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Integrin $\alpha7\beta1$ in Muscular Dystrophy/Myopathy of Unknown Etiology

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To investigate the role of integrin $\alpha7$ in muscle pathology, we used a "candidate gene" approach in a large cohort of muscular dystrophy/myopathy patients. Antibodies against the intracellular domain of the integrin $\alpha7$A and $\alpha7$B were used to stain muscle biopsies from 210 patients with muscular dystrophy/myopathy of unknown etiology. Levels of $\alpha7$A and $\alpha7$B integrin were found to be decreased in 35 of 210 patients (~17%). In six of these patients no integrin $\alpha7$B was detected. Screening for $\alpha7$B mutation in 30 of 35 patients detected only one integrin $\alpha7$ missense mutation (the mutation on the second allele was not found) in a patient presenting with a congenital muscular dystrophy-like phenotype. No integrin $\alpha7$ gene mutations were identified in all of the other patients showing integrin $\alpha7$ deficiency. In the process of mutation analysis, we identified a novel integrin $\alpha7$ isoform presenting 72-bp deletion. This isoform results from a partial deletion of exon 21 due to the use of a cryptic splice site generated by a G to A missense mutation at nucleotide position 2644 in integrin $\alpha7$ cDNA. This spliced isoform is present in about 12% of the chromosomes studied. We conclude that secondary integrin $\alpha7$ deficiency is rather common in muscular dystrophy/myopathy of unknown etiology, emphasizing the multiple mechanisms that may modulate integrin function and stability. *(Am J Pathol 2002, 160:2135–2143)*

Integrins are transmembrane heterodimers of two different subunits, α and β, associated by non-covalent interactions. In humans, at least 18 different α and 8 different β subunits are known, resulting in 22 distinct heterodimers.[1,2] The $\alpha7$ subunit is mainly expressed in skeletal and cardiac muscle, while the $\beta1$ chain is expressed throughout the body and associated with other different α subunits.[3–6] In adults, the alternative $\beta1$D isoform is restricted to skeletal and cardiac muscle.[7–9]

Integrin $\alpha7\beta1$ in skeletal and cardiac muscle binds *via* its extracellular domain to laminin $\alpha2$ and $\alpha4$[10–12] and *via* its cytoplasmic domain possibly to α-actinin and talin ensuring continuity of structure and signaling between the cytoskeleton and the basal lamina.[13,14] Integrins have a prominent role in myogenesis,[15,16] differentiation, cell migration, and cell-cell interactions.[2,17–19]

Integrin $\alpha7$ in skeletal muscle localizes at the sarcolemma, at the neuromuscular junctions, and, most prominently, at the myotendinous junctions where it provides an anchorage for laminin $\alpha2$, conferring mechanical stability and traction resistance to the skeletal muscle fibers.[5,9,20,21] The expression of several integrin $\alpha7$ isoforms modulates the integrin $\alpha7\beta1$ ligand affinity and signaling specificity during myogenesis.[20] All integrin isoforms are encoded by a single $\alpha7$ gene (ITGA7) located on chromosome 12q13[22] and result from alternatively spliced variants.[20,23]

In humans, different extracellular and cytoplasmic domain isoforms have been described.[23,24] The $\alpha7$X1 and $\alpha7$X2 extracellular isoforms result from the alternative splicing of exons 5 or 6, in a variable region nearby the ligand binding site.[5,24,25] The X2 isoform is the main variant expressed in adult muscle fibers.[24,26] The cytoplasmic isoform $\alpha7$A,[20,24,27] corresponding to the normal exon 26 splicing, is temporarily up-regulated during myogenesis, while the $\alpha7$B variant, the longest isoform resulting from the splicing out of exon 26, is the main isoform expressed in skeletal and cardiac muscle, smooth muscle cells, spleen, liver, and brain.[20,28]

To study the involvement of $\alpha7$ integrin during myogenesis and its role in muscle integrity and function, a null allele of the gene for the $\alpha7$ integrin subunit has been generated.[29] Mice homozygous for the mutation showed features of a progressive muscular dystrophy in their muscle, suggesting that integrin $\alpha7$ represents an indispensable linkage between the muscle fiber and the ex-

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tracellular matrix,[29] particularly at the myotendinous junction. To further support the role of integrin in connecting muscle cells with the surrounding extracellular matrix, it has been shown that $\alpha7\beta1$ is up-regulated in Duchenne muscular dystrophy (DMD) patients and in its animal model, mdx mice, to compensate for the reduced dystrophin-mediated linkage of fibers with the basal lamina.[11,30] In merosin-negative congenital muscular dystrophy (CMD) and in its animal model, dy/dy mice, $\alpha7$ integrin appears to be significantly reduced suggesting that lack of laminin $\alpha2$ ($\alpha2\beta1\gamma1$) may down-regulate the expression or change the location of integrin $\alpha7$ gene.[11,12,30]

The cloning of the full-length human ITGA7 cDNA[5,26,31] allowed the identification of the first three patients with primary $\alpha7$ integrin deficiency.[31] The three patients reported were affected with congenital myopathy with variable clinical phenotype, and all showed a complete absence of integrin $\alpha7$ in their muscle biopsies due to primary integrin $\alpha7$ nonsense/splicing mutations or to a down-regulation of integrin $\alpha7$ mRNA.[31] Here we report a study of a large series of muscle biopsies from patients affected with unclassified muscular dystrophy/myopathy tested for integrin $\alpha7$ deficiency by combined integrin $\alpha7$ protein and gene studies.

Materials and Methods

Patients

Our muscle biopsy tissue bank at the Neuromuscular Center of the University of Padova was screened to search for patients meeting the following criteria: muscle weakness and/or hypotonia; muscle histopathology consistent with a myopathic or dystrophic process; and normal dystrophin, α-sarcoglycan, calpain, dysferlin, and laminin $\alpha2$ in their muscle biopsies. Inflammatory myopathies, neurogenic atrophies, and metabolic or mitochondrial myopathies were *a priori* excluded. Two hundred ten patients met the clinical and histopathological selection criteria and were chosen for integrin $\alpha7$ screening. One hundred thirteen were muscular dystrophies, 24 CMD, and 73 carried a histopathological diagnosis of undetermined myopathy.

