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Muscle Lim Protein and myosin binding protein C form a complex regulating muscle differentiation

Demetrios A. Arvanitis[a], Elizabeth Vafiadaki[a], Vasiliki Papalouka[a], Despina Sanoudou[a,b,*]

[a] Molecular Biology Division, Biomedical Research Foundation of the Academy of Athens, Greece
[b] Clinical Genomics and Pharmacogenomics Unit, 4th Department of Internal Medicine, Medical School, National and Kapodistrian University of Athens, Greece

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ABSTRACT

Muscle Lim Protein (MLP) is a protein with multiple functional roles in striated muscle physiology and pathophysiology. Herein, we demonstrate that MLP directly binds to slow, fast, and cardiac myosin-binding protein C (MyBP-C) during myogenesis, as shown by yeast two-hybrid and a range of protein-protein interaction assays. The minimal interacting domains involve MLP inter-LIM and MyBP-C [C4]. The interaction is sensitive to cytosolic Ca^{2+} concentrations changes and to MyBP-C phosphorylation by PKA or CaMKII. Confocal microscopy of differentiating myoblasts showed MLP and MyBP-C colocalization during myoblast differentiation. Suppression of the complex formation with recombinant MyBP-C [C4] peptide overexpression, inhibited myoblast differentiation by 65%. Suppression of both MLP and MyBP-C expression in myoblasts by siRNA revealed negative synergistic effects on differentiation. The MLP/MyBP-C complex modulates the actin activated myosin II ATPase activity in vitro, which could interfere with sarcomerogenesis and myofilaments assembly during differentiation. Our data demonstrate a critical role of the MLP/MyBP-C complex during early myoblast differentiation. Its absence in muscles with mutations or aberrant expression of MLP or MyBP-C could be directly implicated in the development of cardiac and skeletal myopathies.

1. Introduction

The Muscle LIM Protein (MLP), also known as cysteine and glycine-rich protein 3 (CSRP3 or CRP3), belongs to the cysteine-rich protein (CRP) and the LIM-only domain families, and has a key role in striated muscle development, physiology and pathology [1,2]. Briefly, this relative small molecule of 22kD, exhibits a remarkable palette of protein-protein interactions, subcellular localizations and functions. MLP has been demonstrated to bind telethonin [3], α-actinin [4], cofilin 2 [5], calcineurin [6], N-RAP [7], F-actin [8], β-spectrin [9], zyxin [10], MyoD, MRF4 and myogenin [11], while it can also interact with its isoform, MLP-b [12], and oligomerize [13]. It has been found to localize at the sarcomeric Z-disc [3,4,6], the costameres [9,10], the nucleus [11], and the intercalated discs [7]. MLP is involved in: (a) the formation and maintenance of the sarcomeric cytoskeleton [3,7,9,14,15], (b) mechano-signaling and mechano-transduction [16–19], (c) F-actin dynamics [5,20], (d) myofibrillar calcium sensitivity [21], and (e) myogenesis [22].

Mutations in *CSRP3*, the gene encoding for MLP, have been identified and directly linked with dilated and hypertrophic cardiomyopathies [3,23–28], while the MLP knock-out mouse develops dilated cardiomyopathy and heart failure [14]. Severe deregulation of MLP expression is detected in human failing hearts [29] and skeletal myopathies [30–32]. However, many aspects of MLP localization, translocation and functions, including its involvement in striated muscle pathogenesis, remain to be elucidated [33].

Herein, we demonstrate the novel interaction of MLP and MyBP-C (all its three forms: cardiac, fast and slow), which takes place during the differentiation of myoblasts to myotubes, and represents a critical step for the development of fully differentiated myocytes and the proper organization of contractile myofilaments in sarcomeres. Blocking the complex formation leads to severe myocyte dysfunction therefore representing a novel potential mechanism of pathogenesis for cardiac and skeletal myopathies.

