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Identification and mapping of protein kinase A binding sites in the costameric protein myospryn

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Abstract

Recently we identified a novel target gene of MEF2A named *myospryn* that encodes a large, muscle-specific, costamere-restricted α-actinin binding protein. Myospryn belongs to the tripartite motif (TRIM) superfamily of proteins and was independently identified as a dysbindin-interacting protein. Dysbindin is associated with α-dystrobrevin, a component of the dystrophin–glycoprotein complex (DGC) in muscle. Apart from these initial findings little else is known regarding the potential function of myospryn in striated muscle. Here we reveal that myospryn is an anchoring protein for protein kinase A (PKA) (or AKAP) whose closest homolog is AKAP12, also known as gravin/AKAP250/SSeCKS. We demonstrate that myospryn co-localizes with RIIα, a type II regulatory subunit of PKA, at the peripheral Z-disc/costameric region in striated muscle. Myospryn interacts with RIIα and this scaffolding function has been evolutionarily conserved as the zebrafish ortholog also interacts with PKA. Moreover, myospryn serves as a substrate for PKA. These findings point to localized PKA signaling at the muscle costamere.

Keywords: MEF2 target; Muscle-specific; Tripartite motif; Scaffolding protein; Costamere; Protein kinase A

1. Introduction

Myocyte enhancer factor-2 (MEF2) functions as an important regulator of cell proliferation and differentiation in multiple tissues by coordinately regulating the expression of a broad spectrum of genes [1,2]. Studies have revealed that components of signal transduction pathways represent a significant fraction of the total number of genes regulated by MEF2 [3–6]. Consistent with these observations is the finding that expression of the stress-responsive protein kinase MKK6, the G-protein signaling effector RGS2, and the calcium regulated serine/threonine protein phosphatase calcineurin genes is deregulated in hearts lacking MEF2A [7].

The cAMP-protein kinase A (PKA) signal transduction pathway has been extensively characterized and plays an important role in muscle function and disease [8,9]. Despite the ubiquity of PKA signaling in cells, specificity is achieved via the recruitment of the kinase to distinct subcellular regions by scaffolding proteins known as A-kinase anchoring proteins (AKAPs) [10–12]. Tethering of PKA by AKAPs enables PKA to phosphorylate a target within a specific location in the cell. For instance, in striated muscle PKA signaling plays a central role in contractility via the phosphorylation of the ryanodine receptor (RyR) resulting in Ca^{2+}-induced Ca^{2+}-release from the sarcoplasmic reticulum during excitation–contraction coupling [13]. Recruitment of PKA to the sarcoplasmic reticulum is coordinated through the scaffolding function of muscle AKAP (mAKAP) which is localized to the sarcoplasmic reticulum and the neighboring perinuclear membrane where it forms a complex with the ryanodine receptor [14,15]. While additional AKAPs are also expressed in muscle [16], to date, mAKAP is the only known muscle-specific PKA anchoring protein.

Previously we demonstrated that the muscle-specific *myospryn* gene is a direct MEF2 target [17]. The *myospryn* gene product harbors a tripartite motif (TRIM) and is localized to the costamere of striated muscle where it interacts with α-actinin and dysbindin [17–19]. The TRIM domain is encoded by the 550 C-terminal amino acids of the protein and is the only known motif in this large protein of 3739 amino acids. Because myospryn function remains

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largely uncharacterized we searched various protein databases to identify additional biochemical and structural information on the protein. One of these searches revealed similarity between myospryn and a PKA anchoring protein, AKAP12, also known as gravin/AKAP250/SSeCKS (Src-suppressed C kinase substrate) [20,21]. Through functional domain mapping we show that myospryn harbors three bona-fide PKA-anchoring domains that bind to RIIα, a type II regulatory subunit of PKA. Furthermore, we show that myospryn co-localizes with RIIα at the costameric region overlying the Z-disc in striated muscle. Thus, myospryn represents a novel muscle-specific AKAP and the first to be localized to the costamere in striated muscle. Myospryn is also the first example of a protein in the TRIM superfamily that can function as a scaffold for protein kinases. The ability of myospryn to recruit PKA to the costamere may enable the cAMP signal transduction pathway to regulate proteins within this important subcellular structure in muscle.

2. Materials and methods

2.1. Plasmids

For coimmunoprecipitation assays the following expression vectors were constructed:

Flag-tagged PKA-subunit constructs in the pcDNA3.1 vector, Flag-RIα, Flag-RIIα, Flag-RIβ, and Flag-RIIβ, along with various Myc-tagged myospryn constructs (described in Fig. 5) also in the pcDNA3.1 vector backbone. For GST pulldown assays, RIIα was cloned into pGEX-2T-KG. For subcellular location studzies in COS cells, NLS-RIIα was generated by cloning the following nuclear localization signal in pCDNA3-RIIα: MAPKKKRKV; 5′-atg gct cca aag aag aag cgt aag gta-3′.