Case Report

Patient E.S. was delivered by Caesarian section at the 39th gestational week due to the threat of miscarriage. The child was oxygen-dependent and mechanically ventilated since birth. He presented with hypotonia and with hip, wrist, and ankle contractures. Karyotype study was normal. A brain computed tomography (CT) scan showed cortical atrophy and white matter signal abnormalities. Creatine kinase (CK) was 507 U/L (normal $<$250) and electromyography (EMG) myopathic. At one month of age a muscle biopsy was consistent with a congenital muscular dystrophy. Dystrophin, α-sarcoglycan, and laminin $\alpha2$ studies in his muscle biopsy were normal. The patient died at 13 months of age from respiratory failure. Integrin $\alpha7$ was markedly reduced in the patient's muscle biopsy.

Integrin $\alpha7$ Immunohistochemistry

Serial cryosections, 4-μm thick, were fixed for 5 minutes in 100% ice-cold-acetone, air dried, and preincubated with phosphate-buffered saline containing 2% bovine serum albumin and 5% goat serum as blocking agent.[31] Polyclonal anti-integrin $\alpha7$B antibody (1:500) directed against the intracellular domain of the protein,[12] polyclonal anti-integrin $\alpha7$A antibody (1:500) directed against the COOH terminal peptide of the protein, polyclonal anti-$\beta1$D (1:500),[12] and monoclonal antibody directed against the carboxyl terminus region of the laminin $\alpha2$ (1:1000)[32] (Chemicon, Temecula, CA) were used to incubate the sections for 2 hours at room temperature. Appropriate Cy-3-conjugated (anti-rabbit or anti-mouse) secondary antibody (1:100) (Caltag Lab, Burlingame, CA) were used. The visualization of mounted sections was done on Zeiss Axioskop photomicroscope. Hematoxylin and eosin staining was done on parallel cryosections to check tissue integrity and histopathology.

Muscle biopsies were scored as integrin $\alpha7$B negative, integrin $\alpha7$B markedly reduced, or integrin $\alpha7$B slightly reduced (based on the amount of integrin $\alpha7$ immunostaining detected in each muscle biopsy). The same criteria were used to score $\alpha7$A immunostaining.

Integrin $\alpha7\beta$ Mutation Studies

RNA extraction

Approximately 50 mg of frozen muscle biopsy tissue was homogenized using a Kinematica Polytron PT 2100 homogenizer. RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) accordingly to the manufacturer's instructions and stored in RNase-free water at -80°C.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

For cDNA synthesis, $\sim$1–2 micrograms of RNA was reverse transcribed using oligo-dT primers as previously described.[33] cDNA was boiled to denature and inactivate reverse transcriptase. Eighteen sets of overlapping primers were designed to cover the entire 3414 bp of the integrin $\alpha7$ coding sequence and the 5[prime]-UTR region (Table 1). PCR reactions contained about 50 ng of cDNA, 50 ng of each primer, 100 nmol/L of each dNTP, and 0.2 μCi α-[^{32}P] dATP in 12.5 μl total volume. Reaction conditions were 10 minutes at 94°C to denature; 30 seconds at 94°C, 30 seconds at 65°C, 30 seconds at 72°C for 35 cycles, with an extension of 10 minutes at 72°C for all primer sets except primer sets 1212F-1451R and 2068F-2245R where the annealing temperature was 50°C.

Table 1. Integrin α7 Primers Sequence

Primer set	
-56F, 5′-TTGGGGCGTGCGAGATTTC-3′	249R, 5′-CTGCCCAGGAAGAGCCAGGGC-3′
32F, 5′-GGGCCTCCGGGATTTGCTAC-3′	279R, 5′-AGCGAAGAGGCCTCCAGTGC-3′
201F, 5′-GAGCTGGCTGCTGGTGGGTGC-3′	467R, 5′-GTCTCCAGGATCTGGTCCAC-3′
410F, 5′-TTGTTACCTGTGCACACCGAT-3′	676R, 5′-GCAACCCCTTCCAATTATAGG-3′
608F, 5′-CCGCCTTCTCCCCTGATAG-3′	841R, 5′-AGCTCAGCTCTTCTGCACG-3′
807F, 5′-GGGGAAAGGTCTGGTGCG-3′	1022R, 5′-GCACCCACTATCAGGTCTGGCC-3′
980F, 5′-TGACCTCAACAGTGATGGCTG-3′	1282R, 5′-CCTGTGAAGGTTTGGCGAC-3′
1212F, 5′-CTTTGATGGTGATGGGAAAGTC-3′	1451R, 5′-GCAATAGAGACCTCATGGGAG-3′
1391F, 5′-ACACCGCAGTGCTCTTCAGGGC-3′	1648R, 5′-GGTTACGGCTCAGGAACGTC-3′
1581F, 5′-GTTAGATGCGGACACAGACCG-3′	1894R, 5′-AGTGGATCTCTGCCCGCTGG-3′
1850F, 5′-TCCTCAATGCCCACCAGC-3′	2083R, 5′-GCAGGTTGGTGACCATCA-3′
2068F, 5′-GTGGATGGAACAACAGCCC-3′	2245R, 5′-CACACTCAACATGGGAGGC-3′
2207F, 5′-TCTGCCTGTCCAATGAGAATG-3′	2464R, 5′-CAGAGAAGAAGAGTTGCTGGG-3′
2407F, 5′-ATTGAGCTGCCACTGTCCATTG-3′	2644R, 5′-CAACCTGCATTGGGTACAGC-3′
2607F, 5′-TGCCAATGGGAAGTGGTTG-3′	2887R, 5′-GGCAGATGAACACCACACAG-3′
2806F, 5′-CCAGTGTCCTCTGCTGAGAAG-3′	3075R, 5′-GTATACCATCACTGGGATCACTG-3′
3017F, 5′-CCTCCATAAAGAACTTGATGCTCC-3′	3251R, 5′-GGAATCTTCACCGCATGG-3′
3223F, 5′-GTGCCCCAGTACCATGCGGTG-3′	3391R, 5′-GCCCATCGGGGCCCAGCTCG-3′

Single Strand Conformational Polymorphism Analysis

Three different single strand conformational polymorphism (SSCP) conditions[33] were used for screening for integrin α7 mutation. Conformers were re-amplified using the original amplification primer and PCR conditions as previously described[33] and directly sequenced on automatic sequencer (CRIBI Biotechnology Center, Department of Biology, University of Padova).

