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Cib2 Binds Integrin α7Bβ1D and Is Reduced in Laminin α2 Chain-deficient Muscular Dystrophy*[S]

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Mutations in the gene encoding laminin α2 chain cause congenital muscular dystrophy type 1A. 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 Cib2 (calcium- and integrin-binding protein 2) 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 the 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 α7Bβ1D. Thus, our data suggest a role for Cib2 as a cytoplasmic effector of integrin α7Bβ1D 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),[3] 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 data sets 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. Nevertheless, the diaphragm might have different molecular signatures compared with limb muscles. Hence, in this study, we have compared hind limb skeletal muscles from dy^{3K}/dy^{3K} mice, which completely lack expression of laminin α2 chain, with hind limb skeletal muscles from wild-type mice. In the present study, we report that the most strikingly up-regulated genes in laminin α2 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, whereas those being down-regulated mainly participate in diverse metabolic processes and kinase activities.

One of the down-regulated genes, *Cib2*, encodes a protein called Cib2 (calcium- and integrin-binding protein 2). 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 αIIb in platelets and to several protein kinases (FAK and PAK1) (9, 10). Moreover, Cib1 binds the Rho GTPase Rac3 and the antiapoptotic 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 sig-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.

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[3] The abbreviations used are: MDC1A, congenital muscular dystrophy type 1A; WT, wild type; RT, reverse transcription; GST, glutathione *S*-transferase; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; ITF, intrinsic tryptophan fluorescence; EASE, Expression Analysis Systematic Explorer; MTJ, myotendinous junctions; NMJ, neuromuscular junctions.

naling via αIIbβ3 in platelet precursors, possibly by acting as an inhibitor of αIIbβ3 activation (12). Integrin α7β1 is one of the major laminin α2 chain-binding receptors of muscle cells, and the absence of integrin α7 both in humans and mice leads to myopathy (13). Both α7 and β1 chains occur in several splice isoforms. The cytoplasmic domain of integrin α7 has at least two splice forms, A and B, and integrin β1 is also alternatively spliced in the regions encoding intracellular domains, giving rise to the major β1A and β1D isoforms (13). Notably, the signaling cascades induced by laminin α2 chain binding to integrin α7β1 are largely unknown. Investigating cellular signaling pathways that may be changed due to laminin α2 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^{3K}/dy^{3K} muscle, is a novel integrin α7Bβ1D-binding protein. We hypothesize that Cib2 might be involved in outside-in and/or inside-out signaling via integrin α7Bβ1D subunit in skeletal muscle.

EXPERIMENTAL PROCEDURES

Animals—Wild-type (WT), laminin α2 chain-deficient (dy^{3K}/dy^{3K}), and dy^{3K}LNα1TG mice were previously described (14, 15). *Mdx* breeder pairs were purchased from Jackson Laboratories. Animals were maintained in the animal facilities of the 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 hind limb skeletal muscles of WT, dy^{3K}/dy^{3K}, dy^{3K}LNα1TG 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 A_{260}/A_{280} ratios and agarose gel analyses. Isolated total RNA was used for microarray, reverse transcription (RT)-PCR, and Northern blot analyses.

Microarray—Total RNA was isolated independently from all hind limb muscles of three WT and three dy^{3K}/dy^{3K} mice (5 weeks old). After a quality control with an Agilent Bioanalyzer 2100 (Agilent®), 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 criteria were chosen: (i) an absolute and average -fold change of >2, (ii) a *p* value of <0.05 by *t* testing, and (iii) a present call in all WT replicates when computing the dy^{3K}/dy^{3K} down-regulated genes or a present call in all dy^{3K}/dy^{3K} replicates when computing the dy^{3K}/dy^{3K} up-regulated genes. The false discovery rate was assessed by 50 random permutations of the samples. The median false discovery rate 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. Microarray data were deposited in the Gene Expression Omnibus database, accession number GSE12049.

Functional Classification—Expression Analysis Systematic Explorer (EASE) software (17 (available on the World Wide Web) 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 nonredundant GeneIDs. Those probe set IDs lacking a GeneID were not considered in the EASE analysis. The LocusLink-mouse data set 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.

RT-PCR—Total RNA was reverse-transcribed using SuperScript II RT (Invitrogen). PCR amplification of specific fragments was performed with the following primers: *Cib2*, forward (TCTGTGCTCTCTGCGAATCAGC) and reverse (GCGATCATGTCCTCAAAGTCA); *Cib3*, forward (GGCTGTTCTATCGATACCAGGA) and reverse (GGTCTCCATCTGCTTCATCC); *Rps18*, forward (GGGCTGGAGAACTCACGGAGGAT) and reverse (GGCCCAGAGACTCATTTCTTCTT). Amplified fragments were 236, 380, and 300 bp, respectively.

