GenBank™ AF140691), with 92% identity with the human amino acidic 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 carboxyl-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. 1*B*). Moreover, four distinct P*XX*P motifs, representing the minimal consensus sequence recognized by SH3 domains (32, 33) and two Y*XX*I/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. 2*A*). 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. 2*B*).

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. 3*A*, 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. 3*B*). In adult muscles a doublet of protein bands was detected by Western blotting, suggesting possible posttranslational

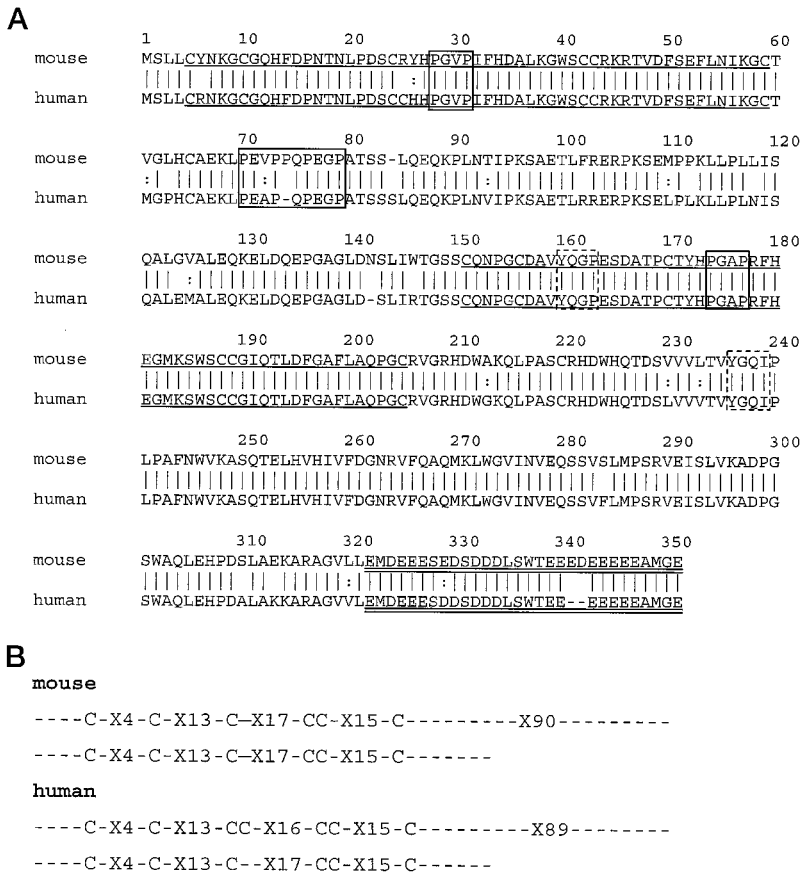

FIG. 1. **Amino acid sequence of Melusin.** *A*, the amino acid sequence was deduced from the full-length human and mouse cDNAs. Identical residues are indicated by *vertical bars* and conservative substitutions by *double dots*. The two cysteine-rich domains are *underlined,* and the acidic amino acid stretch at the extreme carboxyl-terminal is *double underlined*. P*XX*P sequences, putative SH3 domain binding motifs, are *boxed*. Y*XX*I/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.

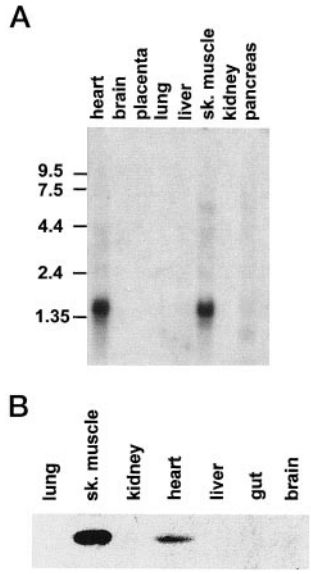

FIG. 2. **Expression of Melusin is restricted to striated muscle tissue.** *A*, poly(A)$^+$ RNA from different human tissues was hybridized with D3–2 rat probe isolated with the two-hybrid screening as described under "Materials and Methods." A single band of approximately 1.4 kilobases was present only 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.