The integrin α7 mutations identified were confirmed with the appropriate restriction endonuclease digestion if a gain or loss of restriction site was detected or with appropriate PCR primers designed to cover the mutated region and PCR product size-fractioned with denaturing polyacrylamide gel electrophoresis. Primers used to amplify exon 15 and exon 21 were as following: ex 15F 5′-TGCCCCTTATCTATGTCTCC-3′; ex 15R 5′-GAGAGGGCTTTCTCTCAATCC-3′; ex 21F 5′-TCCCCTCATACTCTCTTTTTCC-3′, and ex 21R 5′-AACTCCCCATAACATCTGTAACC-3′.

Integrin α7 Gene Expression Studies

RNA extraction and cDNA synthesis were done as described above. Biopsies studied were from the 30 patients screened for integrin α7 mutation and from 10 integrin α7-positive myopathic control muscle biopsies.

Primer sets for co-amplification of integrin α7 RNA and α-sarcoglycan RNA were designed, and for each set the forward primer was synthesized with fluorescein-linked 5′ ends (Invitrogen). Primer sequences were as follows: α-sarcoglycan ADH3F: 5′fluorescein-CTGCTCAACGTCACCTCTG-3′; ADH3R: 5′-CCGGCACTGACTTATCCAC-3′; and integrin α7 ITGA7.9F: 5′fluorescein-TTGATGGTGATGGGAAAGTCTTCA-3′; and ITGA7.12R: 5′-CAGACCCTTAGGTCCACACAGACC-3′.

Multiplex RT-PCR and quantitation of fluorescent PCR products were done as previously described.[34] Briefly, cDNA corresponding to approximately 20 ng of total RNA was amplified with primers mixed for 22 cycles using standard conditions (denaturation at 95°C for 10 minutes, at 94°C for 1 minute, at 62°C for 1 minute, at 72°C for 1 minute, for 22 cycles; and extension at 72°C for 10 minutes). Fluorescent RT-PCR products were denatured and loaded on an ABI automated sequencer (Applied Biosystems, Foster City, CA) using a ROX/fluorescein matrix standard. ROX labeled markers were included as internal size standards. Peak areas corresponding to expected RT-PCR product sizes were determined, and peak area ratios of integrin α7 relative to internal control (α-sarcoglycan) calculated. Two separate RT-PCR measurements were done for each patient's biopsy. Statistical significance was done by Student's *t* test using all calculated ratios.

Results

Histological Features of 35 Integrin α7-Deficient Patients

The entire database, about 5000 muscle biopsies, was searched for patients meeting the histopathological diagnosis of muscular dystrophy of unknown etiology (normal dystrophin, α-sarcoglycan, calpain, dysferlin, and laminin α2). About 560 muscle biopsies, collected over 2 consecutive years, were searched for undetermined myopathy. Two hundred ten patients were selected for integrin α7 screening from the database search of muscle biopsies. One hundred thirty seven muscular dystrophy and 73 myopathies were tested with integrin α7 immunofluorescence. Muscle biopsies from patients diagnosed with muscular dystrophy showed marked fiber size variation, increased central nuclei, degeneration and regeneration, and a marked increase in the endomysial and perimysial connective tissue with fatty infiltration. Common features in the undetermined myopathy group of muscle biopsies included mild fiber size variation and scattered central nuclei, with little or absent muscle fiber degeneration or regeneration. Fibrofatty infiltration was absent or minimal.

Antibodies directed against the α7A, α7B, and the β1D subunit of integrin were used to characterize integrin expression in patients' muscle biopsies. In parallel, lami-

Table 2. Integrin α7 Immunofluorescence Results in 210 Muscle Biopsies

	Undetermined muscular dystrophy (*n* = 137)			Undetermined myopathy (*n* = 73)			
	<2 yr	>2 yr	Total	<2 yr	>2 yr	Total	Total (*n* = 210)
Integrin α7 negative	2 (1%)	4 (3%)	6 (4%)	0	0	0	6 (3%)
Integrin α7 markedly reduced	3 (2%)	5 (4%)	8 (6%)	4 (5%)	7 (10%)	11 (15%)	19 (9%)
Integrin α7 slightly reduced	1 (0.7%)	6 (4%)	7 (5%)	1 (1%)	2 (3%)	3 (4%)	10 (5%)
Integrin α7 normal	13 (9%)	103 (75%)	116 (84%)	4 (5%)	55 (75%)	59 (80%)	175 (83%)

Percentage values were rounded to nearest 5.

nin α2 immunostaining was done in each muscle biopsy to check the integrity of the muscle fibers. Three percent (6 of 210) of the muscle biopsies studied were completely deficient for integrin α7B (Table 2; Figure 1G) and about 14% (29 of 210) showed a (variable) reduction of integrin α7B immunostaining (Table 2; Figure 1, M and Q).