Quantitative Real Time PCR—Quantitative real time PCR was performed on a LightCycler (Roche Applied Science). We used Maxima SYBR Green qPCR Master Mix (Fermentas) to detect amplification products. Amplification efficiency was evaluated through amplification of serially diluted template cDNAs (1:5 to 1:125). We calculated the relative expression of *Cib2* relative to housekeeping gene *Tbp* (encoding TATA box-binding protein) by the formula $2^{\Delta n}$, where $\Delta n = n_{\mathrm{Cib2}} - n_{\mathrm{Tbp}}$. Primer pairs for *Cib2* were as described above, and the primer pair for *Tbp* was GCTCTGGAATTGTACCGCAG (forward) and CTGGCTCATAGCTCTTGGCTC (reverse). The PCR conditions were 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s.

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 (Bio-Rad) 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 [^{32}P]dCTP, using the Megaprime DNA Labeling System (GE Healthcare). Hybridization was performed for 1 h at 68 °C using ExpressHyb hybridization solution (Clontech). After washing (3 × 10 min with 2× SSC, 0.05% SDS at room temperature, and 2 × 20 min with 0.1× SSC, 0.1% SDS at 50 °C), the membrane was exposed to Hyperfilm MP (Amersham Biosciences) and then developed (Curix 60; AGFA).

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 the RNeasy minikit (Qiagen). For whole mount *in situ* hybridization, embryonic day 12.5–13.5 mouse embryos were fixed in 4%

TABLE 1
Top 40 up-regulated genes in laminin α2 chain-deficient skeletal muscle

Gene name	GeneID	Symbol	Change
			-fold
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, α1	12837	*Col8a1*	7.03
Tubulin, β2b	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, β4 polypeptide	67821	*Atp1b4*	4.99
Proteoglycan 4 (megakaryocyte-stimulating factor, articular superficial zone protein)	65022	*Prg4*	4.99
Serine (or cysteine) proteinase inhibitor, clade A, member 3N	20716	*Serpina3n*	4.97
Procollagen, type XIX, α1	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		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

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 prehybridization were performed on ice except for 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 polyvinylidene difluoride strips. Briefly, polyvinylidene difluoride strips containing Cib2-GST and GST, respectively, were blocked in 5% BSA/PBS for 1 h at room temperature. Sera were incubated with strips overnight at 4 °C and washed 3 × 10 min with 500 mM NaCl, 50 mM Tris-HCl, pH 7.4, and 1 × 10 min 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 of 1 M 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 fluorescein isothiocyanate-conjugated α-bungarotoxin (Molecular Probes, Inc., Eugene, OR).

Protein Extraction and Western Blot Analysis—Total protein extracts were isolated from all hind limb skeletal muscles of three WT (3.5 weeks old), three dy^{3K}/dy^{3K} (3.5 weeks old), and three dy^{3K}LNα1TG mice (4.5 weeks old). All samples were immediately frozen in liquid nitrogen and reduced to powder. Samples were homogenized in preheated SDS-lysis buffer (80 mM Tris-HCl, pH 6.8, 10% SDS, 0.12 M sucrose, 5 mM EDTA) and a mixture of proteases inhibitors (Complete; 1:50; Roche Applied Science) and heated for 15 min 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% bromphenol blue, 5% β-mercaptoethanol, 2% SDS, 10% glycerol) and heated to 94 °C for 5 min. 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 min in deionized water and then blocked for 1 h in 1× 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 1× TBS, 0.01% Tween 20, 3% BSA). Blots were washed three times for 20 min each with 1× TBS containing 0.01% Tween 20 and incubated with horseradish peroxidase-conjugated polyclonal swine anti-rabbit immunoglobulins (1:2000 in 1× TBS, 0.01% Tween 20, 3% BSA, P0217; Dako) for 40 min and then exposed to ECL (Amersham Biosciences). Each membrane was