2. Materials and methods

2.1. Yeast two-hybrid screening

Full length MLP cDNA was generated by RT-PCR from human

* Corresponding author at: Clinical Genomics and Pharmacogenomics Unit, 4th Department of Internal Medicine, Attikon Hospital, Medical School, National and Kapodistrian University of Athens, Rimini 1, Chaidari 124 62, Greece.
E-mail address: dsanoudou@med.uoa.gr (D. Sanoudou).

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postmortem muscle tissues using TRIzol (Invitrogen, Carlsbad, CA, USA), as previously described [5]. All the primer sets used in the study are presented in Supplemental Table 1. The MLP cDNA was subsequently cloned in the *Eco*RI/*Sal*I sites of the yeast pGBKT7 vector (Matchmaker System, BD Biosciences Clontech, Erembodegem, Belgium) and was used as a bait to screen a pre-transformed Human Heart Matchmaker cDNA library (BD Biosciences), according to manufacturer's instructions. Positive clones were selected by plating on high-stringency media lacking Trp, Leu, His and Ade in the presence of 0.02 mg/ml X-α-gal. For the identification of the minimal binding sites, subcloned domains of the interacting partners were generated and tested in the Y2H system. In addition, human cMyBP-C constructs were generated using specific primers (Supplemental Table 1). After digestion with the appropriate restriction enzymes the constructs were cloned in the pACT2 vector. pACT2-HAX1 and pGBKT7-p53 were used as negative controls.

2.2. Generation of recombinant proteins

The generation of the GST-MLP (1–194 aa), MBP-MLP (1–194 aa), MBP-MLP-LIM1 (9–94 aa), MBP-MLP-LIM2 (105–188 aa) and MBP-MLP-inter-LIM (64–117 aa) constructs has been previously described [5]. The human cMyBP-C constructs were generated by PCR using the primers presented in Supplemental Table 1. After digestion with the appropriate restriction enzymes the constructs were cloned in pACT2, pGEX-5x-1, pEGFP, pMALc2x or pET28 (BD Biosciences Clontech), depending on the experimentation protocol. The authenticity of all constructs was confirmed by sequence analysis (MacrogenInc). Recombinant protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h and purification was done by affinity chromatography on Glutathione Sepharose™ 4B (Amersham Biosciences) for GST fusion proteins, amylose resin beads (New England Biolabs) for MBP recombinant proteins and Ni-NTA agarose resin (Qiagen) for His-tagged proteins. For the blot overlay assays the fusion-peptides were eluted from the beads in accordance to manufacturer's instructions.

2.3. Immunoprecipitation

For the immunoprecipitation experiments, protein lysates were obtained from post-mortem human quadriceps and cardiac muscle homogenates [5], followed by pre-clearing with pre-washed protein-A/G agarose beads (#17-0756-01, Amersham Biosciences) overnight (o/n) on a rotary wheel at 4 °C. Rabbit polyclonal anti-MLP (#ab42504, AbCam) was conjugated to pre-washed protein-A/G Sepharose (#17-0756-01, Amersham Biosciences) bead mixture o/n (1 μl antibody/10 μl agarose beads). The antibody-bound beads were incubated with the pre-cleared protein lysates for 3 h at room temperature. Immunoprecipitates were collected, washed three times in PBS and boiled for 5 min, followed by a 5 min spin at 2000 rpm, to remove the beads.

2.4. Western blot analysis

Protein extracts from cardiac and skeletal muscle tissues were prepared using the Ultra-Turrax® tissuemizer (IKA-Werke GmbH & Co., Staufen, Germany) in ice cold lysis buffer (10 mM $NaPO_4$, pH 7.2, 2 mM EDTA, 10 mM NaN_3, 120 mM NaCl and 1% Nonidet P-40) supplemented with a mixture of protease inhibitors (Sigma-Aldrich ChemieGmbh, Munich, Germany). The protein extracts were analyzed by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell Bioscience GmbH, Dassel, Germany). The membranes were probed with the chicken polyclonal anti-MLP (#ab14013, AbCam, Cambridge, UK), rabbit polyclonal anti-MLP (#ab42504, AbCam), or mouse monoclonal GAPDH antibodies (#G9295, Sigma-Aldrich). They were then washed in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween-20, and incubated with a peroxidase-conjugated anti-chicken (#ab6753, Abcam), anti-rabbit (#170–6515, Bio-Rad Laboratories Ltd) or anti-mouse (#A9917, Sigma-Aldrich) secondary antibodies. Immunoreactive bands were visualized using electrogenerated chemiluminescence (ECL) reagents, according to the manufacturer's protocol (Amersham Biosciences).

