Cib2 BINDS INTEGRIN α7β1D AND IS REDUCED IN LAMININ α2 CHAIN DEFICIENT MUSCULAR DYSTROPHY

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Mutations in the gene encoding laminin α2 chain cause congenital muscular dystrophy, MDC1A. In skeletal muscle, laminin α2 chain binds at least two receptor complexes; the dystrophin-glycoprotein complex and integrin α7β1. To gain insight into the molecular mechanisms underlying this disorder, we performed gene expression profiling of laminin α2 chain deficient mouse limb muscle. One of the down-regulated genes encodes a protein called calcium and integrin binding protein 2 (Cib2) whose expression and function is unknown. However, the closely related Cib1 has been reported to bind integrin αIIb and may be involved in outside-in-signaling in platelets. Since Cib2 might be a novel integrin α7β1 binding protein in muscle, we have studied Cib2 expression in the developing and adult mouse. Cib2 mRNA is mainly expressed in the developing central nervous system and in developing and adult skeletal muscle. In skeletal muscle Cib2 colocalizes with integrin α7B subunit at the sarcolemma and at the neuromuscular- and myotendinous junctions. Finally, we demonstrate that Cib2 is a calcium binding protein that interacts with integrin α7β1D. Thus, our data suggest a role for Cib2 as a cytoplasmic effector of integrin α7β1D signaling in skeletal muscle.

Muscular dystrophy is a general term that describes a group of inherited and gradually debilitating myogenic disorders (1). The genetic defects underlying many muscular dystrophies have been elucidated and mutations in the gene encoding laminin α2 chain cause congenital muscular dystrophy type 1A (MDC1A), which accounts for about 40% of the classical congenital muscular dystrophies. MDC1A shows autosomal recessive inheritance and is characterized by neonatal onset of muscle weakness, hypotonia, muscle fiber degeneration and defects in central and peripheral nervous systems (2).

To increase the understanding of the molecular mechanisms underlying various muscular dystrophies, gene expression profiling on human and mouse limb muscles has been performed (3-6). However, only limited microarray datasets have been published on MDC1A (7). Recently, gene expression profiling of diaphragm muscle from laminin α2 chain deficient dy/dy dystrophic mice was reported (8). Predominantly augmented gene expression was reported and approximately half of the genes that were shown to be up-regulated in dystrophic muscle encode proteins involved in muscle development and cell motility.
Yet, diaphragm might have different molecular signatures compared with limb muscles. Hence, in this study, we have compared hindlimb skeletal muscles from dy\textsuperscript{3K/dy}\textsuperscript{3K} mice, which completely lack expression of laminin \(\alpha\)2 chain, with hindlimb skeletal muscles from wild-type mice. In the present study, we report that the most strikingly up-regulated genes in laminin \(\alpha\)2 chain deficient leg muscle encode specific isoforms of proteins that are transiently expressed during normal muscle development and regeneration, and genes that encode cell adhesion and extracellular matrix proteins, while those being down-regulated mainly participate in diverse metabolic processes and kinase activities.

One of the down-regulated genes, Cib2, encodes a protein called calcium and integrin binding protein 2 (Cib2). Virtually nothing is known about the expression and function of this protein. However, it is closely related to Cib1, which interacts with a diverse range of biological targets. For example, Cib1 binds to integrin \(\alpha\)IIb in platelets and to several protein kinases (FAK and PAK1) (9, 10). Moreover Cib1 binds the Rho GTPase Rac3 and the anti-apoptotic caspase-2 splice variant caspase-2S (11). The functional consequences of these interactions are to a large extent unknown, but Cib1 has been implicated in hemostasis, DNA damage response and apoptosis (11). Of particular interest is the finding that Cib1 participates in outside-in signaling via \(\alpha\)IIb\(\beta\)3 in platelet precursors, possibly by acting as an inhibitor of \(\alpha\)IIb\(\beta\)3 activation (12). Integrin \(\alpha\)7\(\beta\)1 is one of the major laminin \(\alpha\)2 chain binding receptors of muscle cells and absence of integrin \(\alpha\)7 both in man and mouse leads to myopathy (13). Both \(\alpha\)7 and \(\beta\)1 chains occur in several splice isoforms. The cytoplasmic domain of integrin \(\alpha\)7 has at least two splice forms, A and B and integrin \(\beta\)1 is also alternatively spliced in the regions encoding intracellular domains giving rise to the major \(\beta\)1A and \(\beta\)1D isoforms (13). Notably, the signaling cascades induced by laminin \(\alpha\)2 chain binding to integrin \(\alpha\)7\(\beta\)1 are largely unknown. Investigating cellular signaling pathways that may be changed due to laminin \(\alpha\)2 chain deficiency offers an approach to identifying molecular targets for MDC1A therapy. Here, we demonstrate that Cib2, whose expression is down-regulated in dystrophic dy\textsuperscript{3K/dy}\textsuperscript{3K} muscle, is a novel integrin \(\alpha\)7\(\beta\)1D-binding protein. We hypothesize that Cib2 might be involved in outside-in and/or inside-out signaling via integrin \(\alpha\)7\(\beta\)1D subunit in skeletal muscle.

**EXPERIMENTAL PROCEDURES**

**Animals**- Wild-type (WT), laminin \(\alpha\)2 chain deficient (dy\textsuperscript{3K/dy}\textsuperscript{3K}) and dy\textsuperscript{3K LN}\textsuperscript{\alpha}TG mice were previously described (14, 15). Mdx breeder pairs were purchased from Jackson Laboratories. Animals were maintained in the animal facilities of Biomedical Center (Lund) according to animal care guidelines, and permission was given by the regional ethical board.

**Preparation of total RNA**- Total RNA was isolated from normal adult mouse tissues and from all hindlimb skeletal muscles of WT, dy\textsuperscript{3K/dy}\textsuperscript{3K}, dy\textsuperscript{3K LN}\textsuperscript{\alpha}1TG and mdx mice (frozen mdx skeletal muscle was also generously provided by Dr. Rachelle Crosbie, UCLA) using TRIzol reagent (Invitrogen) and further purified using the RNeasy Mini Kit (Qiagen) according to the manufacturers’ instructions. RNA quality was analyzed by OD\textsubscript{260}/OD\textsubscript{280} ratios and agarose gel analyses. Isolated total RNA was used for microarray, RT-PCR and Northern blot analyses.