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

Gene name	GeneID	Symbol	Change
			-fold
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*	−3.4
Solute carrier family 37 (glycerol 6-phosphate transporter), member 4	14385	*Slc37a4*	−3.36
Tubulin, α8	53857	*Tuba8*	−3.35
Calcium/calmodulin-dependent protein kinase II α	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-protein 1	14545	*Gdap1*	−3.05
HRAS-like suppressor	27281	*Hrasls*	−3
Protein kinase, AMP-activated, β2 noncatalytic subunit	108097	*Prkab2*	−2.92
Nuclear receptor subfamily 4, group A, member 1	15370	*Nr4a1*	−2.89
ADP-ribosylation factor-like 6-interacting protein 2	56298	*Arl6ip2*	−2.79
Leucine-rich repeat containing 38	242735	*Lrrc38*	−2.76
Laminin, α2	16773	*Lama2*	−2.65
ATP-binding cassette, subfamily D (ALD), member 2	26874	*Abcd2*	−2.61
Phosphatidic acid phosphatase type 2 domain containing 3	227721	*Ppapdc3*	−2.6
Solute carrier family 41, member 3	71699	*Slc41a3*	−2.6
Mitochondrial tumor suppressor 1	102103	*Mtus1*	−2.6
Kyphoscoliosis	16716	*Ky*	−2.56
Glycerol phosphate dehydrogenase 2, mitochondrial	14571	*Gpd2*	−2.55
DIS3 mitotic control homolog (*S. cerevisiae*)-like 2	208718	*Dis3l2*	−2.54
Coiled-coil domain-containing 58	381045	*Ccdc58*	−2.49
Small nuclear ribonucleoprotein N	20646	*Snrpn*	−2.47
Solute carrier family 25 (mitochondrial thiamine pyrophosphate carrier), member 19	67283	*Slc25a19*	−2.44
RIKEN cDNA 1110006G14 gene	68469	*1110006G14Rik*	−2.44
RIKEN cDNA 9630033F20 gene	319801	*9630033F20Rik*	−2.44
Phosphorylase kinase γ1	18682	*Phkg1*	−2.43

also hybridized with mouse monoclonal anti-α-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, Inc., Santa Cruz, CA). The quantifications were performed using the ImageJ 1.40 software (available on the World Wide Web; developed by Wayne Rasband, National Institute of Health, Bethesda, MD).

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 AAGAATTCAATGGGGAACAAGCAGACCAT (forward) and TTGCGGCCGCAGGCCCCCACGGCCTTGGCA (reverse) containing restriction sites EcoRI and NotI, respectively. The amplified product (590 bp) was digested with EcoRI and NotI 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 a BCA assay (Pierce).

$^{45}Ca^{2+}$ Binding—GST-Cib2 and GST were loaded and separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Hybond; 0.45-μm pore size; Amersham Biosciences). Blots were overlaid with $^{45}Ca^{2+}$ (1 μCi/ml) for 10 min as previously described (9) with or without an excess of unlabeled $CaCl_2$ (10 mM). The membranes were exposed to Hyperfilm MP for 5 days at −70 °C and then developed (Curix 60; AGFA).

Solid Phase Binding Assay—Microtiter wells (Nunc) were coated with 50 μl of protein solution (5 μg/ml GST-Cib2 in PBS) or with BSA (40 μg/ml in PBS) or with laminin-111 (5 μg/ml in PBS; Chemicon) and incubated at 4 °C overnight. Wells were blocked with 100 μl of 1% BSA (1 mg of BSA/ml of PBS) at room temperature for 2 h and then washed three times with wash solution (0.5% BSA, 0.1% Tween 20). Fifty μl of 1% Nonidet P-40-extracted protein lysates from adult mouse skeletal muscle (5 μg/ml protein lysates in 0.5% BSA, 0.1% Tween 20) were added and incubated at room temperature for 60 min. 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 min at room temperature. After washing three times, 50 μl of secondary antibody (rabbit IgG antibody, ab6722 Abcam, 1:500 in 0.5% BSA, 0.1% Tween 20) was added and incubated for 60 min. 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 A_{405}. Data were analyzed using an unpaired *t* test.

Intrinsic Tryptophan Fluorescence (ITF)—N-terminally acetylated peptides corresponding to the C-terminal portions of integrin α7A (GWDSSSGRSTPRPPCPSTTG) and α7B (LAADWHPELGPDGHPVPATA) were synthesized and purified (Innovagen). GST was cleaved off from GST-Cib2 using PreScission Protease (Amersham Biosciences) according to the manufacturer's protocol. The binding of Cib2 to the integrin peptides was studied under the following experimental conditions: 20 mM Hepes, 140 mM NaCl, 5 mM $CaCl_2$, pH 7.5, at 20 °C. Increased intrinsic tryptophan fluorescence of the integrin peptides was monitored as a function of Cib2 concentration using a SPEX (Jobin Yvon) FluoroMax-3 spectrofluorometer in a 1-cm quartz cell. Fluorescence emission spectra were collected between 320 and 430 nm, selectively exciting the only present tryptophan residue at 295 nm. Point titrations were performed on 100 nM integrin peptides at increasing concentrations of Cib2, monitoring the fluorescence intensity change (increase) at the wavelength corresponding to the peak of the emission spectrum of the integrin peptide. Three independent titration data sets were collected, with no significant differences in the results. The data were analyzed according to the model of a single class of binding sites (21) upon correction for the amount of Cib2 actually free in solution.