Six muscle biopsies showing complete integrin α7B deficiency were all undetermined muscular dystrophy (Figure 1G). Three were diagnosed as CMD and 3 as limb-girdle muscular dystrophy. Marked integrin α7B reduction (ie, variable and barely detectable integrin α7 immunostaining in the majority of the muscle fibers with or without some scattered myofibers showing patchy but positive immunostaining) (Figure 1M) was present in about 6% (8 of 137) of the muscular dystrophies and in 15% (11 of 73) of the myopathies studied (Table 2). A slight reduction in integrin α7B (ie, variable and faint integrin α7 immunostaining and/or a mosaic-like pattern) (Figure 1Q) was detected in 5% (7 of 137) of muscular dystrophies and in about 4% (3 of 73) of unclassified myopathy (Table 2). Interestingly, the integrin α7-positive muscle fibers, in the subset of α7B partial deficiency, often showed a integrin α7-positive cytoplasmic immunostaining. Integrin α7A was reduced (Figure 1, H, N, R), and integrin β1D mildly reduced (Figure 1, F, L, P) in all muscle biopsies showing integrin α7B deficiency. In the

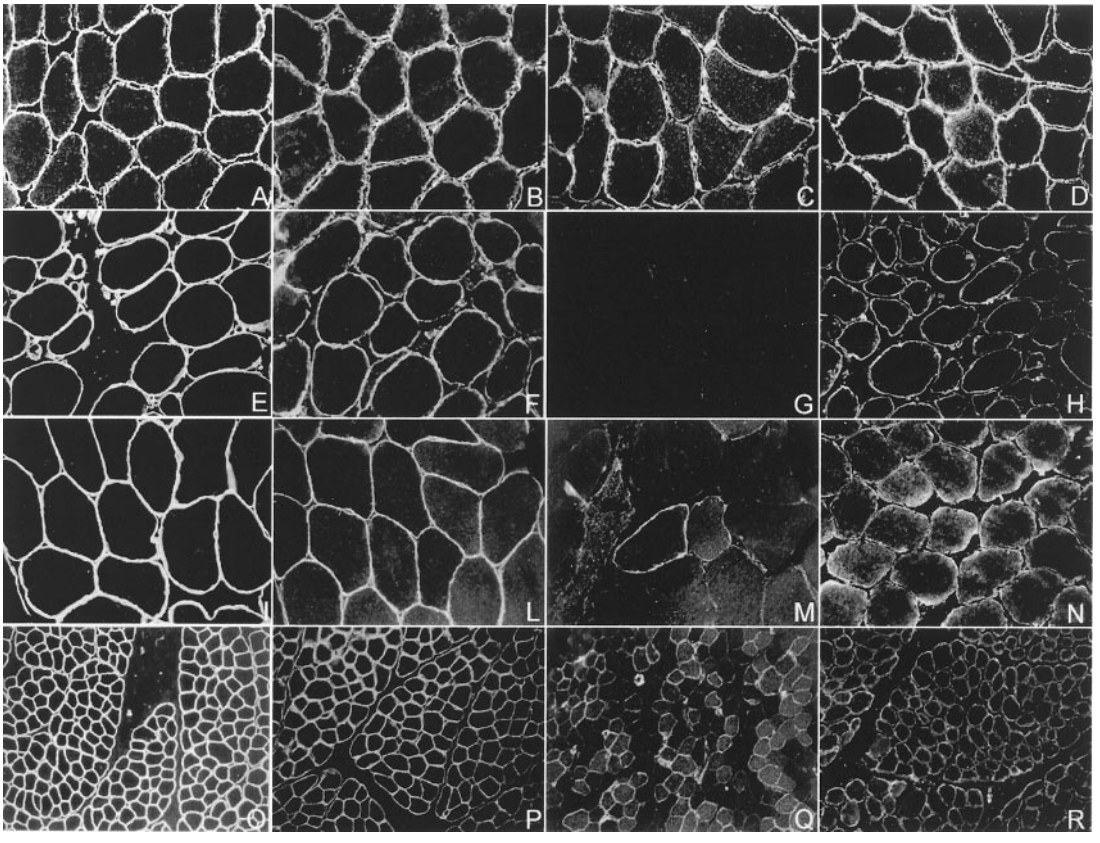

Figure 1. Integrin immunofluorescence studies. Transverse cryosections of muscle-biopsy specimen from a normal control and three patients with integrin deficiency were stained with antibodies directed against the intracellular domain of integrin α7B, integrin α7A, integrin β1D, and laminin α2. **A–D** show a normal muscle biopsy, **E–H** show a muscle biopsy showing complete integrin α7 deficiency, and **I–R** show muscle biopsies from two partial integrin α7-deficient patients. Integrin α7B immunostaining shows a uniform staining of the periphery of each myofiber in the control muscle (**C**), where a muscle biopsy showing a complete absence of immunoreactivity is shown in **G**. **M:** Integrin α7 is severely reduced. The majority of the muscle fibers show a barely detectable immunostaining, but a few, scattered fibers are strongly integrin α7-positive and show a slight cytoplasmic α7B-positive immunostaining. **Q:** A different pattern of integrin α7 partial deficiency is shown. Integrin α7-positive and -negative fibers are scattered in a mosaic-like pattern. The integrin-positive fibers show a α7B-positive cytoplasmic staining. Integrin α7A was reduced (**H**, **N**, **R**) and integrin β1D mildly reduced (**F**, **L**, **P**) in the complete integrin α7 deficiency muscle biopsy and in both the partial integrin α7 deficiency specimens in comparison with the control muscle (**D** and **B**). Laminin α2 was normal in all muscle biopsies (**A**, **E**, **I**, **O**).