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. 3*C*, 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 β_1 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 β_1. Using the protein fragment coded by plasmid D3–2, corresponding to the carboxyl-terminal of Melusin (tail domain) as a bait, interaction occurred with β_1 but not with β_2 and β_3 cytoplasmic domains (Table I). Moreover, no interaction occurred with the integrin α_5 cytoplasmic domain used as control (Table I). The β_1 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 (β_1A, β_1B, and β_1D) suggesting that the interaction involved the common subdomain. To further test this hypoth-

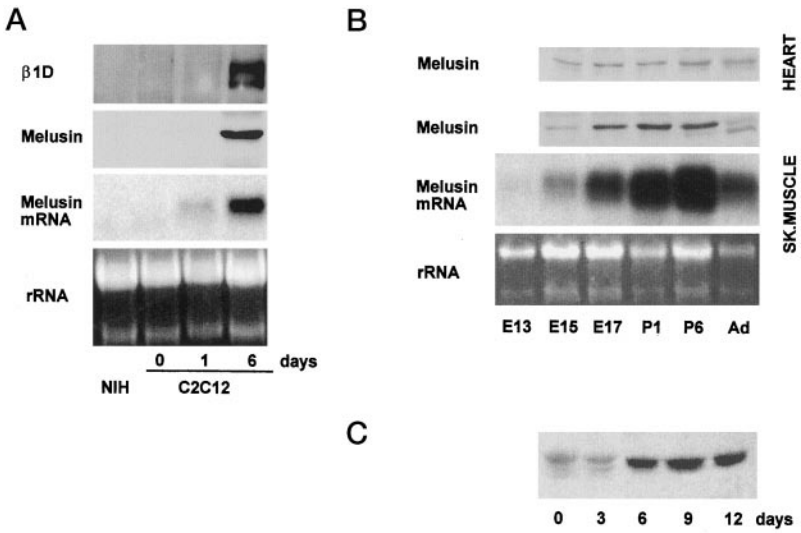

FIG. 3. **Melusin expression is regulated during *in vitro* and *in vivo* myogenesis and during muscle regeneration.** *A*, C2C12 mouse myoblasts (*0*) were allowed to differentiate in serum-free medium for 1 or 6 days. NIH 3T3 fibroblasts were used as negative control. Protein extracts were analyzed by Western blotting with antibodies to Melusin and to β_1D integrin as marker for differentiation (two *upper panels*). Total RNA was also extracted and hybridized with the Melusin probe, or stained with ethidium bromide as gel loading control (two *lower panels*). *B*, protein extracts (two *upper panels*) from heart and limb muscles of mouse embryos at 15 (*E15*) and 17 (*E17*) days of fetal life, newborn mice (*P1*), 1-week old (*P6*), and adult (*Ad*) mice were analyzed by Western blotting with Melusin polyclonal antibodies. Total RNA was also extracted from limb muscles at different stages of development and analyzed by Northern blotting with Melusin probe. Total RNA stained with ethidium bromide (*lower panel*) is shown as gel loading control. *C*, degeneration of tibialis anterior muscle of adult mice was induced by liquid nitrogen freezing, and regeneration was allowed to proceed for 3, 6, 9, and 12 days. The muscles were excised and protein extracts were analyzed by Western blotting for the expression of Melusin with specific polyclonal antibodies. Day 0 (*0*) is untreated tibialis anterior muscle.