RESULTS

Array Data—In order to investigate the gene expression differences between WT and laminin α2 chain-deficient muscle, we compared hind limb muscles at 5 weeks of age. A gene was considered differentially expressed if it met the criteria described under "Experimental Procedures." We present the top 40 genes with the highest significant positive and negative -fold changes, respectively (Tables 1 and 2). The clustering dendogram (supplemental Fig. 1) revealed that the WT samples comprised a distinct subgroup, whereas the dy^{3K}/dy^{3K} 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 overrepresented biological processes were muscle development, cell motility, cell adhesion, muscle contraction, and acute phase response, whereas the most overrepresented cellular components were extracellular, extracellular space, extracellular matrix, and actin cytoskeleton. The molecular function 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 overrepresented molecular functions were calmodulin-regulated protein kinase activity, kinase activity, catalytic activity, oxidoreductase activity, and calmodulin binding (Table 2) (data not shown). It is interesting to note that laminin α2 chain mRNA was only reduced ~2.6-fold (Table 2). However, Guo *et al.* (22) have shown that dy^{3K}/dy^{3K} muscles are completely devoid of laminin α2 chain, in contrast to other mouse models for laminin α2 chain deficiency. Thus, the remaining mRNA appears not to encode a protein.

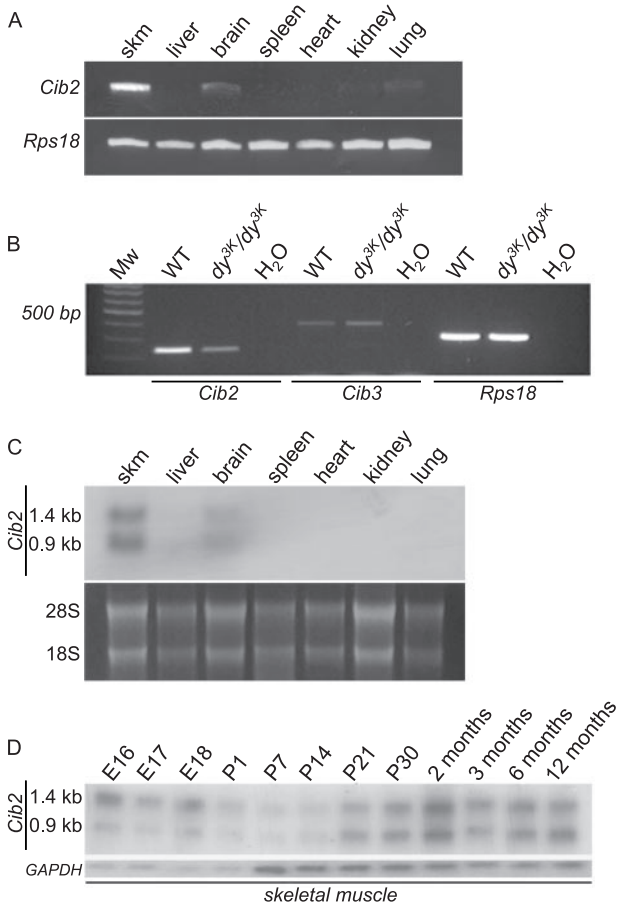

FIGURE 1. **Characterization of *Cib2* mRNA expression.** *A*, RT-PCR analyses (24 cycles) on RNA isolated from various mouse tissues. *Cib2* mRNA is strongly expressed in skeletal muscle, and lower amounts are seen in brain and lung. *Skm*, skeletal muscle. *B*, RT-PCR analyses of RNA isolated from skeletal muscle of WT and dy^{3K}/dy^{3K} 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, 28 and 18 S ribosomal RNAs were monitored. *D*, Northern blot analysis on RNA isolated from mouse skeletal muscles from embryonic day 16 into adulthood. *Cib2* mRNA is expressed in developing, postnatal and adult skeletal muscle. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