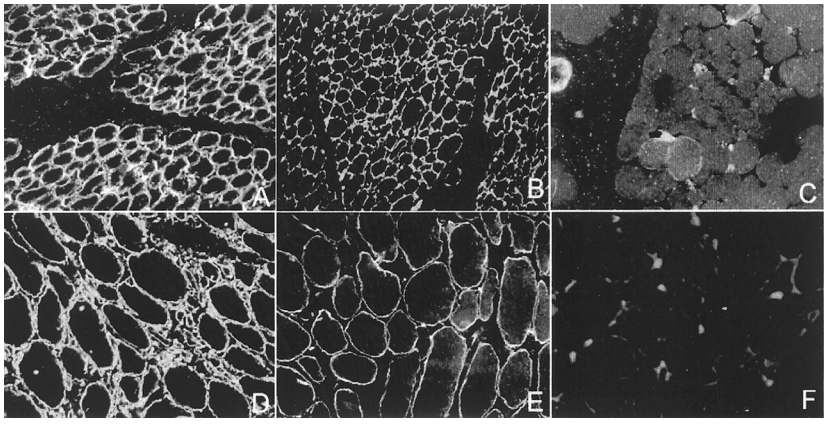

Figure 2. Integrin α7 deficiency in a two-year-old patient is not rescued over time. Cryosections from muscle biopsies performed at 2 years (**A–C**), and 6.3 years of age (**D–F**) in the same patient were stained with an antibody directed against the intracellular domain of the integrin α7B (**C** and **F**), integrin β1D (**B** and **E**), and laminin α2 (**A** and **D**). In **C** (a muscle biopsy performed at 2 years of age), integrin α7B is faintly expressed in a minority of the patient's muscle fibers. In the muscle biopsy performed at 6.3 years of age the number of integrin α7-positive fibers is dramatically reduced and strongly integrin α7-positive fibers are no longer detectable (**F**). Integrin β1D was mildly reduced in both of the patient's muscle biopsies (**B** and **E**) and laminin α2 was normal (**A** and **D**).

six muscle biopsies showing complete integrin α7B deficiency, integrin α7A was more severely decreased than in the partial deficiency. Laminin α2 was normal in all biopsies studied (Figure 1, E, I, O).

Integrin α7B deficiency was present in about 39% (11 of 28) (Table 2) of muscle biopsies from patients younger than 2 years, and only in 13% (24 of 182) (Table 2) from patients older than 2 years. To determine whether integrin α7B deficiency in younger patients was a manifestation of integrin α7B being expressed at a lower level at a younger age, we selected a patient with a marked reduction of integrin α7B in muscle at 1 year of age who had two subsequent muscle biopsies obtained at 2 and 6.3 years of age. Integrin α7B immunostaining was performed in parallel with laminin α2 and integrin β1D in the three-muscle biopsy. As shown in Figure 2, the patient's muscle biopsy performed at 2 years of age (Figure 2C) contained scattered myofibers with a faint and variable integrin α7B immunostaining. Rare fibers showed a patchy and barely detectable integrin α7B immunostaining in the muscle biopsy performed at 6.3 years of age (Figure 2F). Integrin β1D was mildly reduced (Figure 2, B and E), and laminin α2 was normal in these biopsies (Figure 2, A and D).

Integrin α7 Mutation Studies

Adequate muscle tissue for RNA extraction was available for 30 of 35 muscle biopsies showing integrin α7B deficiency. Twenty were muscular dystrophies (including the six samples showing complete α7B deficiency) and 10 were myopathies. These 30 muscle biopsies were selected for integrin α7 mutation screening. RNA was extracted and RT-PCR/SSCP was performed using 18 sets of overlapping primers covering the entire integrin α7 coding sequence.

Integrin α7 Polymorphisms

Several conformers were identified and directly sequenced. Seven integrin α7 polymorphisms were identified. All of the identified polymorphisms were in the extracellular domain of the protein. Of the seven polymorphisms identified, six were silent nucleotide changes: G285T (Pro95Pro), G351A (Glu117Glu), A366G (Gln122Gln), G810A (Gly270Gly), T2307C (Ser769Ser), and C3018G (Ser1006Ser). An A1952G nucleotide change resulted in a histidine to an arginine amino acid change (His651Arg). The A1952G nucleotide change was present in 32 of 70 (46%) of the chromosomes studied.

Integrin α7 Alternatively Spliced Isoform

A unique conformer was detected with primer set 2607F-2868R in a single patient. Sequencing showed an in-frame deletion mutation starting at nucleotide position 2641 to nucleotide position 2712 of the integrin α7 coding sequence (Figure 3). The heterozygous in-frame deletion encompassed 72 bp in exon 21. This in-frame deletion detected at the cDNA level was absent at the genomic DNA level suggesting that the recognition of a cryptic splice site within exon 21 may be responsible for the skipping of about 40% of exon 21 directly to exon 22. To further characterize this in-frame deletion we performed SSCP on exon 21 in genomic DNA from the patient and

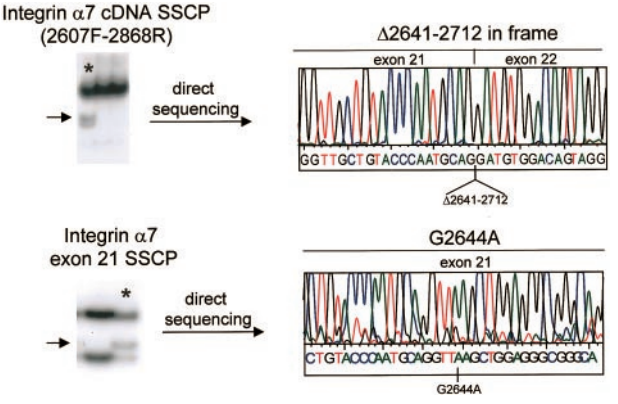

Figure 3. Identification of a novel alternatively spliced integrin α7. **Top:** SSCP analysis of RT-PCR product for primer set 2607F-2868R showed a unique conformer in a patient (**asterisk**). Direct sequencing of this conformer showed a 72-bp in-frame deletion mutation starting at nucleotide position 2641 to nucleotide 2712 (Δ2641–2712) of the integrin α7 coding sequence corresponding to about 40% of exon 21. The Δ2641–2712 deletion was absent in the genomic DNA of the patient. SSCP analysis of exon 21 (**bottom**) showed a G to A substitution at position 2644 of the integrin α7 cDNA. The G2644A nucleotide change caused the creation of a cryptic donor splice site within exon 21.