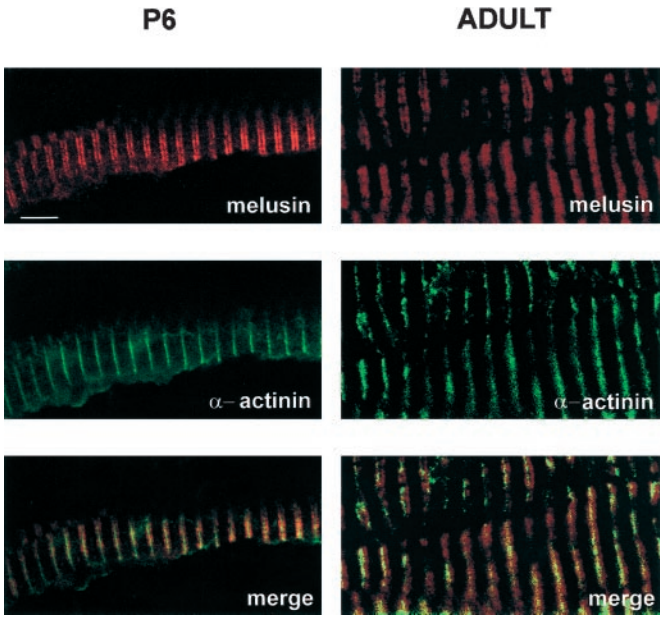

FIG. 4. **Melusin is enriched at costameres.** *Panels* in *left column* show longitudinal cryosections of 1-week old (*P6*) mouse limb co-stained with affinity purified Melusin polyclonal antibody and α-actinin monoclonal antibody. The two images are superimposed in the *lower panel* (*merge*). Soleus muscle from 6-month old mouse was also stained and is shown in panels of *right column*. Note the striated localization of Melusin and the reduced staining intensity in adult muscle. *Scale bar*, 10 μm.

esis, we used a truncated bait consisting only of the common subdomain. As shown in Table I, the common β_1 subdomain interacted with the tail region of Melusin with an intensity comparable with the one observed for the entire cytoplasmic regions.

Different Melusin constructs were also tested for their ability to interact with the β_1 integrin cytoplasmic domain. As mentioned above, the interaction occurred with preys consisting of the tail domain plus a large fragment of the second cysteine-rich repeat (D7–2) or the tail domain alone (D3–2) (Table II). Further deletion of the acidic amino acid stretch did not affect the interaction, allowing mapping the binding site for the β_1 integrin in a restricted portion of the Melusin tail domain from amino acid residues 211 to 320 (Table II). Interestingly the full-length Melusin construct showed no interaction (Tables I and II), suggesting that the tail domain is unavailable for binding to β_1 in the full-length protein.

Binding of Melusin to β_1 Integrins in Vitro—To further test the interaction of Melusin with integrin cytoplasmic domains, we performed *in vitro* binding assays. Either the full-length or a fragment (Cterm), consisting of the second cysteine-rich repeat and the tail domain, were fused to GST and bound to Sepharose beads (see "Materials and Methods" and Table II). When detergent extract of COS cells in Ca^{2+}-containing buffer was incubated with the Sepharose beads, β_1 integrins bound strongly to the Cterm Melusin fragment but not to the full-length protein. Because Melusin contains a putative Ca^{2+} binding domain, we investigated whether Ca^{2+} ions are inhibiting the interaction of the full-length Melusin with β_1 integrin. As shown in Fig. 5, Ca^{2+} chelator (EDTA) strongly enhanced binding of Melusin to β_1 integrin. Interestingly, Ca^{2+} concentration did not affect binding of the Cterm portion of Melusin to β_1 integrin (Fig. 5).

DISCUSSION

In this work we describe a new muscle-specific protein, Melusin, interacting with the cytoplasmic domain of the β_1 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

TABLE I

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 211–350) and the full-length melusin protein. β_1A, β_1B and β_1D are different splicing variant of β_1 integrin. β_1Com is a mutant containing the cytoplasmic subdomain common to all splicing variants (35). α_5 integrin subunit cytoplasmic domain was used as control.

Baits	Cytoplasmic domain sequences	D3–2	Melusin
β_1A	KLLMIIHDRREFAKFEKEKMNAKWDTGENPIYKSAVTTVVNPKYEGK	+++	–
β_1B	KLLMIIHDRREFAKFEKEKMNAKWDTVSYKTSKKQSGL	+++	–
β_1D	KLLMIIHDRREFAKFEKEKMNAKWDTQENPIYKSPINNFKNPNYGRKAGL	+++	–
β_1Com	KLLMIIHDRREFAKFEKEKMNAKWDT	+++	–
β_2	KALIHLSDLREYRRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAES	–	–
β_3	KLLITIHDRKEFAKFEEERARAKWDTANNPLYKEATSTFTNITYRGT	–	–
α_5	KLGFFKRSLPYGTAMEKAQLKPPATSDA	–	–

TABLE II

Interaction of different melusin constructs with β_1 integrin

Different melusin constructs used are schematized. The ability of the constructs to bind the β_1 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	
				Ca^{2+}	EDTA
	Melusin full-length	1-350	-	-	+
	Cterm	149-350	nt	+	+
	D7-2	164-350	+	nt	nt
	D3-2	211-350	+	nt	nt
	D3-2Δ	211-320	+	nt	nt

Cysteine-rich domain; Intervening sequence; Tail domain containing the acidic amino acid stretch (DD).