2.5. Immunofluorescence studies

Human quadriceps muscle specimens were sectioned at 10 μm thickness, placed onto polysine-coated microscope slides (Sigma-Aldrich) and fixed in ice-cold methanol for 20 min. Samples were washed in phosphate-buffered saline (1x PBS) and permeabilized for 30 min at 25 °C in PBS containing 0.1% (v/v) Triton X-100. Following three washes with PBS, the samples were incubated in blocking buffer (1 × PBS, 1 mg/ml BSA, 10 mM NaN_3) for 1 h at 25 °C. Primary antibodies, including chicken or rabbit anti-MLP (#ab14013 or #ab42504, AbCam), mouse anti-α-actinin (#A7811, Sigma-Aldrich), mouse anti-MYH (#M4276, Sigma-Aldrich) or mouse or rabbit anti MyBP-C (#sc-137,237 [G-7] or #sc-67,354 [H-120], Santa Cruz) were then applied to the sections and incubated for 1 h at 25 °C. Following washes with 1 × PBS, the samples were counterstained for 1 h with appropriate secondary antibodies Alexa Fluor anti-rabbit 488 (#A11008, Invitrogen), Alexa Fluor anti-chicken 568 (#A11041, Invitrogen) or Alexa Fluor anti-mouse 633 (#A21050, Invitrogen) diluted 1:500 in blocking buffer. Following washes with 1 × PBS, the samples were mounted with Vectashield medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) and analyzed with a Leica confocal laser scanning microscope (TCS SP5, DMI6000, inverted with the acquisition software LAS-AF). All confocal images obtained were subject to co-localization Pearson analysis [Coloc2, ImageJ].

2.6. Blot overlay assays

Blot overlay assays were performed as previously described [5,34]. Briefly, ~2.5 μg of affinity-purified recombinant proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Nonspecific sites on the membrane were blocked by incubation in buffer A (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween-20, 2 mM dithiothreitol, 0.5% NP-40, 5% non-fat milk) for 16 h at 4 °C. The samples were then incubated with 3 μg/ml GST- or MBP-interacting fused peptide in buffer A, in the presence of 1 mM ATP, for 4 h at 25 °C. At the end of this incubation period, the blots were washed in buffer C (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween-20, 2 mM dithiothreitol, 1% NP-40) at 25 °C and were subsequently probed with anti-GST antibody (#RPN1236, Amersham) for 1 h at 25 °C. The immunoreactive bands were visualized using ECL reagents.

2.7. Pull down assays

Pull down assays were performed as previously described [5]. Briefly, equivalent amounts of recombinant GST and GST-MyBP-C or MBP and MBP-MLP, bound on to glutathione-Sepharose4B™ (#17-0756-01, Amersham Biosciences) or amylase resin (#E8021S, New England Biolabs) respectively, were mixed with cardiac or skeletal muscle homogenates and incubated at 4 °C overnight, except for the experiments performed to study the effect of calcium in which incubation were for 2-h. The beads were then washed three times with 10 mM $NaPO_4$, pH 7.2, 10 mM NaN_3, 120 mM NaCl, 3.5% (v/v) Tween-20, analyzed by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with anti-MLP or anti-MyBP-C respectively, washed in 50 mM Tris-HCl pH 7.5, 150 mMNaCl, 0.05% (v/v) Tween-20, and incubated with a peroxidase-conjugated anti-mouse (Sigma-Aldrich) or anti-rabbit (Bio-Rad) secondary antibodies. Immunoreactive bands were visualized with ECL, as described above.