**Microarray**- Total RNA was isolated independently from all hindlimb muscles of three WT and three dy\textsuperscript{3K/dy}\textsuperscript{3K} mice (5-weeks-old). After a quality control with Agilent Bioanalyzer 2100 (Agilent\textsuperscript{®}), RNA was processed for microarray hybridization to Affymetrix MOE430 2.0 mouse GeneChips at Swegene Microarray Resource Centre (Lund, Sweden).

**Data analysis and visualization**- dChip 2004 software was used for the analysis of microarray raw-data, which was normalized and the model-based expression values were calculated using the PM-only model (16). To identify differentially expressed genes the following criterias were chosen: (i) an absolute and average fold change > 2, (ii) a \(p\)-value < 0.05 by t-testing, and (iii) a present call in all WT replicates.
computing the \( \frac{dy^{3K}}{dy^{3K}} \) down-regulated genes or a present call in all \( \frac{dy^{3K}}{dy^{3K}} \) replicates when computing the \( \frac{dy^{3K}}{dy^{3K}} \) up-regulated genes. The False Discovery Rate (FDR) was assessed by 50 random permutations of the samples. The median FDR of 50 permutations was 1.2%. After normalization and calculation of expression value the samples were hierarchically clustered, which provides a quality control for the three independent biological replicates.

**Functional classification - Expression Analysis Systematic Explorer (EASE) software** (http://david.niaid.nih.gov/david/upload.asp) was used to classify genes into and find overrepresented categories of biological processes, cellular components and molecular functions. In order to make an EASE analysis for a set of differentially expressed genes, the Affymetrix probe set IDs were translated to a list of non-redundant GeneIDs. Those probe set IDs lacking a GeneID were not considered in the EASE analysis. LocusLink-mouse dataset was used as background. GeneIDs not present in the background were removed from the analysis. An EASE score of \(< 0.05\) was considered significantly overrepresented.

**Reverse-transcriptase PCR** Total RNA was reverse-transcribed using SuperScript II RT (Invitrogen). PCR amplification of specific fragments was performed with the following primers: Cib2; for-TCTGTGCTCTCTGCGAATCAGC and rev-GCGATCATGTCCTCAAGTCA; Cib3; for-GGCCTGTCTTATCGATACCAGGA and rev-GGTCTCCATGTCCTTCATCC; Rps18; for-GGGCTGGAGAACTCACGGAGGAT and rev-GGCCACGACAGAAGACTTCTC. Amplified fragments were 236, 380 and 300 bp respectively.

**Northern blot analysis** The Cib2 specific probe was amplified by PCR using cDNA prepared from adult mouse skeletal muscle. Total RNA (20 µg) from different tissues was fractionated on a standard formaldehyde gel (1.2% agarose) and transferred to a nylon membrane (BioRad) by capillary blotting. The ready made membrane with RNA isolated from skeletal muscle at various ages was from Zyagen. Twenty-five ng of the Cib2 probe was labeled with \([32P]\)-dCTP using the Megaprime DNA Labeling System (GE Healthcare). Hybridization was performed for one hour at 68°C using ExpressHyb hybridization solution (Clontech). After washing (3x10 minutes with 2xSSC, 0.05% SDS at room temperature, and 2x20 minutes with 0.1x SSC, 0.1% SDS at 50°C), the membrane was exposed to Hyperfilm MP (Amersham Biosciences) and then developed (AGFA, Curix 60).

**Whole-mount in situ hybridization** The entire coding region of Cib2 cDNA was subcloned into pYX-Asc vector (RZPD GmbH). Digoxigenin-labeled (Roche Applied Science) sense and antisense Cib2 riboprobes were synthesized by linearization with NotI and EcoRI and transcription with T7- and T3 polymerases, respectively. Probes were purified with RNeasy Mini Kit (Qiagen). For whole-mount in situ hybridization, E12.5-13.5 mouse embryos were fixed in 4% paraformaldehyde overnight, dehydrated in methanol series and kept at -20°C in 100% methanol until used. Whole-mount RNA in situ hybridization was carried out as described previously (18). All steps before pre-hybridization were calculated the relative expression of Cib2 relative to house keeping gene Tbp (encoding TATA box binding protein) by the formula \(2^{(-\Delta \Delta C)}\), where \(\Delta C = n_{Cib2} - n_{Tbp}\). Primer pairs were for Cib2 as described in the previous section and for Tbp; for-GCTCTGGGAATTGTACCGCAG and rev-CTGCTCATAGCTCCTGGGCTC. The PCR conditions were 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds.
performed on ice except the proteinase K treatment.

**Generation of antibodies-** Polyclonal antibodies (PEDRO and TANNI) were produced against a GST-Cib2 fusion protein (for expression of the fusion protein, see below). Immunization was performed by Agrisera AB (Vännäs, Sweden). Antibodies were purified from the sera using PVDF strips. Briefly, PVDF strips containing Cib2-GST and GST, respectively, were blocked in 5% BSA-PBS for one hour at room temperature. Sera were incubated with strips overnight at 4°C and washed 3x10 minutes with 500 mM NaCl, 50 mM Tris-HCl, pH 7.4 and 1x10 minutes with 100 mM NaCl, 50 mM Tris-HCl, pH 7.4. GST-Cib2- and GST-strips were separated and antibodies were eluted with 2 ml of 50 mM glycine, pH 2.5 at 4°C. 200 µl 1M Tris-HCl, pH 8.0 was added to bring the pH to 7.4.

**Immunofluorescence-** Immunofluorescence was performed as previously described (15). Quadriceps muscles were collected and analyzed from three mice of each genotype. Sections were incubated with rabbit polyclonal antibodies against Cib2 and integrin α7B chain (19), respectively. For detection of neuromuscular junctions, samples were simultaneously incubated with FITC-conjugated α-bungarotoxin (Molecular Probes).