2.2. Cell culture, co-immunoprecipitations and GST pulldown assays

COS1 cells were grown in 6-cm dishes using DMEM supplemented with 10% Fetal Bovine Serum, 1% Penicillin/Streptomycin, and 1% L-Glutamine. COS1 cells were transfected with 6.0 μg total DNA using Mirus TransIT-LT1 transfection reagent. Forty-eight hours post-transfection, cells were washed in 1× PBS, pelleted and subsequently homogenized in 500 μl ELB Buffer (0.05 M HEPES, 0.25 M NaCl, 0.005 M EDTA, 0.1% NP40, 1 mM PMSF, 1 mM DTT, and 1× Roche protease inhibitor cocktail solution). Homogenized cells were incubated on ice for 10 min, and centrifuged at 4 °C for 10 min at 13,000 rpm. Protein extracts were added to 20 μl of pre-washed Protein G-Sepharose beads (Amersham Biosciences) pre-incubated with 2.0 μg of either anti-Flag or anti-Myc antibodies and immunoprecipitated for 2 h at 4 °C. Beads were washed three times in ELB Buffer and re-suspended in one bed volume of SDS sample loading buffer containing 1% β-mercaptoethanol. Samples were fractionated on 10% SDS-PAGE gels and blotted on Immun-blot PVDF membrane (BioRad). Membranes were immunoblotted with 0.2 μg/ml primary antibody, followed by HRP-conjugated secondary antibodies and reacted with Western Lightning chemiluminescent reagent (Perkin Elmer). Anti-FLAG® M2 monoclonal antibody (Sigma) was used to detect Flag-tagged PKA constructs. c-Myc (9E10) mouse monoclonal antibody (Santa Cruz Biotechnology) was used to detect Myc-tagged myospryn constructs. Goat-anti-mouse HRP conjugated secondary antibodies (Perkin Elmer) were used for detection of proteins for western blotting.

For endogenous immunoprecipitations, COS cells were transfected with pcDNA3-Myc or Myc-Spe as described above. Immunoprecipitated protein extracts were analyzed for the presence of PKA by immunoblotting using mouse-anti-PKA-C (catalytic) antibody (BD Transduction Laboratories) to detect endogenous protein.

GST pulldown assays. GST alone and GST-RIIα were expressed in *Escherichia coli* DH5α cells and purified. Thirty micrograms of protein was added to glutathione sepharose beads and incubated at 4 °C for 2 h. Total protein from COS cells transfected with Myc-Spe was added to GST protein and beads and incubated at 4 °C for 2 h, washed with ELB buffer to remove non-specific interactions and loaded and immunoblotted with the anti-Myc antibody.

2.3. Immunohistochemistry

Adult mice were perfused with 4% paraformaldehyde, hindlimb muscles were dissected and cryoprotected in 30% sucrose (in 1× PBS) at 4 °C prior to embedding. Hindlimb muscle was embedded in OCT compound (Tissue-Tek), sectioned at 15 μM, and air-dried onto Superfrost plus glass slides (Fisher). Primary antibody to detect PKA RII-subunits was added at a 1:40 dilution in blocking solution (1× PBS containing 3% BSA) and allowed to incubate overnight at 4 °C. Following overnight incubation, primary antibodies for myospryn were diluted 1:100 in blocking solution (3% normal horse serum in 1× PBS) and incubated for 1 h at room temperature. Secondary antibodies (1:500 dilution) were added to each slide and incubated for 1 h at room temperature. Slides were washed in 1× PBS, followed by addition of Vectashield mounting medium (Vector Labs), and cover slips were applied and sealed before imaging on light microscope. Goat polyclonal anti-PKA-RII subunit antibodies (Upstate Biotechnology) were used to detect RII protein in both the co-immunostain and RII-alone stained control. Rabbit polyclonal antibodies for myospryn were generated as previously described [17]. Donkey anti-rabbit-Texas Red antibodies (Santa Cruz Biotechnology) were used to detect primary rabbit anti-myospryn antibodies on skeletal muscle sections. Chicken anti-goat FITC-conjugated antibodies (Santa Cruz Biotechnology) were used to detect goat polyclonal anti-PKA RII antibodies.

COS cells were transfected with 2.0 μg of RIIα, NLS-RIIα, or Myc-Spe or cotransfected with 1.0 μg each of NLS-RIIα and Myc-Spe. Forty eight hours post-transfection, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, washed with 1× PBS and incubated for 10 min with blocking solution at room temperature. Primary antibody to detect PKA-RII or Myc was added at a 1:200 dilution in blocking buffer and incubated for 1 h at room temperature. Cells were washed with 1× PBS and incubated with chicken anti-goat IgG FITC (Santa Cruz Biotechnology) to detect primary goat-anti-PKA-RII antibodies or Texas Red anti-mouse IgG (Vector Labs) to detect mouse-anti-Myc antibodies at a 1:200 dilution in 1× PBS with 0.1% IGEPAL for 1 h. Cells were washed with 1× PBS and followed by addition of Vectashield mounting medium containing DAPI (Vector Labs), and cover slips were applied and sealed before imaging on light microscope.

2.4. In vitro kinase assay

Epitope-tagged plasmids were transfected into COS cells grown in 6-cm dishes. Forty-eight hours post-transfection cells were harvested, lysed and immunoprecipitated using the desired antibody as described. Samples were incubated with 2 μl of supplied 10× Reaction Buffer, 10 μCi of γ-P^{32}ATP, and 1 μl purified cAMP-dependent protein kinase (PKA) catalytic subunit (New England Biolabs), and incubated at 30 °C for 30 min. For PKA specificity experiments reactions were treated with the PKA peptide inhibitor, PKI (Promega). Samples were fractionated in 10% SDS-PAGE, dried onto Whatman paper and exposed to a phosphoimager.

3. Results

3.1. Sequence similarity between myospryn and AKAP12

The muscle-specific protein, myospryn harbors a TRIM region [19] located in the carboxy-terminal 550 amino acids of the full-length protein (3,739 amino acids) leaving well over 3,000 amino acids of the protein uncharacterized. In an effort to gain insight into the potential function of myospryn in muscle we searched various protein motif databases for further structural information on the protein. One such protein family