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 nonquantitative 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 semiquantitative RT-PCR

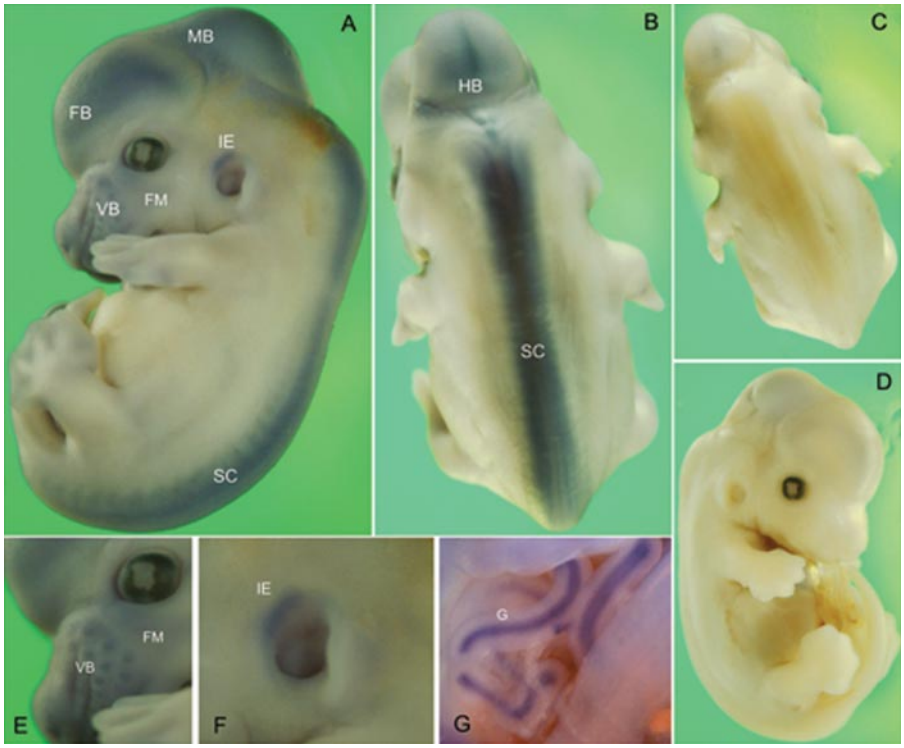

FIGURE 2. **Expression of *Cib2* mRNA in WT embryos at embryonic day 13.5 detected by nonradioactive whole mount *in situ* hybridization.** *A*, a distinct expression pattern of *Cib2* mRNA is observed in forebrain (*FB*), midbrain (*MB*), spinal cord (*SC*), vibrissae (*VB*), inner ear (*IE*), and facial 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* and *D*, embryos hybridized with the sense strand control probe are negative.

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 the brain and lung (Fig. 1*A*). Quantitative real time PCR also demonstrated that *Cib2* mRNA is mainly expressed in skeletal muscle, and lower amounts were seen in the brain (supplemental Fig. 2). Semiquantitative RT-PCR confirmed that expression of *Cib2* mRNA was down-regulated in laminin α2 chain-deficient dy^{3K}/dy^{3K} muscle (Fig. 1*B*). 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. 1*B*). 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. 1*B*) (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. 1*C*, two bands of 0.9 and 1.4 kb, respectively, were strongly detected in skeletal muscle. Weaker bands were detected in brain (Fig. 1*C*). We also confirmed by Northern blot analysis that expression of *Cib2* mRNA is reduced in 4-week-old laminin α2 chain-deficient muscle (see Figs. 3*C* and 5*A*). 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. 1*D*).

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 embryonic day 13.5 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. A similar expression pattern was detected in embryonic day 12.5 embryos (data not shown). These findings suggest that Cib2 could play an important 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 (see Fig. 5*B*). Next, we stained cross-sections of 4-week-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 dy^{3K}/dy^{3K} muscle. A slight reduction of Cib2 expression was noted in MTJ, and a moderate reduction was seen in NMJ (Fig. 3*A*). Overall, an ~2-fold down-regulation of Cib2 was noted in dy^{3K}/dy^{3K} muscle (see Fig. 5, *B* 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. 3*A*). 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 $^{45}Ca^{2+}$ blot overlay assays. Recombinant Cib2 had a mass of about 48 kDa. The results demonstrated in Fig. 3*B* showed that $^{45}Ca^{2+}$ bound recombinant Cib2 but not GST alone and that binding was abolished upon inclusion of 10 mM unlabeled Ca^{2+} (Fig. 3*B*, *right*). Hence, Cib2 is indeed a calcium-binding protein. Abnormal Ca^{2+} handling