**Protein extraction and Western blot analysis-** Total proteins extracts were isolated from all hindlimb skeletal muscles of three WT (3.5-weeks-old), three dy3K/dy3K (3.5-weeks-old) and three dy3K LNα1TG mice (4.5-weeks-old). All samples were immediately frozen in liquid nitrogen and reduced to powder. Samples were homogenized in pre-heated SDS-lysis buffer (80 mM Tris-HCl, pH 6.8, 10% SDS, 0.12 M sucrose, 5 mM EDTA) and a cocktail of proteases inhibitors (Complete, 1:50; Roche) and heated 15 minutes at 56°C. Protein extracts were quantified using the BCA assay kit (Pierce) following the manufacturer’s instructions. Prior to loading on the gel, 30 µg of proteins were freshly diluted in loading buffer (61 mM Tris-HCl pH 6.8, 0.05 % bromophenol blue, 5% β-mercaptoethanol, 2% SDS, 10% glycerol) and heated to 94°C for 5 minutes. Proteins were separated by SDS-PAGE using 15% acrylamide gels and blotted on nitrocellulose membranes (Hybond-C, Amersham Biosciences). The membranes were boiled for 30 minutes in deionized water then blocked for one hour in 1X-TBS containing 0.01% Tween-20, 5% milk, 3% BSA and incubated overnight at 4°C with rabbit polyclonal serum anti-Cib2 (1:100, in 1X-TBS, 0.01% Tween-20, 3% BSA). Blots were washed 3x20 minutes with 1X-TBS containing 0.01% Tween-20 and incubated with horseradish peroxidase-conjugated polyclonal swine anti-rabbit immunoglobulins (1:2000 in 1X-TBS, 0.01% Tween-20, 3% BSA, P0217, Dako) for 40 minutes and then exposed to ECL (Amersham Biosciences). Each membrane was also hybridized with mouse monoclonal anti-alpha-actinin (1:3000, clone EA-53, Sigma) for loading normalization following the same procedure as above using a secondary horseradish peroxidase-conjugated goat anti-mouse antibody (1:4000, sc-2005, Santa Cruz Biotechnology). The quantifications were performed using the ImageJ 1.40 software (http://rsb.info.nih.gov/ij/download.html, developed by Wayne Rasband, National Institute of Health, Bethesda, USA).

**Expression of GST-fusion protein-** Mouse Cib2 cDNA (GenBank Accession Number BC005739) was used as a template for PCR. The Cib2 cDNA was amplified by PCR using primers for-AAGAATTCAATGG-GGAACAAGCAGACCAT and rev-TTGCCGGCGAGGCCACGCGGCTTTGCA containing restriction sites EcoRI and Not1, respectively. The amplified product (590 bp) was digested with EcoRI and Not1 and ligated into the vector pGEX-6P-1 (Amersham Biosciences). The in-frame fusion was confirmed by DNA sequencing. The GST fusion protein and GST were purified by glutathione-sepharose according to the manufacturer’s instructions as previously described (20). Protein concentration was determined using BCA assay (Pierce).

**45Ca2+ Binding-** GST-Cib2 and GST were loaded and separated by SDS-PAGE and
transferred to a PVDF membrane (Amersham Biosciences, Hybond 0.45 µm pore size). Blots were overlaid with $^{45}\text{Ca}^{2+}$ (1 µCi/ml) for 10 minutes as previously described (9) with or without excess of unlabeled CaCl$_2$ (10mM). The membranes were exposed to Hyperfilm MP for 5 days at -70°C and then developed (AGFA, Curix 60).

**Solid-phase binding assay** - Microtiter wells (Nunc) were coated with 50 µl protein solution (5 µg/ml GST-Cib2 in PBS) or with BSA (40 µg/ml in PBS) or with laminin-111 (Chemicon; 5 µg/ml in PBS) and incubated at 4°C overnight. Wells were blocked with 100 µl 1% BSA (1 mg BSA/ml PBS) at room temperature for two hours and then washed 3 times with wash solution (0.5% BSA, 0.1% Tween-20). Fifty µl of 1% NP40 extracted protein lysates from adult mouse skeletal muscle (5µg/ml protein lysates in 0.5% BSA, 0.1% Tween-20) were added and incubated at room temperature for 60 minutes. The wells were washed three times with wash solution and then primary antibodies against integrin α7A, α7B, β1A and β1D (diluted 1:200 in 0.5% BSA, 0.1% Tween-20) were applied to separate wells and incubated for 60 minutes at room temperature. After washing three times, 50 µl secondary antibody (rabbit IgG antibody, ab6722 Abcam, 1:500 in 0.5% BSA, 0.1% Tween-20) was added and incubated for 60 minutes. After additional washings the amount of bound proteins was detected with phosphatase substrate $p$-nitrophenyl phosphate (Sigma). Protein binding to GST-Cib2/BSA was measured at OD405. Data was analyzed using unpaired t-test.

**RESULTS**

**Array data.** In order to investigate the gene expression differences between WT and laminin α2 chain deficient muscle, we compared hindlimb muscles at 5 weeks of age. A gene was considered differentially expressed if it met the criteria described in Experimental Procedures. We present the top 40 genes with the highest significant positive and negative fold changes, respectively (Table 1 and 2). The clustering dendogram (Supplemental Figure 1) revealed that the WT samples comprised a distinct subgroup whereas the $dy^{Kk}/dy^{Kk}$ muscle samples were subgrouped together. Thus, hierarchical clustering correctly grouped the replicates by their appropriate genotype. We used EASE to facilitate the biological interpretations from the results of the microarray experiments and genes with altered expression were classified within specific Gene Ontology categories of 1) biological processes, 2) cellular components and 3) molecular functions. Among the up-regulated genes, the most over-represented biological processes were muscle development, cell motility, cell adhesion, muscle contraction and acute-phase response, while the most over-represented cellular components were extracellular, extracellular space, extracellular matrix and actin cytoskeleton. The molecular functions...
categories with the highest overrepresentation involved the theme of binding (heparin, glycosaminoglycan, metal ion and calcium ion binding and enzyme regulator activity) (Table 1 and data not shown). Among the genes with decreased expression, themes concerning metabolism were overrepresented in the category of biological processes (oxidation of organic compounds, energy pathways, carbohydrate metabolism, glucose metabolism and main pathways of carbohydrate metabolism). The most overrepresented cellular components were cytoplasm, mitochondrion and unlocalized. The most over-represented molecular functions were calmodulin regulated protein kinase activity, kinase activity, catalytic activity, oxidoreductase activity and calmodulin binding (Table 2 and data not shown).