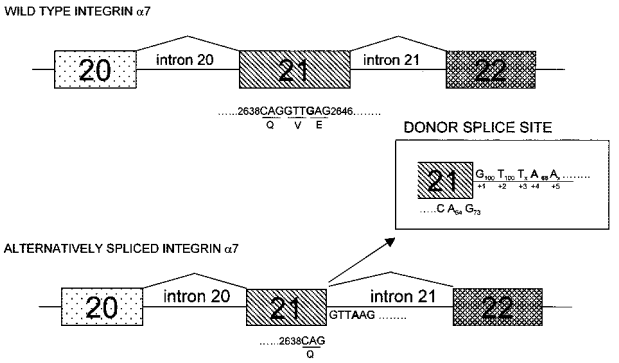

Figure 4. Identification of a novel cryptic donor splice site in integrin α7 exon 21. Full length and alternatively spliced integrin α7 are shown. Full length integrin α7 is the major transcript in adult skeletal muscle and results from the expected splicing from exon 21 to exon 22 (wild-type integrin α7 in the figure). The alternatively spliced integrin α7 derives from the use of a cryptic splice site starting at position 2639 (AG/GTT**A**$_{2644}$AG) of the integrin cDNA resulting in the skipping of about 40% of the exon 21. PCR/SSCP of exon 21, in the original patient where the alternatively spliced isoform was identified, showed a G to A nucleotide change at position 2644 (**bold**). Nucleotide sequence 5[prime] of the G2644A nucleotide substitution were consistent with a consensus donor splice site sequence: $2639A^{-2}{}_{62}G^{-1}{}_{77}/G^{+1}{}_{100}T^{+2}{}_{100}T^{+3}{}_{xx}$. The G to A substitution at position + 4 of the intron 21 created a weak donor sequence according to an algorithm to identify splice site based on a Shapiro and Senapathy matrix. In the alternatively spliced integrin α7 the donor splice site sequence is shown. Numbers below each base give the frequency of occurrence.[49]

from 60 controls. Identical conformers were detected in the patient and in 14 of 120 chromosomes studied (~12%). Sequence analysis of the conformers showed a G to A substitution at position 2644 in cDNA (Figure 3). The G2644A base change resulted in a glutamic acid to lysine amino acid change (E822K) and it caused a gain of *Mse*I restriction digestion site. Appropriate restriction digestion and RFLP analysis confirmed the mutation in the genomic DNA of the patient.

Interestingly, when the G2644A mutated exon 21 sequence was searched for a splice site using a Splice Site Finder program (supported by Genet.sickkids.on.ca) a cryptic donor splice site sequence was identified (AG/GTTA$_{2644}$AG) (Figure 4). When the score of the cryptic donor sequence was calculated using an algorithm based on the matrix compiled by Shapiro and Senapathy,[35] the cryptic donor splice site AG/GTTA$_{2644}$AG) scored 71.0 where the wild-type donor sequence (TG$_{2712}$/GTGAGG) obtained 84.3 (accordingly to a Splice Site Score Calculator program supported by Genet. sickkids.on.ca).

Integrin α7 Mutation

One of the 30 patients studied showed a heterozygous integrin α7 mutation. Patient E.S. showed a unique conformer with primer set 1850F-2083R (Figure 4). Direct sequencing of the aberrant conformers showed an heterozygous C to G nucleotide change at position 1969 in this patient (Figure 5). The C1969G nucleotide change resulted in a arginine to glycine amino acid change at position 657 of the integrin α7 protein. The Arg657Gly is located in the region containing the major ADP-ribosylation site. The C1969G nucleotide change resulted in a loss of a *Sma*I restriction-enzyme site. To verify the frequency of this mutation in the control population, integrin α7 exon 15 was PCR-amplified, and the PCR products were digested with *Sma*I. Digestion fragments were present in all of 200 control chromosomes but only in one allele of the patient's DNA (heterozygous mutation) (Figure 5, bottom).