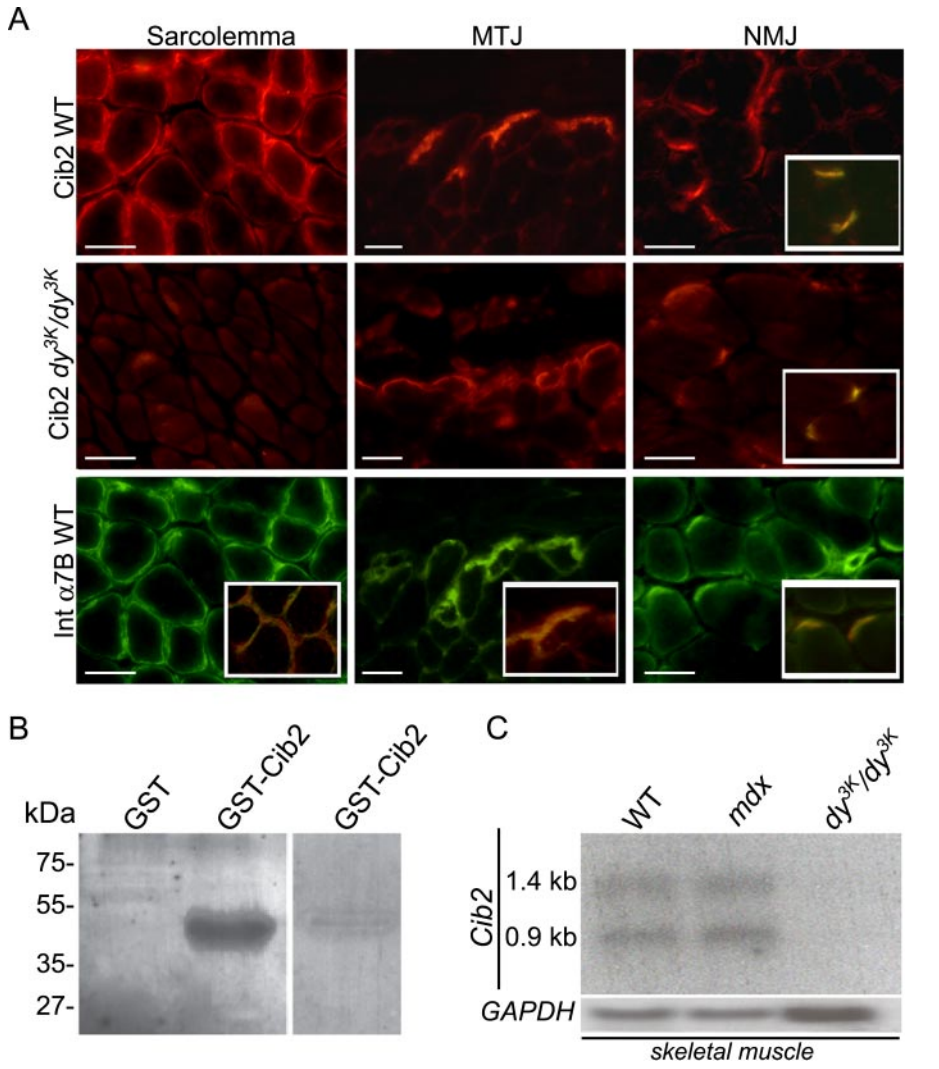

FIGURE 3. **Cib2 colocalizes with integrin α7B subunit in skeletal muscle and binds calcium.** *A*, cross-sections of quadriceps muscles from WT and dy^{3K}/dy^{3K} mice were stained with rabbit antibodies against Cib2 and integrin α7B, respectively. Cib2 is expressed in association with the sarcolemma and is enriched at the MTJ and NMJ. The *inset* in the *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. The *inset* in the *middle NMJ panel* shows double staining with Cib2 antibodies and α-bungarotoxin. Integrin α7B is also expressed at the sarcolemma (the *inset* is a digital overlay of the images of Cib2 and integrin α7B stainings and hence co-localization in *yellow*). The integrin α7B subunit is enriched at the MTJ (the *inset* is a digital overlay of the images of Cib2 and integrin α7B stainings) and at the NMJ (the *inset* shows double staining with integrin α7B antibodies and α-bungarotoxin). *Bars*, 50 μm. *B*, calcium binding assay. Purified GST and GST-Cib2 were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes, and the membranes were overlaid with $^{45}Ca^{2+}$ (1 μCi/ml) for 10 min with (*right*) or without (*left*) an excess of unlabeled $CaCl_2$ (10 mM). GST-Cib2 but not GST binds calcium. *C*, Northern blot analysis of *Cib2* mRNA expression in WT, *mdx*, and dy^{3K}/dy^{3K} hind limb skeletal muscles (25 day old). *Cib2* mRNA expression appears normal in *mdx* muscle but is reduced in dy^{3K}/dy^{3K} skeletal muscle. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