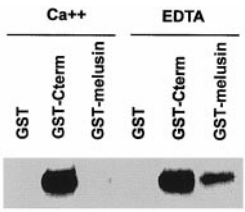

FIG. 5. **Interaction of Melusin with integrins is Ca^{2+} dependent.** GST fusion proteins containing either the Cterm portion or the full-length Melusin protein (see "Materials and Methods" section and Table II) were bound to glutathione-Sepharose. GST protein alone was used as control. COS cells were detergent-extracted in buffer containing 1 mM $CaCl_2$ or 5 mM EDTA. Cell extracts were incubated with GST fusion protein-Sepharose, and β_1 integrin binding was determined by Western blot analysis of eluted material. While β_1 integrin binds to the Cterm region of Melusin both in the presence or absence of Ca^{2+} ions, binding to the intact Melusin occurred only in the absence of divalent cations.

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. 3*B*). 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 β_1A and β_1D integrin isoforms. These two isoforms are differentially expressed during muscle development (36). β_1A is expressed in muscles during embryonic development and is down-regulated after birth. On the other hand, the β_1D isoform starts to appear in skeletal muscle in 17-day embryos and becomes the only β_1 isoform in adult muscles. The ability to bind β_1A and β_1D 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 β_1 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.

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 because codons coding these double cysteine residues were found in human Melusin cDNA fragments present in the dbEST data base. An intervening sequence of 89–90 amino acid residues is present between the two cysteine-rich regions. The carboxyl-terminal portion of the molecule consists of a tail domain of 143/145

residues and contains a stretch of 18/20 negatively charged amino acids at the extreme carboxyl-terminal. Similar acidic carboxyl-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 β_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 β_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. This, however, is not the case, in fact, the presence of Ca^{2+} ions did not prevent binding of the truncated Melusin Cterm 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 β_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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REFERENCES

