Biochimica et Biophysica Acta 1820 (2012) 76–83

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Microphthalmia-associated transcription factor is required for mature myotube formation

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ARTICLE INFO

Article history:
Received 15 May 2011
Received in revised form 29 October 2011
Accepted 10 November 2011
Available online 20 November 2011

Keywords:
Mitf
Myogenesis
Integrin
p21
Myotube

ABSTRACT

Background: The roles of microphthalmia-associated transcription factor (Mitf) in the skeletal muscle and during myogenesis are unclear.
Methods: Expression of *Mitf* in mouse tissues and during myogenesis was evaluated. Effects of *Mitf* knockdown on myogenesis and gene expression related to myogenesis were subsequently explored. Furthermore, effects of *p21*, a cyclin-dependent kinase inhibitor, and *integrin α9* (*Itga9*) were examined.
Results: Mitf was highly expressed in the skeletal muscle; *Mitf-A* and *-J* were expressed. Mitf expression increased after differentiation stimulation in C2C12 myogenic cells. Down-regulation of *Mitf* expression by transfection of siRNA for common *Mitf* inhibited myotube formation, which was reproduced by *Mitf-A* knockdown. Morphometric analyses indicated that both multinucleated cell number and the proportion of myotubes with more than 6 nuclei were decreased in *Mitf*-knockdown cells, suggesting that Mitf is required for not only the formation of nascent myotubes but also their maturation. Searching for genes positively regulated by Mitf revealed *p21* and *Itga9*; decreasing *Mitf* expression inhibited up-regulation of *p21* expression after differentiation stimulation and blocked the induction of *Itga9* expression in response to differentiation. Knockdown of *p21* decreased the number of multinucleated cells, whereas *Itga9* knockdown did not affect the myotube number. Both *p21* knockdown and *Itga9* knockdown decreased the proportion of myotubes with more than 6 nuclei.
General significance: Mitf positively regulates skeletal muscle formation; Mitf is significantly expressed during myogenesis, and is required for efficient myotube formation through expression of p21 and Itga9.

1. Introduction

Skeletal muscle formation consists of a complex set of differentiation steps: commitment of mesenchymal stem cells to myoblast lineage cells, progression of differentiation with the expression of muscle-cell-specific proteins, and fusion of myoblasts into multinucleated myotubes. Mammalian myotube formation occurs in two phases [1,2]. In the first phase, differentiated myoblasts fuse together to form small myotubes; to accomplish this process, proliferating myoblasts exit cell cycle, and some myoblasts undergo apoptosis. In the second phase, additional myoblasts subsequently fuse with myotubes to form large myotubes. Although the roles of myogenic regulatory factors (MRFs), including Myf5, Myod, Myogenin and Mrf4, in myogenic differentiation are unquestionable [3–5], a number of factors, such as secreted proteins, membrane proteins and transcriptional regulators, are also involved in myogenesis [2].

Microphthalmia-associated transcription factor (Mitf) is a member of the basic helix–loop–helix leucine zipper (bHLH-LZ) family of transcription factors [6–8]. Expression levels of Mitf, evaluated by Western blotting and Northern blotting, vary among tissues; it is highly expressed in melanocytes, mast cells, osteoclasts, and the heart [9–11]. Thus, the roles of Mitf have been mainly examined in these cells [11–14], and Mitf activities in other tissues are largely unknown. Of the *Mitf* variants that are not the result of genetic mutation, nine *Mitf* isoforms have been identified in mice that differ in their transcriptional initiation site: *Mitf-A*, *-B*, *-C*, *-D*, *-E*, *-H*, *-J*, *-M* and *-mc*. The *Mitf* variants contain an isoform-specific first exon, while exons 2 to 9 of all *Mitf* isoforms examined to date are identical. *Mitf* isoforms are expressed in a cell type-specific manner, and their transcriptional activities are slightly but significantly different depending on the target gene [15–19]. In addition, two types of *Mitf* mRNAs with or without an 18-base insert (exon 6a) are generated by alternative use of the two acceptor sites located at the 5′ end of exon 6 in *Mitf-A*, *-H*, *-J*, *-M* and *-mc* [6,9,20,21]. In the present study, we show the relatively higher expression of *Mitf-A* in the skeletal muscle, and present the potential role of Mitf in the progression of myogenesis.