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 affected or unaffected animal models (dysferlin-deficient, 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. 3*C*) (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. 4, *A* 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 Fig. 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 (λ_{max}), and the intensity, but not the wavelength, of this peak increased upon addition of Cib2, as shown in Fig. 4*C*, where the absence of fluorescence emission from Cib2 alone is also evident. Fig. 4*D* 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 determination of the binding affinity of the two species, with an equilibrium dissociation constant (K_d) equal to 304 nM. Interestingly, it was shown that CIB1 binds an integrin αIIb peptide with a K_d 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 fluo-

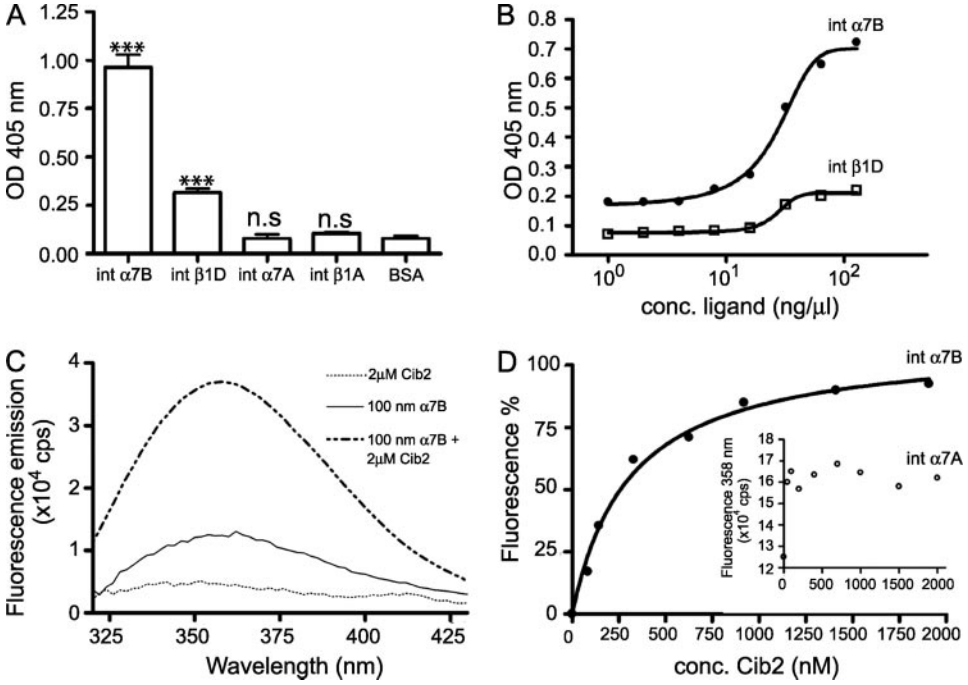

FIGURE 4. ***In vitro* binding of Cib2 to different integrin isoforms.** *A*, binding of integrins α7B, β1D, α7A, and β1A to GST-Cib2 or BSA (control) coated wells. Each value is the mean of 16 wells. A significant interaction between Cib2-integrin α7B and Cib2-integrin β1D is observed. Results are shown as means ± S.E. ***, 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 behavior, which indicates true bindings. *C*, comparison of the fluorescence emission spectrum of Cib2 alone with that of the integrin α7B peptide alone and upon the 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 $\lambda_{max} = 358$ 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 under "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 $\lambda_{max} = 358$ nm for the integrin α7A 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 α7A peptide and Cib2.