1. Reszka, A. A., Hayashi, Y., and Horwitz, A. F. (1992) *J. Cell Biol.* **117,** 1321–1330
2. Marcantonio, E. E., Guan, J. L., Trevithick, J. E., and Hynes, R. O. (1990) *Cell Regul.* **1,** 597–604
3. Sastry, S. K., and Horwitz, A. F. (1993) *Curr. Opin. Cell Biol.* **5,** 819–831
4. Hemler, M. E. (1998) *Curr. Opin. Cell Biol.* **10,** 578–585
5. Hannigan, G. E., Leung-Hagesteijn, C., Fitz-Gibbon, L., Coppolino, M. G., Radeva, G., Filmus, J., Bell, J. C., and Dedhar, S.(1996) *Nature* **379,** 91–96
6. Chang, D. D., Wong, C., Smith, H., and Liu, J. (1997) *J. Cell Biol.* **138,** 1149–1157
7. Zhang, X. A., and Hemler, M. E. (1999) *J. Biol. Chem.* **274,** 11–19
8. Lilental, J., and Chang, D. D. (1998) *J. Biol. Chem.* **273,** 2379–2383
9. Fornaro, M., and Languino, L. R.(1997) *Matrix Biol.* **16,** 185–193
10. Belkin, A. M., Zhidkova, N. I., Balzac, F., Altruda, F., Tomatis, D., Maier, A., Tarone, G., Koteliansky, V. E., and Burridge, K. (1996) *J. Cell Biol.* **132,** 211–226
11. Belkin, A. M., Retta, S. F., Pletjushkina, O. Y., Balzac, F., Silengo, L., Fassler, R., Koteliansky, V. E., Burridge, K., and Tarone, G. (1997) *J. Cell Biol.* **139,** 1583–1595
12. Bozyczko, D., Decker, C., Muschler, J., and Horwitz, A. F. (1989) *Exp. Cell Res.* **183,** 72–91
13. Swasdison, S., and Mayne, R.(1989) *Cell Tissue Res.* **257,** 537–543
14. Volk, T., Fessler, L. I., Fessler, J. H. (1990) *Cell* **63,** 525–536
15. Hirsch, E., Lohikangas, L., Gullberg, D., Hohansson, S., and Fassler, R. (1998) *J. Cell Sci.* **111,** 2397–2409
16. Fassler, R., Rohwedel, J., Maltsev, V., Bloch, W., Lentini, S., Guan, K., Gullberg, D., Hescheler, J., Addicks, K., and Wobus, A. M. (1996) *J. Cell Sci.* **109,** 2989–2999
17. Mayer, U., Saher, G., Fassler, R., Bornemann, A., Echtermeyer, F., von der Mark, H., Miosge, N., Poschl, E., and von der Mark, K. (1997) *Nat. Genet.* **17,** 318–323
18. McDonald, K. A., Lakonishok, M., and Horwitz, A. F. (1995) *J. Cell Sci.* **108,** 2573–2581
19. Martin-Bermudo, M. D., Brown, N. H. (1996) *J. Cell Biol.* **134,** 217–226
20. Gyuris, J., Golemis, E., Chertkov, H., and Brent, R. (1993) *Cell* **75,** 791–803
21. Gietz, D., St Jean, A., Woods, R. A., and Schiestl, R. H. (1992) *Nucleic Acids Res.* **20,** 1425
22. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* **215,** 403–410
23. Boguski, M. S., Lowe, T. M., and Tolstoshev, C. M. (1993) *Nat. Genet.* **4,** 332–333
24. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
25. Zornig, M., Klett, C., Lovec, H., Hameister, H., Winking, H., Adolph, S., and Moroy, T. (1995) *Cytogenet. Cell Genet.* **71,** 37–40
26. Lichter, P., Tang Chang, C. J., Call, K., Hermanson, G., Evans, G. A., Housman, D., and Ward, D. C. (1990) *Science* **247,** 64–69
27. Toyota, N., and Shimada, Y. (1984) *Cell Tissue Res.* **236,** 549–554
28. Hegyi, H., and Pongor, S. (1993) *Comput. Appl. Biosci.* **9,** 371–372
29. Bairoch, A., and Boeckmann, B. (1993) *Nucleic Acids Res.* **21,** 3093–3096
30. Baksh, S., and Michalak, M. (1991) *J. Biol. Chem.* **266,** 21458–21465
31. Fliegel, L., Ohnishi, M., Carpenter, M. R., Khanna, V. K., Reithmeier, R. A., and MacLennan, D. H. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 1167–1171
32. Rickles, R. J., Botfield, M. C., Weng, Z., Taylor, J. A., Green, O. M., Brugge, J. S., and Zoller, M. J. (1994) *EMBO J.* **13,** 5598–5604
33. Yu, H., Chen, J. K., Feng, S., Dalgarno, D. C., Brauer, A. W., and Schreiber, S. L. (1994) *Cell* **76,** 933–945
34. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., *et al.* (1993) *Cell* **72,** 767–778
35. Retta, S. F., Balzac, F., Ferraris, P., Belkin, A. M., Fassler, R., Humphries, M. J., De Leo, G., Silengo, L., and Tarone, G. (1998) *Mol. Biol. Cell* **4,** 715–731
36. Brancaccio, M., Cabodi, S., Belkin, A. M., Collo, G., Koteliansky, V. E., Tomatis, D., Altruda, F., Silengo, L., and Tarone, G. (1998) *Cell Adhes. Commun.* **5,** 193–205
37. Pardo, J. V., Siliciano, J. D., and Craig, S. W. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 1008–1012
38. Koteliansky, V. E., and Gneushev, G. N (1983) *FEBS Lett.* **159,** 158–160

Melusin Is a New Muscle-specific Interactor for β_1 Integrin Cytoplasmic Domain

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