It is interesting to note that laminin α2 chain mRNA was only reduced approximately 2.6-fold (Table 2). Yet, Guo et al., have shown that dy3K/dy3K muscles are completely devoid of laminin α2 chain, in contrast to other mouse models for laminin α2 chain deficiency (22). Thus, the remaining mRNA appears not to encode a protein.

Cib2 mRNA is mainly expressed in skeletal muscle and expression is reduced in laminin α2 chain deficient muscle. One of the down-regulated genes encodes a protein, Cib2 that is highly homologous to Cib1 (37% similarity). We were interested in further characterizing the expression and function of Cib2, since we hypothesized that it might be a novel integrin binding protein. Previous non-quantitative RT-PCR experiments demonstrated CIB2 mRNA expression in a wide variety of human tissues (23). We isolated total RNA from several mouse tissues and subjected it to semi-quantitative RT-PCR with primers corresponding to exon 4 and 5, respectively. A distinct band of the expected size was noted with RNA isolated from skeletal muscle and weaker amplicons were noted in brain and lung (Fig. 1A). Quantitative real-time PCR also demonstrated that Cib2 mRNA is mainly expressed in skeletal muscle and lower amounts were seen in brain (Supplemental Figure 2). Semiquantitative RT-PCR confirmed that expression of Cib2 mRNA was down-regulated in laminin α2 chain deficient dy3K/dy3K muscle (Fig. 1B). The CIB family includes four members in humans and orthologs have also been found in some other species (11, 24). The closest homologue to Cib2 is the as yet uncharacterized Cib3 (62% similarity). RT-PCR experiments revealed that Cib3 mRNA is also expressed in skeletal muscle, but that the expression is not significantly altered upon laminin α2 chain deficiency (Fig. 1B). Moreover, Cib3 mRNA is only weakly expressed in skeletal muscle. A distinctive RT-PCR product was only found after ~30 cycles of amplification and Cib3 mRNA could not be detected by Northern blot analyses in skeletal muscle (Fig. 1B and data not shown). To determine the transcript size of Cib2 mRNA we performed Northern blot analysis of RNA from different tissues. Gene structure analyses predict six exons in the Cib2 gene and a transcript size of 0.9-1.4 kb. As shown in Fig. 1C, two bands of 0.9 kb and 1.4 kb, respectively, were strongly detected in skeletal muscle. Weaker bands were detected in brain (Fig. 1C). We also confirmed by Northern blot analysis that expression of Cib2 mRNA is reduced in 4-weeks-old laminin α2 chain deficient muscle (Fig. 3C and 5A). Hence, skeletal muscle appears to be the major source of Cib2 mRNA in the adult mouse. To uncover if Cib2 mRNA is expressed in developing and postnatal skeletal muscle, we probed a ready made membrane with RNA isolated from skeletal muscle at various ages. Cib2 transcripts were detected from embryonic day 16, throughout postnatal development and in adult muscle (Fig. 1D).

Cib2 mRNA expression in the mouse embryo. To investigate the expression of Cib2 mRNA during embryonic development, we performed whole-mount in situ hybridization in E13.5 mouse embryos. Cib2 mRNA was expressed strongly in the developing forebrain, midbrain, hindbrain, spinal cord, somites, inner ear, vibrissae and gut. In addition, Cib2 mRNA expression was detected in embryonic musculature. Similar expression pattern was detected in E12.5 embryos (data not shown). These findings suggest that Cib2 could play an important role in embryonic development.
role in the development of the central nervous system and musculature (Fig. 2).

Cib2 colocalizes with integrin α7B in skeletal muscle. To determine the localization of Cib2 in skeletal muscle we generated rabbit anti mouse antibodies. These antibodies detected a 22 kDa protein corresponding to Cib2 in WT muscles (Fig. 5B). Next, we stained cross-sections of 4-weeks-old quadriceps muscles. In WT muscles, Cib2 was associated with the sarcolemma and enriched at the myotendinous junctions (MTJ) and at the neuromuscular junctions (NMJ). The sarcolemmal Cib2 staining was severely reduced in dy3K/dy3K muscle. A slight reduction of Cib2 expression was noted in MTJ and a moderate reduction was seen in NMJ (Fig. 3A). Overall, an approximately 2-fold down-regulation of Cib2 was noted in dy3K/dy3K muscle (Fig. 5B and C). Interestingly, Cib2 had a similar expression pattern as integrin α7B subunit in skeletal muscle. Integrin α7B is also expressed at the sarcolemma and enriched at the MTJ and NMJ (13; Fig. 3A). In summary, these findings imply that Cib2 is expressed at the NMJ (13; Fig. 3A). These antibodies detected a 22 kDa protein corresponding to Cib2 in WT muscles (Fig. 5B). Next, we stained cross-sections of 4-weeks-old quadriceps muscles. In WT muscles, Cib2 was associated with the sarcolemma and enriched at the myotendinous junctions (MTJ) and at the neuromuscular junctions (NMJ). The sarcolemmal Cib2 staining was severely reduced in dy3K/dy3K muscle. A slight reduction of Cib2 expression was noted in MTJ and a moderate reduction was seen in NMJ (Fig. 3A). Overall, an approximately 2-fold down-regulation of Cib2 was noted in dy3K/dy3K muscle (Fig. 5B and C). Interestingly, Cib2 had a similar expression pattern as integrin α7B subunit in skeletal muscle. Integrin α7B is also expressed at the sarcolemma and enriched at the MTJ and NMJ (13; Fig. 3A). In summary, these findings imply that Cib2 is expressed at the sarcolemma, MTJ and NMJ, where it colocalizes with integrin α7B chain. Furthermore, Cib2 expression depends on the presence of laminin α2 chain.