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doi:10.1016/j.bbagen.2011.11.005

2. Materials and methods

2.1. Animals and cell culture

Nine C57BL/6 mice aged 7–8 wk were used to examine the tissue distribution of Mitf. The experiment was approved by Azabu University Animal Experiment Committee. C2C12 myoblasts were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in growth medium, i.e., Dulbecco's modified Eagle's medium (DMEM) with heat-inactivated 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, at 37 °C under a humidified 5% CO_2 atmosphere. To induce differentiation from myoblasts to myotubes, the medium was replaced at confluence (day 0) with differentiation medium consisting of DMEM with 2% horse serum supplementing the antibiotics. To isolate myotubes, cells on day 7 were trypsinized for a short time under a microscope until detachment of multinucleated cells (~2 min), followed by centrifugation to obtain a myotube-rich fraction.

2.2. RT-PCR, restriction fragment length polymorphism and quantitative RT-PCR

Total RNA isolation from tissues and cells, RT-PCR and restriction fragment length polymorphism (RFLP) were performed as described previously [21]. Quantitative RT-PCR (qRT-PCR) was carried out as described previously [22]. The used PCR primers in qRT-PCR are presented in Table 1. To compare tissue *Mitf* expression, the appropriate corrected gene was chosen using a mouse housekeeping gene primer set (TaKaRa, Otsu, Japan). The relative mRNA level was expressed as a ratio with *β-actin* to evaluate tissue distribution and *Gapdh* was used as a reference gene to examine regulatory expression in C2C12 cells.

2.3. Western blotting

To examine expression of Mitf in the skeletal muscle, thigh muscle and heart as a positive control were homogenized in RIPA buffer. After 30 min on ice, the debris was removed by centrifugation at 2500 ×*g* for 2 min at 4 °C. After centrifugation at 12,000 ×*g* for 5 min at 4 °C, the supernatant was recovered and the protein concentration was measured by the bicinchoninic acid method [23]. Fifty micrograms of the protein was loaded on 10% SDS-polyacrylamide gel. Western blotting was performed as described previously [24,25]. Expression of Mitf and myosin heavy chain (Myhc) was examined by use of mouse monoclonal antibodies against Mitf (X1405M; Exalpha Biologicals) and Myhc (MY-32; Sigma), respectively. There are two types of muscle fiber, i.e., fast-twitch and slow-twitch; fast-twitch muscle fibers express Myhc2a, Myhc2b and Myhc2x, whereas slow-twitch muscle fibers do Myhc1 [26]. According to the manufacturer's

Table 1
Oligonucleotide PCR primers for qRT-PCR.

