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Biochemical and Biophysical Research Communications 329 (2005) 1001–1009
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Characterization of mouse myotilin and its promoter

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Received 1 February 2005

Abstract

Myotilin is a sarcomeric protein mutated in two forms of muscle disease, limb-girdle muscular dystrophy type 1A and myofibrillar myopathy. Myotilin is expressed late during human myofibrillogenesis and localizes to Z-discs in mature sarcomere. It interacts with α-actinin, actin, and filamin C, and has strong F-actin-bundling activity. These features suggest an important role for myotilin in sarcomere organization. In our effort towards the construction of a genetic model for myotilin-related muscle disorders, we have cloned mouse myotilin, including its promoter region, and studied the expression in various tissues. Mouse myotilin is 90% identical with the human orthologue. Northern blot analysis revealed strong mRNA transcripts in skeletal and cardiac muscle, and weak expression in liver and lung tissue. Western blot and RT-PCR analysis showed the presence of one major product in mouse tissues. Analysis of the 5′-flanking region revealed a number of putative regulatory elements that drive expression in differentiating myoblasts. Finally, endogenous myotilin is induced at later stages of Z-disc assembly in C_2C_{12} cells indicating conservation between mouse and human promoter region.

Keywords: Muscular dystrophy; LGMD; MFM; Myotilin; Z-disc

Striated muscle cells have a highly specialized cytoskeleton. According to the ''three-filament-sarcomere'' model, the giant protein titin acts as a ruler, along which thin and thick filaments align [1]. Several other proteins are required for the correct assembly of this formidable structure. At both ends of the sarcomere, actin filaments are anchored to the Z-discs, thereby transmitting the actomyosin sliding force through the length of the fiber. At the sarcolemma, the dystrophin–glycoprotein complex (DGC) provides a link between the actin-based cytoskeleton and the extracellular matrix, and protects the cell from contraction-induced damage [2].

Myotilin [3,4] is a structural component of the Z-discs in human skeletal and cardiac muscle. Its C-terminus contains two immunoglobulin (Ig)-like C2 domains highly homologous to those of palladin and myopalladin, and, to a lesser extent, homologous to those of Ig-repeats 7 and 8 found in the Z-line-associated region of titin [5–8]. The unique N-terminal half contains several serine pairs and a stretch of hydrophobic amino acids. Numerous potential phosphorylation sites are present in the serine-rich region. Myotilin forms homodimers and binds to α-actinin [3], F-actin [9], and filamin C [10]. Overexpressed full-length myotilin induces the formation of actin bundles in non-muscle cells [9], whereas truncated myotilin perturbs sarcomeric assembly in differentiating muscle cells [9,10]. Interestingly, human myotilin is also found underneath the sarcolemma and in intramuscular nerves, indicating that it may have other functions, not related to sarcomeric organization. To a lower extent, myotilin is also

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.02.074

expressed in other human tissues, such as bone marrow, liver, and thyroid gland ([4] and unpublished results).

Genetic defects in both sarcomeric and sarcolemmal proteins cause muscular and cardiac diseases. Limb-girdle muscular dystrophies (LGMD) are a heterogeneous group of muscle disorders that include both dominant and recessive forms [11–13]. Mutations in *myotilin* gene cause an autosomal dominant form of LGMD, LGMD1A, a progressive muscle disease presenting with proximal limb weakness, dysarthric speech, and reduced tendon reflexes [14,15]. Mutated forms of myotilin also cause MFM, a genetically heterogeneous group of myopathies, also called desmin-storage or desmin-related myopathy [16]. All characterized patients, irrespective of disease phenotype, carry a single missense mutation leading to a change of an N-terminal Ser or Thr-residue.

In view of its localization, interactions, disease association, and its ability to modify actin-containing structures, myotilin is likely to play an important role in the structural organization of muscle cytoskeleton. In order to better understand the role of myotilin, and to gain insight into the pathogenesis of myotilin-related disorders, we identified the mouse orthologue of *myotilin* and studied its expression pattern in adult tissues. Furthermore, we characterized the mouse *myotilin* promoter sequence, which, as its human counterpart, induces myotilin expression very late during myofibrillogenesis.

Materials and methods

cDNA cloning, gene structure, and probes. A cDNA library (Uni-ZAP XR library, Stratagene) derived from mouse skeletal muscle was screened using human full-length myotilin cDNA as a probe. Positive clones were analyzed by PCR with myotilin-specific primers, and a partial cDNA clone was identified and used as a probe for subsequent screening. Full-length mouse myotilin cDNAs were then isolated and sequenced by ABI 310 Genetic Analyzer (Perkin-Elmer). Sequence alignments were done with the MegAlign software (DNAstar). Analysis of myotilin gene was performed by long-range PCR on high-molecular weight genomic DNA extracted from mouse tissue. Ten nanograms of genomic DNA was used in 25 μl reactions under the following thermocycle conditions: template was denatured for 5 min at 94 °C and then four ''touchdown'' miniprograms of four cycles each were performed (94 °C, 30 s; annealing step, 30 s; 68 °C, 4 min) with annealing temperatures 62, 60, 58, and 56 °C, followed by 32 standard cycles (94 °C, 30 s; 55 °C, 30 s; and 68 °C, 4 min) and a final extension step at 68 °C for 10 min. PCR products were purified from agarose gel and sequenced to determine exon/intron boundaries. To generate myotilin probes, two sequences from the coding region (nt 531–1056) and the 3′-end (nt 1614–2150) of myotilin cDNA were amplified and cloned into pBluescript vector to give mCOD and mEND plasmids, respectively. Both plasmids were sequenced in order to verify that no mutation was introduced during the cloning process. Titin probe [17] was a kind gift from Dr. S. Labeit (Heidelberg, Germany).