Cib2 is a calcium-binding protein. To determine whether Cib2 specifically binds Ca^{2+}, we expressed Cib2 as a GST fusion protein and performed 45Ca^{2+} blot overlay assays. Recombinant Cib2 had a mass of about 48 kDa. The results demonstrated in Fig. 3B showed that 45Ca^{2+} bound recombinant Cib2, but not GST alone, and that binding was abolished upon inclusion of 10 mM unlabeled Ca^{2+} (Fig 3B, right panel). Hence, Cib2 is indeed a calcium-binding protein. Abnormal Ca^{2+} handling may contribute to fiber destruction in dystrophic skeletal muscle (25). Since Cib2 is a Ca^{2+} binding protein, Cib2 down-regulation might be a feature of other muscular dystrophies too. By microarray analyses, Cib2 mRNA has been shown to be differentially expressed between severely affected animal models (dystrophin and sarcoglycan deficient mice) and mildly or non-affected animal models (dysferlin, sarcospan deficient and WT mice) (6). However, by Northern blot analyses Cib2 mRNA expression appeared normal in muscles of dystrophin deficient mdx animals (both at 4 weeks and 4 months of age; Fig. 3C and data not shown). Thus, it is possible that Cib2 expression is regulated mainly by laminin α2 chain expression.

Cib2 binds integrin α7Bβ1D. Since Cib2 and integrin α7B expression overlapped in skeletal muscle, in vitro binding assays were used to uncover whether Cib2 interacts with integrin α7β1. Purified recombinant Cib2 was immobilized to microtiter wells and integrin α7β1 from muscle extracts was allowed to bind. The amount of bound integrin was quantified using antibodies against α7A, α7B, β1A and β1D. Immobilized Cib2 bound α7B and β1D but not α7A or β1A (Fig. 4A and B). To exclude the possibility that the extraction method accidentally affected the activity of integrins, we tested whether the extracted integrins could bind laminin-111. Indeed, extracted integrins α7A, α7B, β1A and β1D all bound to laminin-111 (Supplemental Figure 3). To further validate the Cib2-integrin α7B interaction, we measured Cib2 binding to an integrin α7B peptide by ITF. The Cib2 sequence does not contain a tryptophan residue whereas the C-terminal peptide of integrin α7B does and therefore Cib2 binding could be illustrated by changes in the ITF of the integrin α7B upon titration with Cib2. The emission spectrum of the integrin α7B peptide showed a maximum at ~358 nm (λmax) and the intensity, but not the wavelength, of this peak increased upon addition of Cib2, as shown in Fig. 4C, where the absence of fluorescence emission from Cib2 alone is also evident. Fig. 4D shows the saturation binding curve obtained by titrating the integrin α7B peptide with increasing amounts of Cib2, as measured by ITF: fitting the data to a single class of tight binding sites allowed determining the binding affinity of the two species, with an equilibrium dissociation constant (Kd) equal to 304 nM. Interestingly, it was shown that CIB1 binds an integrin α1b peptide with a Kd of ~300 nM, using a similar assay (26). In order to rule out the possibility that the
interaction of integrin of α7B and Cib2 is not specific, we tested another tryptophan containing peptide corresponding to integrin α7A (which should not bind Cib2 according to the solid-phase assay) by ITF for its binding capacity to Cib2. Although the presence of Cib2 resulted in an increase of the peptide fluorescence, this increase was consistently independent from Cib2 concentration, indicating that Cib2 does not bind integrin α7A subunit specifically (inset in Fig. 4D). In summary, our findings demonstrate an in vitro interaction of Cib2 and integrin α7Bβ1D.

Transgenic expression of laminin α1 chain in laminin α2 chain deficient muscles restores integrin α7B and Cib2 expression

Immunofluorescence data supported an interaction between Cib2 and integrin α7B in vivo. To further assess whether Cib2 and integrin α7Bβ1D interact in vivo, we analyzed mice deficient in laminin α2 chain but instead expressing laminin α1 chain in muscle (dyα1LNα1TG mice). Integrin α7B is reduced at the sarcolemma of laminin α2 chain deficient muscle. However, transgenic expression of laminin α1 chain restores integrin α7B expression (27). If Cib2 interacts with integrin α7B in vivo, we reasoned that Cib2 expression should also be restored upon transgenic laminin α1 chain expression. Indeed, Cib2 mRNA expression was partially normalized in skeletal muscles of dyα1LNα1TG animals (Fig. 5A). This finding was also confirmed at the protein level. Western blot analyzes revealed that expression of Cib2 was partially reconstituted in skeletal muscles of dyα1LNα1TG animals (Fig. 5B and C).

DISCUSSION

Mutations in the gene that encodes the laminin α2 chain cause laminin α2 deficient congenital muscular dystrophy (MDC1A) (2). Here, we have compared the gene expression in laminin α2 chain deficient hindlimb muscles with that of WT muscles. The genes reported to be up-regulated in dy/dy diaphragm (8) and in muscles from other distinct muscular dystrophy models (3-6) are very similar to the genes up-regulated in dyα1LNα1TG limb muscle. The most up-regulated genes in the most over-represented groups are genes that encode specific isoforms of proteins that are transiently expressed during normal muscle development and regeneration (for example, Myh3, Myl4, Tnnt2) and genes that encode cell adhesion, extracellular and extracellular matrix proteins (for example, Postn, Ctgf, Dlk1, Thbs4, Ncam, Aspn). This is not surprising in view of the fact that expression of a large number of genes was down-regulated in dyα1LNα1TG limb muscle compared to dy/dy diaphragm (8). A majority of the decreased genes was assigned to metabolism themes. Thus, these data indicate a probable metabolic crisis in dyα1LNα1TG limb muscle and similar observations have also been made in previous studies of other muscular dystrophies (6). In addition, genes such as Map2k6 and Cib2 are down-regulated, suggesting that signaling cascades mediated by laminin α2 chain are affected in dystrophic muscle. Yet, signaling cascades induced by laminin α2 chain binding to integrin α7B1 and dystroglycan (the main laminin α2 chain receptors in muscle) are largely unknown (13). Therefore, we studied the uncharacterized Cib2 gene in more detail.