	Oligonucleotide		GenBank accession number
	5′-primer	3′-primer	
Mitf			
Common *Mitf*	5′-GCCTTGCAAATGGCAAATAC-3′	5′-GCTGGACAGGAGTTGCTGAT-3′	
Mitf-A	5′-GAGGAGTTTCACGAAGAACC-3′	5′-GCTGGCGTAGCAAGATGCGTGA-3′	NM_001113198
Mitf-H	5′-GAGGAGTTTCACGAAGAACC-3′	5′-GCTGGCGTAGCAAGATGCGTGA-3′	NM_001178049
Mitf-J	5′-CCGTGTCTCTGGGCATCTGAAG-3′	5′-GCTGGCGTAGCAAGATGCGTGA-3′	AY632575
Mitf-M	5′-ATGCTGGAAATGCTAGAATACAG-3′	5′-CATACCTGGGCACTCACTCTC-3′	NM_008601
Myogenic regulatory factors			
Myf5	5′-GCCAGTTCTCCCCTTCTGAGTA-3′	5′-TGGTCCCCAAACTCATCCTCT-3′	NM_008656
Myod1	5′-GTGGCAGCGAGCACTA-3′	5′-GGGCCGCTGTAATCCATC-3′	NM_010866
Myogenin	5′-AGAAGCGCAGGCTCAAGAAA-3′	5′-ATCTCCACTTTAGGCAGCCG-3′	NM_031189
Mrf4	5′-GGGCCTCGTGATAACTGCT-3′	5′-AAGAAAGGCGCTGAAGACTG-3′	NM_008657
Myosin heavy chains			
Myhc2a	5′-AAAGCTCCAAGGACCCTCTT-3′	5′-AGCTCATGACTGCTGAACTCAC-3′	NM_001039545
Myhc2b	5′-CCGAGCAAGAGCTACTGGA-3′	5′-TGTTGATGAGGCTGGTGTTC-3′	NM_010855
Myhc2x	5′-TCGCTGGCTTTGAGATCTTT-3′	5′-CGAACATGTGGTGGTTGAAG-3′	NM_030679
Target candidates			
Adam12	5′-CAGAGCATCCCAGCCAAG-3′	5′-CAGGCTGAGGATCAGGTCTC-3′	NM_007400
Calpastatin	5′-CCTTCAGCTGGTGGAGAGAG-3′	5′-GCTGGTGTGAATGGTTTCTG-3′	NM_009817
Caveolin3	5′-CACAAGGCTCTGATCGCCTC-3′	5′-TCCGTGTGCTCTTCGGTCA-3′	NM_007617
Ccna2	5′-CTTGGCTGCACCAACAGTAA-3′	5′-CAAACTCAGTTCTCCCAAAAACA-3′	NM_009828
Cdh15	5′-TGACATTGCCAACTTCATCAG-3′	5′-GATGAGAGCTGTGTCGTAGGGAG-3′	NM_007662
Ctsb	5′-AAGCTGTGTGGCACTGTCCTG-3′	5′-GATCTATGTCCTCACCGAACGC-3′	NM_007798
Itga3	5′-CATCAACATGGAGAACAAGACC-3′	5′-CCAACCACAGCTCAATCTCA-3′	NM_013565
Itga4	5′-ACCAGACCTGCGAACAGC-3′	5′-CCCCCAGCCACTGGTTAT-3′	NM_010576
Itga5	5′-TACTCTGTGGCTGTGGGTGA-3′	5′-GCCATTAAGGACGGTGACAT-3′	NM_010577
Itga6	5′-GGTTGAGAGGCCATGAAAAG-3′	5′-TTCTTTTGTTCTACACGGACGA-3′	NM_008397
Itga9	5′-TGATGCCAACGTGTCCTTTA-3′	5′-GAAATGCCCATCTCCTCCT-3′	NM_133721
Itgb1	5′-ATGCAGGTTGCGGTTTGT-3′	5′-CATCCGTGGAAAACACCAG-3′	NM_010578
Myof	5′-CATTACTGGCTTCTAAGCTGTCG-3′	5′-AAATTTACTCCACCAGTCAACG-3′	NM_001099634
p21	5′-TGGGCCCGGAACATCTC-3′	5′-TGCGCTTGGAGTGATAGAAA-3′	NM_007669
p57	5′-CAGGACGAGAATCAAGAGCA-3′	5′-TGCGCTTGGCGAAGAAGT-3′	NM_009876
Ptger4	5′-TCCAGATGGTCATCTTACTCAT-3′	5′-TAACTGGTTAATGAACACTCGCA-3′	NM_001136079
Rb1	5′-GCTGCAAATACAGAGACACAAGCAG-3′	5′-CGGAGATATGCTAGACGGTACACTT-3′	NM_009029
v-Cam1	5′-TGATTGGGAGAGACAAAGCA-3′	5′-AACAACCGAATCCCCAACT-3′	NM_011693
Vcl	5′-GATAGCTGCTCTGACCTCTAA-3′	5′-TAGTGCCGTCGCCACTTGTTT-3′	NM_009502
Reference genes			
β-actin	5′-CTAAGGCCAACCGTGAAAAG-3′	5′-ACCAGAGGCATACAGGGACA-3′	X03672
Gapdh	5′-CGTGTTCCTACCCCCAATGT-3′	5′-TGTCATCATACTTGGCAGGTTTCT-3′	NM_008084