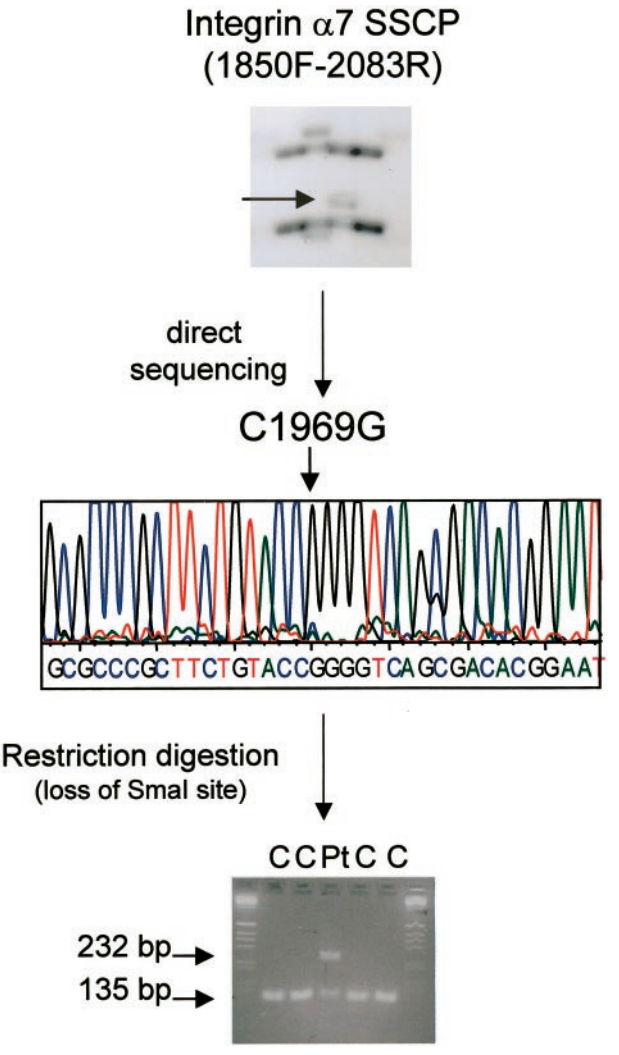

Figure 5. Screening of the integrin α7 coding sequence for potential mutations by SSCP showed a C1969G heterozygous nucleotide change resulting in an Arg657Gly amino acid change in patient E.S. **Top:** SSCP analysis of RT-PCR product for primer set 1850F-2083R showed a unique conformer in patient E.S. (The second conformer detected resulted in a A1952G nucleotide change (His651Arg). This polymorphism was present in 46% of the chromosomes studied). Direct sequencing of the aberrant conformer showed a C to G nucleotide change at position 1969 of the integrin α7 coding sequence (**middle**) resulting in an arginine to glycine amino acid change at position 657 of the integrin α7 protein. Nucleotide change C1969G resulted in a loss of *Sma*I restriction-enzyme site. Primers were designed to amplify exon 15 of the integrin α7 gene and the PCR product was digested with *Sma*I. Digestion fragments were present in 200 control chromosomes and only in one of the patient's chromosomes (heterozygous mutation) (**bottom**).

Integrin α7B Gene Expression Studies

The same 30 patients used for integrin α7 mutation studies were selected for RNA studies. Ten muscle biopsies with myopathic histopathology but with normal integrin α7B immunofluorescence were chosen as controls.

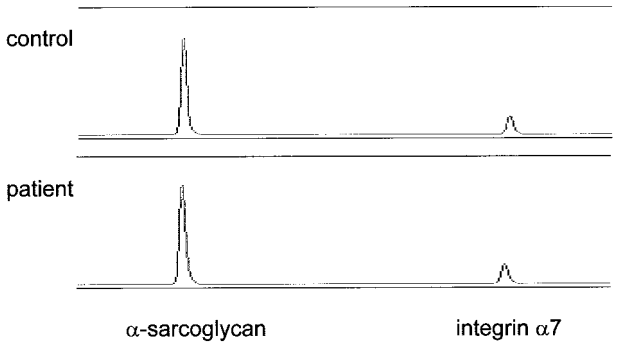

Figure 6. Integrin α7-deficient patients show normal amount of integrin α7 mRNA. Shown are examples of automated sequencer traces of quantitative multiplex fluorescent RT-PCR of the integrin α7 relative to α-sarcoglycan. The levels of the integrin α7 RNA was similar in the patients relative to controls.

RNA was isolated from patients' muscle biopsies and 100 ng of total RNA was reverse transcribed into cDNA using oligo dT^{10} primers. Approximately 20 ng of total cDNA was amplified using both integrin α7 and α-sarcoglycan primers. Quantitative multiplex fluorescent RT-PCR (QMF-RT/PCR) products (22 cycles) were done in duplicate for each sample and electrophoresed on an ABI automatic sequencer (373A) to quantitate signals using peak area. Integrin α7 *versus* α-sarcoglycan RNA ratios were determined. The patients' muscle biopsies showed a normal amount of total integrin α7 RNA relative to controls in their muscles (Figure 6). Mean integrin α7 *versus* α-sarcoglycan ratios were 24.2 ± 10 in the controls and 24.3 ± 11 in the patients' group. Student's *t*-test did not show any significant difference between patients' and controls' integrin α7 RNA levels ($P = 0.8$).

Discussion

Despite the recent advances in our understanding of the molecular basis of neuromuscular disorders, the underlying molecular defect can be identified only in a subset of the cases. A large cohort of patients with muscular dystrophy/myopathy cannot be assigned a specific molecular diagnosis. The goal of this study was to pursue an integrin α7 candidate protein and gene analysis in our large cohort of muscular dystrophy/myopathy patients of unknown etiology. This approach was chosen since candidate protein and gene studies have shown impressive progress in dissecting the heterogeneous limb-girdle dystrophy group. To date, dystrophies caused by α-, β-, and δ-sarcoglycan have all been defined *via* candidate gene and protein approaches.[36–40]

The cloning of the integrin α7 gene,[31] the preferential expression of integrin α7 in skeletal muscle,[41–43] and the identification of causative integrin α7 mutations in a subset of Japanese patients presenting with congenital myopathy[31] made integrin α7 an ideal candidate gene for screening a large bank of patient muscle biopsies. Candidate protein and gene analyses were conducted by using integrin α7 immunofluorescence in 210 muscle biopsies of patients affected with muscular dystrophy or myopathy of unknown etiology and followed by direct mutation screening of muscle biopsy RNA in 30 patients in which integrin α7 deficiency was identified. No integrin α7 mutations were identified in 29 of 30 patients showing various degrees of integrin α7 deficiency ranging from complete to slight deficiency. Only in one patient presenting at birth with CMD and showing, by immunofluorescence, a marked reduction of integrin α7 in his muscle biopsy, an heterozygous C1969G missense mutation resulting in an arginine to a glycine amino acid change (Arg657Gly) was identified. Despite our efforts, we were unable to identify in this patient a second mutation, consistent with the presumed recessive inheritance observed in CMD. We feel that the C1969G nucleotide change may be a causative mutation based on several lines of evidence. First, the C1969G results in an arginine to a glycine amino acid change (Arg657Gly). Arginine is a large, charged amino acid with a molecular weight of 174, whereas glycine is a small, (molecular weight 75) uncharged, polar amino acid. It is unlikely that such a dramatic amino acid change would have no functional consequences in an heterodimeric protein. Second, the C1969G mutation was not observed in 200 control chromosomes and it is located in a region of documented functional importance. The Arg657Gly mutation lies in the region containing the major ADP-ribosylation site[44] that it is likely involved in the mechanisms modulating α7 integrin-mediated signaling pathways.