Four CIB homologues are found in the human genome; CIB1, CIB2, CIB3 and CIB4 (24). So far, only CIB1 has been studied in greater detail. CIB1 is widely expressed and binds to several effectors and has therefore been proposed to be involved in integrin αIIb activation, DNA damage response, apoptosis, embryogenesis and regulation of Ca2+ signals (11). Recently, the role of Cib1 in vivo was elucidated by the generation of Cib1 null mice and male Cib1 deficient mice display defective spermatogenesis (28). The other members of the CIB family have not been studied up to now. Here, we report that Cib2 is mainly expressed in adult skeletal muscle, where it
is co-expressed with integrin α7B subunit. Furthermore, we show that it binds to integrin α7β1D. Integrin α7β1 was originally identified in myoblasts and is crucial for normal muscle function. The expression of the cytoplasmic variants α7A, α7B, β1A and β1D appears to be developmentally regulated in muscle. Both α7A and α7B isoforms are expressed in adult muscle. However, only the α7B isoform is expressed in proliferating myoblasts. β1A is also expressed in myoblasts but is replaced by β1D, which is exclusively expressed in mature muscle (13). Hence, Cib2 could interact with α7B in myoblasts (Cib2 is indeed expressed in myoblasts; data not shown) and α7Bβ1D in mature muscle. Cib2 expression is reduced in laminin α2 chain deficient muscle and this is presumably a consequence of the secondary reduction of integrin α7B expression at the sarcolemma (19, 27, 29, 30), since Cib2 does not appear to bind laminin α2 chain directly (data not shown). Transgenic expression of laminin α1 chain restores integrin α7B at the sarcolemma of laminin α2 chain deficient muscle (27) and Cib2 expression is also partly reconstituted, strengthening the view that integrin α7Bβ1D and Cib2 interact. Further support of this notion comes from the fact that integrin α7B and Cib2 mRNAs are partially co-expressed not only in muscle but also in the embryonic nervous system (31, 32). Notably, Cib2 mRNA is also reduced in 5-weeks-old laminin α2 chain deficient brains (data not shown), suggesting that Cib2 may also interact with integrin α7B in the central nervous system. However, it is possible that Cib2 interacts with other molecules both in muscle and central nervous system and we are currently investigating this.

The exact role of Cib2 remains elusive, but it is tempting to speculate that it has major functions in skeletal muscle and central nervous system, perhaps as a regulator of integrin α7B activation. In humans, the CIB2 gene is localized to the q24 region of chromosome 15 (23). Interestingly, patients with an interstitial deletion of chromosome 15q24 have clinical manifestations such as hypotonia, marked developmental delays and abnormalities of the ears (33). Also, a syndrome of severe mental retardation, spasticity and visual impairment has been linked to chromosome 15q24 (34). Thus, CIB2 is a good candidate gene for these disorders.

In summary, knowledge of the differences between normal and laminin α2 chain deficient skeletal muscle is essential for designing future therapies for MDC1A. Here, we report a catalogue of differentially expressed genes in dystrophic muscle. Moreover, we have identified a new integrin α7Bβ1D binding protein. Through future Cib2 analyses we wish to assemble an accurate picture of signal cascades mediated by laminin α2 chain in the hope that we can apply this information to the treatment of MDC1A.

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FOOTNOTES

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FIGURE LEGENDS

FIG. 1. Characterization of Cib2 mRNA expression. A, RT-PCR analyses (24 cycles) on RNA isolated from various mouse tissues. Cib2 mRNA is strongly expressed in skeletal muscle and lower amounts are seen in brain and lung. Skm, skeletal muscle. B, RT-PCR analyses of RNA isolated from skeletal muscle of wild-type (WT) and dy3K/dy3K mice (25-days-old) with Cib2 (24 cycles) and Cib3 (33 cycles) primers. Cib2 but not Cib3 mRNA expression is reduced in laminin α2 chain deficient muscle. Rps 18, ribosomal protein S18. C, Northern blot analysis of Cib2 mRNA expression in various WT tissues. Cib2 mRNA is mainly expressed in skeletal muscle. Weaker bands are detected in brain. To ascertain equal loading and RNA integrity, 28S and 18S ribosomal RNAs were monitored. D, Northern blot analysis on RNA isolated from mouse skeletal muscles from E16 into adulthood. Cib2 mRNA is expressed in developing, postnatal and adult skeletal muscle.

FIG. 2. Expression of Cib2 mRNA in WT embryos at E13.5 detected by non-radioactive whole-mount in situ hybridization. A, Distinct expression pattern of Cib2 mRNA is observed in forebrain (FB), midbrain (MB), spinal cord (SC), vibrissae (VB), inner ear (IE) and facial muscles (FM). B, Posterior view demonstrates the expression of Cib2 mRNA along the spinal cord and in hindbrain (HB). Magnifications show the expression of Cib2 in vibrissae and facial muscles (E), inner ear (F) and gut (G). C;D, Embryos hybridized with the sense strand control probe are negative.