rescence, this increase was consistently independent from Cib2 concentration, indicating that Cib2 does not bind the integrin α7A subunit specifically (Fig. 4*D*, *inset*). 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 and instead expressing laminin α1 chain in muscle (dy^{3K}LNα1TG 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 dy^{3K}LNα1TG animals (Fig. 5*A*). This finding was also confirmed at the protein level. Western blot analyzes revealed that expression of Cib2 was partially reconstituted in skeletal muscles of dy^{3K}LNα1TG animals (Fig. 5, *B* 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 hind limb 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 dy^{3K}/dy^{3K} limb muscle. The most up-regulated genes in the most overrepresented groups are genes that encode specific isoforms of proteins that are transiently expressed during normal muscle development and regeneration (for example, *Myh3*, *Myl4*, and *Tnnt2*) and genes that encode cell adhesion and extracellular and extracellular matrix proteins (*e.g. Postn*, *Ctgf*, *Dlk1*, *Thbs4*, *Ncam*, and *Aspn*). This is not surprising in view of muscular dystrophies being manifested by degeneration/regeneration of muscle fibers and subsequent replacement of muscle fibers by connective tissue. However, the roles of, for example, periostin, connective tissue growth factor, delta-like 1 homolog, thrombospondin 4, and asporin in muscular dystrophies remain to be elucidated. More surprising is the fact that expression of a large number of genes was down-regulated in dy^{3K}/dy^{3K} limb muscle compared with *dy/dy* diaphragm (8). A majority of the decreased genes was assigned to metabolism themes. Thus, these data indicate a probable metabolic crisis in dy^{3K}/dy^{3K} 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 α7β1 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 Ca^{2+} 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 sper-

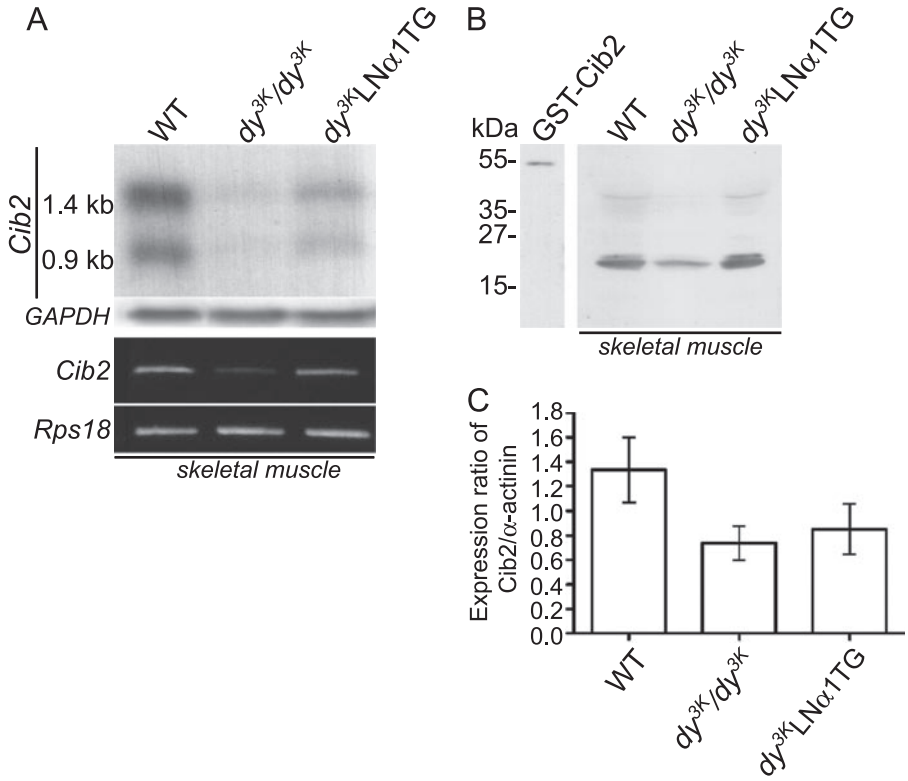

FIGURE 5. **Expression of Cib2 in WT, *dy*^{3K}/*dy*^{3K}, and *dy*^{3K}LNα1TG hind limb skeletal muscles.** *A*, Northern blot and RT-PCR analyses of *Cib2* mRNA expression in 25-day-old hind limb muscles. *Cib2* mRNA expression in skeletal muscle from *dy*^{3K}LNα1TG mice is partially normalized. *B*, immunoblotting of recombinant Cib2 and of total protein lysates from WT, *dy*^{3K}/*dy*^{3K}, and *dy*^{3K}LNα1TG hind limb 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}LNα1TG muscles. *C*, quantitative measurements of Cib2 expression from three independent experiments. α-Actinin was used as a protein loading control with the graph showing the ratio of Cib2/α-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}LNα1TG muscles.

matogenesis (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 α7Bβ1D. 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 the 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-week-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 the 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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