5′-RACE. Total RNA was extracted from skeletal muscle as described [18]. RACE amplification was performed using a kit (Gibco), according to instructions. For first strand synthesis, a myotilin-specific primer was used. A nested primer was used for successive amplification in combination with adapter primer. The products were isolated from agarose gel and sequenced.

RT-PCR. Poly(A)$^+$ RNA was purified from adult dissected organs using the QuickPrep mRNA purification kit (Pharmacia) according to manufacturer's instructions. Briefly, tissues were homogenized in extraction buffer and the lysate was incubated with oligo(dT)-cellulose. After washing, the mixture was applied to a SpinColumn, washed again, and finally poly(A)$^+$ RNA was eluted. About 100 ng mRNA was reverse transcribed and subsequently amplified (forward primer: 5′-ATGG CTCGCAGGTTGTTA-3′; reverse: 5′-TTCTGCCGAATGGAC-3′) with the following protocol: 94 °C, 30 s; 55 °C, 30 s; and 68 °C 1 min, for a total of 40 cycles. RT-PCR products were run in 1.5% agarose gel. Semiquantitative RT-PCR was done using forward 5′-AATGCT TCTCCCTTCTC-3′ and reverse 5′-AGATCTCAGCAAAGAGGG-3′ primers. The same PCR cycling profile was used, and the products were assessed after 35 cycles and 41 cycles. As control, titin-specific primers (a kind gift from Dr. P. Hackman, Helsinki, Finland) were used to evaluate the amplification of muscle-specific transcripts. To verify the RNA integrity, a 348 bp fragment of β-actin was amplified using forward 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ and reverse 5′-TAAAA CGCAGCTCAGTAACAGTCCG-3′ primers.

Preparation of tissue specimens. All the experiments were approved by the Animal Welfare Committee of the Haartman Institute, University of Helsinki. Adult mice were sacrificed by cervical dislocation and organs were dissected in PBS. For in situ hybridization, the samples were fixed in 4% paraformaldehyde, dehydrated in ethanol and xylene, and embedded in paraffin. The tissues that were selected for immunostaining were frozen in isopentane and stored at −80 °C.

Northern blot and in situ hybridization. Northern blot analysis was performed on a mouse multiple tissue mRNA filter from Clontech, using ^{32}P-labeled mEND, mCOD or full-length myotilin cDNA probe following the manufacturer's protocol. For in situ hybridization, sense and antisense riboprobes were generated from linearized myotilin plasmids mCOD and mEND by in vitro transcription in the presence of digoxigenin-conjugated UTP and purified through NICK columns (Pharmacia Biotech). Labeling efficiency was checked using DIG Quantification Teststrips (Boehringer) according to instructions. Hybridizations were performed as described [19]. Tissues were counterstained with hematoxylin.

Immunodetection of myotilin. The 151 rabbit polyclonal antibody was raised against purified His-tagged N-terminal mouse myotilin (amino acids 1–150) and affinity purified using GST-mouse myotilin coupled to Sepharose 4B resin. The 231 rabbit polyclonal antibody was raised against a fragment spanning amino acids 231–342 of human myotilin. The 231–342 sequence was expressed as a GST-tagged fragment and subsequently GST moiety was removed with TEV protease. The specificity of the antibodies was tested in Western blot analysis.

C_2C_{12} cells were cultured in DMEM containing 10% fetal calf serum. Differentiation was induced by shifting the culture to medium containing 2% horse serum. The cells plated on glass coverslips were immunostained using antibody 151. The antibody or preimmune rabbit IgG was diluted 1:100 in the presence of blocking agent (2% goat normal serum) and applied to the samples for 1 h at 4 °C. For Western blotting, mouse tissues were homogenized in reducing Laemmli buffer and the lysates were run on SDS–PAGE and probed with antibody 151 diluted 1:500 in 5% non-fat milk. α-actinin (sarcomeric) mAb clone EA-53 (Sigma) diluted 1:1000 and myosin heavy chain mAb MF20 (a kind gift from Dr. Marianne Tiainen, [20]) diluted 1:100 in blocking solution were used for Western blotting. Extraction of myotilin protein was done as follows: tissues were homogenized in 20 mM Tris, pH 8.0, 500 mM NaCl, and 1% Triton X-100, and centrifuged for 30 min at 20,000*g*. The supernatant is referred to as Triton-soluble fraction. The pellet (Triton-insoluble) was resuspended in equal volume of 1% SDS. Twenty micrograms of the soluble extracts and a corresponding amount of pellet were run on SDS–PAGE and probed with anti-myotilin antibody as above.

Reporter assay. The 5′-upstream genomic region was cloned from BAC DNA containing myotilin locus and sequenced. The different