FIG. 3. Cib2 colocalizes with integrin α7B subunit in skeletal muscle and binds calcium. A, Cross-sections of quadriceps muscles from WT and dy3K/dy3K mice were stained with rabbit antibodies against Cib2 and integrin α7B, respectively. Cib2 is expressed in association with the sarcolemma and is enriched at the MTJ and NMJ. Inset in NMJ upper panel shows double staining with Cib2 antibodies and fluorescein α-bungarotoxin (α-BTX). Cib2 is severely reduced at the laminin α2 chain deficient sarcolemma, but some staining remains in laminin α2 chain deficient MTJ and NMJ, respectively. Inset in middle NMJ panel shows double staining with Cib2 antibodies and α-BTX. Integrin α7B is also expressed at the sarcolemma (inset is a digital overlay of the images of Cib2 and integrin α7B stainings and hence colocalization in yellow). Integrin α7B subunit is enriched at the MTJ (inset is a digital overlay of the images of Cib2 and integrin α7B stainings) and at the NMJ (inset shows double staining with integrin α7B antibodies and α-BTX). Bars, 50 μm. B, Calcium binding assay. Purified GST and GST-Cib2 were separated by SDS-PAGE and transferred to PVDF membranes, and the membranes were overlaid with 45Ca2+ (1 µCi/ml) for 10 minutes with (right panel) or without (left panel) excess of unlabeled CaCl 2 (10mM). GST-Cib2 but not GST binds calcium. C, Northern blot analysis of Cib2 mRNA expression in WT, mdx and dy3K/dy3K hindlimb skeletal muscles (25-days-old). Cib2 mRNA expression appears normal in mdx muscle, but is reduced in dy3K/dy3K skeletal muscle.

FIG. 4. In vitro binding of Cib2 to different integrin isoforms. A, Binding of integrins α7B, β1D, α7A and β1A to GST-Cib2 or BSA (control) coated wells. Each value is the mean of 16 wells. A significant interaction between Cib2-integrin α7B and Cib2-integrin β1D is observed. Results are shown as means ± SEM. ***, Significantly different from BSA (p < 0.0001) (t-test). N.s., not significant. B, Dose response curves of Cib2-integrin α7B (top) and β1D (bottom) display a saturation behaviour, which indicates true bindings. C, Comparison of the fluorescence emission spectrum of Cib2 alone with that of the integrin α7B peptide alone and upon addition of saturating amounts of Cib2, as indicated on the panel side. D, Titration of the α7B integrin peptide (100 nM) with Cib2 recorded by fluorescence change. The increase in fluorescence at λmax = 358 nm is plotted as a function of Cib2 concentration, and expressed as percentage of fluorescence relative to the signal at saturation. The saturation of the integrin α7B peptide as a function of free Cib2 concentration was calculated as described in Experimental Procedures. The continuous line was obtained by nonlinear least-
squares fitting of the experimental data, resulting in a $K_d$ of 304 nM. Inset, the fluorescence signal at $\lambda_{\text{max}} = 358$ nm for the integrin $\alpha7A$ peptide is plotted as a function of Cib2 concentration, expressed in arbitrary units. The independence of the signal from Cib2 concentration confirms the absence of specific interactions between the integrin $\alpha7A$ peptide and Cib2.

FIG. 5. Expression of Cib2 in WT, $dy^{3K}/dy^{3K}$ and $dy^{3K}LN\alpha1TG$ hindlimb skeletal muscles. A, Northern blot and RT-PCR analyses of Cib2 mRNA expression in 25-days-old hindlimb muscles. Cib2 mRNA expression in skeletal muscle from $dy^{3K}LN\alpha1TG$ mice is partially normalized. B, Immunoblotting of recombinant Cib2 and of total protein lysates from WT, $dy^{3K}/dy^{3K}$ and $dy^{3K}LN\alpha1TG$ hindlimb skeletal muscles. The Cib2 antibody detects recombinant Cib2, which has a mass of about 48 kDa. A 22 kDa protein corresponding to Cib2 is detected in WT muscles. Reduced expression is seen in $dy^{3K}/dy^{3K}$ muscles, whereas expression appears normalized in $dy^{3K}LN\alpha1TG$ muscles. Quantitative measurements of Cib2 expression from three independent experiments. $\alpha$-Actinin was used as a protein loading control with the graph showing the ratio of Cib2/$\alpha$-actinin expression. Cib2 is significantly down-regulated in $dy^{3K}/dy^{3K}$ muscles ($p < 0.0168$) whereas no significant difference in expression is seen between WT and $dy^{3K}LN\alpha1TG$ muscles.

### TABLES

Table 1. Top 40 up-regulated genes in laminin $\alpha2$ chain deficient skeletal muscle.

| Gene Name                                                                 | GeneID | Symbol | Fold Change |
|--------------------------------------------------------------------------|--------|--------|-------------|
| myosin, heavy polypeptide 3, skeletal muscle, embryonic                  | 17883  | Myh3   | 55.35       |
| sarcolipin                                                               | 66402  | Sln    | 29.21       |
| myosin, light polypeptide 4, alkali; atrial, embryonic                   | 17896  | Myl4   | 21.21       |
| troponin T2, cardiac                                                     | 21956  | Tnnt2  | 18.79       |
| ankyrin repeat domain 1 (cardiac muscle)                                | 107765 | Ankrd1 | 17.15       |
| angiotensin II receptor, type 2                                          | 11609  | Agtr2  | 15.94       |
| metallothionein 2                                                        | 17750  | Mt2    | 15.63       |
| myosin, heavy polypeptide 8, skeletal muscle, perinatal                  | 17885  | Myh8   | 10.84       |
| delta-like 1 homolog (Drosophila)                                        | 13386  | Dlk1   | 8.81        |
| lectin, galactose binding, soluble 3                                     | 16854  | Lgals3 | 7.66        |
| C1q and tumor necrosis factor related protein 3                          | 81799  | C1qtnf3| 7.33        |
| metallothionein 1                                                        | 17748  | Mt1    | 7.12        |
| procollagen, type VIII, alpha 1                                          | 12837  | Col8a1 | 7.03        |
| tubulin, beta 2b                                                         | 73710  | Tubb2b | 6.95        |
| leukocyte immunoglobulin-like receptor, subfamily B, member 4            | 14728  | Lilrb4 | 6.92        |
| macrophage expressed gene 1                                              | 17476  | Mpeg1  | 6.83        |
| S100 calcium binding protein A4                                           | 20198  | S100a4 | 6.80        |
| C-type (calcium dependent, carbohydrate recognition domain) lectin, superfamily member 12 | 56644  | Clec7a | 6.62        |
| GTL2, imprinted maternally expressed untranslated mRNA                    | 17263  | Meg3   | 6.28        |
| double cortin and calcium/calmodulin-dependent protein kinase-like 1    | 13175  | Dclk1  | 6.08        |
| ectodysplasin A2 isoform receptor                                        | 245527 | Eda2r  | 6.03        |
| RIKEN cDNA 6030416H16 gene                                               | 77712  | 6030416H16Rik | 6.02 |
expressed sequence AW551984 244810 AW551984 5.53
cathepsin S 13040 CtsS 5.24
cartilage intermediate layer protein, nucleotide pyrophosphohydrolase 214425 Cilp 5.21
ATPase, (Na+)/K+ transporting, beta 4 polypeptide proteoglycan 4 (megakaryocyte stimulating factor, articular superficial zone protein) 67821 Atp1b4 4.99
serine (or cysteine) proteinase inhibitor, clade A, member 3N 20716 Serpina3n 4.97
procollagen, type XIX, alpha 1 12823 Col19a1 4.65
CD44 antigen 12505 Cd44 4.63
RIKEN cDNA 1110006E14 gene 76286 1110006E14Rik 4.63
periostin, osteoblast specific factor 50706 Postn 4.58
insulin-like growth factor 2 16002 Igf2 4.54
thrombospondin 4 21828 Thbs4 4.26
glycoprotein (transmembrane) nmb 93695 Gpnmb 4.23
RIKEN cDNA 1110002H13 gene 66139 Kene1l 4.20
potassium voltage-gated channel, Isk-related family, member 1-like 66240 Kcne1l 4.12
cytochrome P450, family 2, subfamily f, polypeptide 2 13107 Cyp2f2 4.09
Kruppel-like factor 5 12224 Klf5 3.87
a disintegrin and metalloprotease domain 8 11501 Adam8 3.85