Several integrin α7 polymorphisms were also identified. In particular, we report a novel alternatively spliced integrin α7 isoform, generated from the use of a cryptic splice site in exon 21 (AG/GTTA_{2644}AG), that was derived from a G2644A nucleotide change. The use of this cryptic splice site resulted in the skipping of about 40% of exon 21 directly to exon 22. This splice variant is present in about 12% of the chromosomes studied. A Senpathy score of at least 70 is required for donor splice site recognition.[45] The AG/GTTA_{2644}AG cryptic donor site sequence scored only 71, thus the splicing theory would predict that the AG/GTTA_{2644}AG sequence would be a weak donor site. Since both normal and cryptic mRNA are observed, both splice sites are used. However, the ratio between the normal and cryptic RNA favors the normal transcript (Figure 3), suggesting that the utilization of the cryptic AG/GTTA_{2644}AG splice is incomplete. It is difficult to predict the functional importance of this alternatively spliced integrin α7 isoform that removes 27 amino acids at a region where a major ADP-ribosylation site and a potential integrin leucyl-aspartyl-valine (LDV) binding site have been located.[26] While it is possible that the spliced variant may act as a modifying factor in a predisposing genetic background, it is also possible that it is without any functional and/or structural importance. Among the several integrin α7 isoforms previously reported,[5,20,23–28] the α7D, resulting from a partial deletion of exon 15 and the entire exon 16, is particularly interesting encompassing the major ADP-ribosylation site.[23,26] Unfortunately, the exact role of ADP-ribosylation on α7 integrins remains to be determined,[26] thus the potential functions of these isoforms are still largely unknown.

It has been reported that the expression of the cytoplasmic splice variant α7B is developmentally regulated,

and that it is detectable in skeletal muscle only after the age of 2 years.[30] Since we used an antibody against the integrin α7B variant, we checked if the integrin α7 deficiency was related to the age of the patients at the time of the biopsy. Indeed, 39% (11 of 28) of the muscle biopsies of patients younger than 2 years compared to 13% (24 of 182) of patients older than 2 years showed integrin α7 deficiency. Even if this difference is significant, we feel that factors other than developmental stage may affect the sarcolemmal expression of the α7B integrin subunit in diseased skeletal muscle, as suggested by Cohn et al.[30] In the only patient where consecutive muscle biopsies were available, the integrin α7 deficiency detected in the muscle biopsy performed at 1 year of age was not rescued in the subsequent muscle biopsies done at 2 and 6.3 years of age. Moreover, 60% (17 of 28) of patients younger than 2 years showed normal integrin α7 immunostaining. The positive integrin α7 immunostaining detected in patients younger than 2 years of age may also be interpreted as some yet undefined compensatory mechanisms acting in dystrophic/myopathic muscle as suggested by the expression of integrin α7B in DMD and in the hypertrophic muscle fibers in SMA (spinal muscular atrophy) patients younger than 2 years.[30] To better investigate the discrepancy between the integrin α7 protein deficiency detected through immunofluorescence analysis of patients' muscle biopsies and the lack of identification of integrin α7 gene mutations, we conducted a series of experiments.

Our inability to detect integrin α7 gene mutation in integrin α7-deficient muscle samples is not likely due to technical reasons. We cannot rule out that mutations in the promotor region or in some intronic sequences that we did not analyze, underlie the observed integrin α7 deficiency. However, we feel that our mutation detection system is quite sensitive (we calculated about 93% sensitivity in a previous study).[46] Since laminin α2 deficiency, both partial or total, is a well-known cause of secondary integrin α7 deficiency,[11,12,30] all of the 210 muscle biopsies included in this study were checked by laminin α2 immunofluorescence, and all were normal.

To determine whether the decreased accumulation of integrin α7 protein might be the result of protein instability or due to the lack of integrin α7 gene expression or RNA processing, we quantitated total RNA from muscle biopsies of integrin α7-deficient patients. QMF-RT/PCR has been shown to have a relatively low experimental error, and has the additional advantage of using internal control RNA for each test.[47] Our results showed normal integrin α7 mRNA levels in the patients studied suggesting that the loss of integrin α7 in their muscle biopsies occurs at the protein level. It is possible that the observed secondary integrin α7 deficiency may be the result of an incorrect processing and/or location of a normally synthesized integrin α7 protein in the patients' muscles. The cytoplasmic integrin α7-positive immunostaining observed in some of our patients' muscle biopsies further support the hypothesis of a integrin α7 moiety that is properly produced but is mislocated or unstable at the membrane level in absence, for example, of some yet unknown ligand. Quantitation of integrin α7, such as by immunoblotting analysis, may be informative, but unfortunately the small size of our diagnostic biopsies is a limiting factor. The biopsies are not sufficient to obtain a membrane-enriched pellet, and this limitation did not allow us to perform such an analysis in this study.

Taken together, our results suggest that secondary integrin α7 deficiency is rather common in muscular dystrophy/myopathy of unknown etiology. This is not surprising considering the central role played by α7β1D integrin in anchoring the intracellular cytoskeleton *via* actin, to the extracellular matrix, *via* laminin, and the potential for signal transduction possibly mediated by extracellular matrix, soluble growth factors, and/or associated transmembrane molecules. "Inside-out" and "inside-in" signals can mutually modulate the activation of cell proliferation and migration, and if this signaling is malfunctioning, the lack of appropriate cell matrix interaction can lead to cell death *via* apoptosis.[48] Analyses of mutant phenotypes provide further evidence of the key role of integrins in adhesion-mediated events in vertebrates. Germ-line mutations in genes coding for other laminin binding integrin result in disease. For example, mutations in either the α6 or the β4 subunit of the epithelial hemidesmosomes integrin result in a variant of the usually lethal skin blistering disorder, epidermolysis bullosa. α3 integrin in epithelia plays a direct role in the assembly of the basement membrane as shown by the α3-null mice that show basement membrane abnormalities in the kidney, lung, and skin and die soon after birth.[48] Further work is necessary to obtain more insight into the role of integrin-mediated adhesion and signaling and their modulating factors.

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