Table 2. Top 40 down-regulated genes in laminin α2 chain deficient skeletal muscle.

| Gene Name                                                   | GeneID   | Symbol          | Fold Change |
|-------------------------------------------------------------|----------|-----------------|-------------|
| RIKEN cDNA 2310065F04 gene                                  | 74184    | 2310065F04Rik   | -6.2        |
| spermine oxidase                                            | 228608   | Smox            | -4.84       |
| immunoglobulin heavy chain 6 (heavy chain of IgM)           | 16019    | Igh-6           | -4.53       |
| D-aspartate oxidase                                         | 70503    | Ddo             | -4.29       |
| exocyst complex component 3-like 2                         | 74463    | Exoc3l2         | -4.04       |
| RIKEN cDNA 9330159F19 gene                                  | 212448   | 9330159F19Rik   | -4.03       |
| glutamic pyruvate transaminase (alanine aminotransferase) 2 | 108682   | Gpt2            | -3.82       |
| mitogen activated protein kinase kinase 6                   | 26399    | Map2k6          | -3.81       |
| RIKEN cDNA 2310010M20 gene                                  | 69576    | 2310010M20Rik   | -3.73       |
| calcium and integrin binding family member 2                | 56506    | Cib2            | -3.69       |
| aquaporin 4                                                 | 11829    | Aqp4            | -3.66       |
| prostaglandin E receptor 3 (subtype EP3)                    | 19218    | Ptger3          | -3.59       |
| RIKEN cDNA 6430571L13 gene                                  | 235599   | 6430571L13Rik   | -3.3        |
| solute carrier family 37 (glycerol-6-phosphate transporter), member 4 | 14385    | Slc37a4         | -3.36       |
| tubulin, alpha 8                                            | 53857    | Tuba8           | -3.35       |
| calcium/calmodulin-dependent protein kinase II alpha        | 12322    | Camk2a          | -3.34       |
| expressed sequence AU040377                                 | 268780   | Egflam          | -3.27       |
| cDNA sequence BC018222                                      | 235135   | Tmem45b         | -3.26       |
| JTV1 gene                                                   | 231872   | Jtv1            | -3.18       |
| RIKEN cDNA 2010110I21 gene                                  | 70260    | 2010110I21Rik   | -3.18       |
| ganglioside-induced differentiation-associated-              | 14545    | Gdap1           | -3.05       |
| Protein Name                                                                 | Accession | Regulation |
|----------------------------------------------------------------------------|-----------|------------|
| Protein 1                                                                  | 27281     | Hrasls     |
| HRAS-like suppressor                                                        | 108097    | Prkab2     |
| Protein kinase, AMP-activated, beta 2 non-catalytic subunit                 |           |            |
| Nuclear receptor subfamily 4, group A, member 1                             | 15370     | Nr4a1      |
| ADP-ribosylation factor-like 6 interacting protein 2                        | 56298     | Arl6ip2    |
| Leucine rich repeat containing 38                                           | 242735    | Lrrc38     |
| Laminin, alpha 2                                                            | 16773     | Lama2      |
| ATP-binding cassette, sub-family D (ALD), member 2                           | 26874     | Abcd2      |
| Phosphatidic acid phosphatase type 2 domain containing 3                    | 227721    | Ppapdc3    |
| Solute carrier family 41, member 3                                           | 71699     | Slc41a3    |
| Mitochondrial tumor suppressor 1                                             | 102103    | Mts1       |
| Kyphoscoliosis                                                              | 16716     | Ky         |
| Glycerol phosphate dehydrogenase 2, mitochondrial                           | 14571     | Gpd2       |
| DIS3 mitotic control homolog (S. cerevisiae)-like 2                         | 208718    | Dis3l2     |
| Coiled-coil domain containing 58                                            | 381045    | Ccdc58     |
| Small nuclear ribonucleoprotein N                                           | 20646     | Snrpn      |
| Solute carrier family 25 (mitochondrial thiamine pyrophosphate carrier), member 19 | 67283     | Slc25a19   |
| RIKEN cDNA 1110006G14 gene                                                  | 68469     | 1110006G14Rik |
| RIKEN cDNA 9630033F20 gene                                                  | 319801    | 9630033F20Rik |
| Phosphorylase kinase gamma 1                                                | 18682     | Phkg1      |
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Cib2 binds integrin α7β1D and is reduced in laminin α2 chain deficient muscular dystrophy

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