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The mAKAPβ scaffold regulates cardiac myocyte hypertrophy via recruitment of activated calcineurin

Jinliang Li [a,1], Alejandra Negro [a,1], Johanna Lopez [a], Andrea L. Bauman [a], Edward Henson [a], Kimberly Dodge-Kafka [b], Michael S. Kapiloff [a,*]

[a] Cardiac Signal Transduction and Cellular Biology Laboratory, Interdisciplinary Stem Cell Institute, Departments of Pediatrics and Medicine, University of Miami Miller School of Medicine, R198, P.O. Box 016960, Miami, FL 33101, USA
[b] Calhoun Center for Cardiology, University of Connecticut Health Center, Farmington, CT 06030, USA

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ABSTRACT

mAKAPβ is the scaffold for a multimolecular signaling complex in cardiac myocytes that is required for the induction of neonatal myocyte hypertrophy. We now show that the pro-hypertrophic phosphatase calcineurin binds directly to a single site on mAKAPβ that does not conform to any of the previously reported consensus binding sites. Calcineurin–mAKAPβ complex formation is increased in the presence of Ca^{2+}/calmodulin and in norepinephrine-stimulated primary cardiac myocytes. This binding is of functional significance because myocytes exhibit diminished norepinephrine-stimulated hypertrophy when expressing a mAKAPβ mutant incapable of binding calcineurin. In addition to calcineurin, the transcription factor NFATc3 also associates with the mAKAPβ scaffold in myocytes. Calcineurin bound to mAKAPβ can dephosphorylate NFATc3 in myocytes, and expression of mAKAPβ is required for NFAT transcriptional activity. Taken together, our results reveal the importance of regulated calcineurin binding to mAKAPβ for the induction of cardiac myocyte hypertrophy. Furthermore, these data illustrate how scaffold proteins organizing localized signaling complexes provide the molecular architecture for signal transduction networks regulating key cellular processes.

1. Introduction

Cardiac myocyte hypertrophy is the major intrinsic mechanism by which the heart may counterbalance chronically elevated demands for pumping power. Myocyte hypertrophy is controlled by a network of intracellular signaling pathways that are activated by G-protein coupled, growth factor and cytokine receptors and by mechanical and oxidative stress [1]. These signals are transduced by MAPK, cyclic nucleotide, Ca^{2+} and phosphoinositide-dependent pathways. Although much progress has been made over the last 20 years to define this network, it is still unclear how the various constituent pathways act in concert to regulate the overall cellular phenotype [2]. Moreover, while individual signaling pathways may regulate specific cellular functions, the molecules that comprise these signaling pathways often serve multiple functions in the same cells. Therefore, an important question in the field of signal transduction has been how pleiotropic signaling molecules such as protein kinases and phosphatases can specifically regulate individual downstream effectors in response to different upstream stimuli. One mechanism by which specificity in signal transduction is conferred is the formation of multimolecular signaling complexes by scaffold proteins of different combinations of common signaling enzymes [3].

While signaling enzymes may be broadly distributed within the cell, scaffold proteins, such as A-kinase anchoring proteins (AKAPs), recruit small pools of these enzymes to discrete multimolecular complexes that are sequestered in distinct intracellular compartments and that serve different cellular functions [4]. mAKAP (muscle AKAP) was initially identified in a screen for protein kinase A (PKA) binding proteins. mAKAPα and mAKAPβ are the two known isoforms encoded by the single *mAKAP (AKAP6)* gene and are expressed in neurons and striated myocytes, respectively [5]. As a consequence of alternative mRNA splicing, mAKAPβ is identical to residues 245–2314 (the C-terminus) of mAKAPα. In adult and neonatal cardiac myocytes, mAKAPβ is primarily localized to the outer nuclear membrane through its association with nesprin-1α [6,7]. In addition to PKA, proteins that have been shown to associate with the mAKAPβ scaffold in myocytes include adenylyl cyclase type 5 [8], the cAMP-specific phosphodiesterase PDE4D3 [9], the cAMP-activated guanine nucleotide exchange factor Epac1 [10], ERK5 and MEK5 mitogen-activated protein kinases (MAPK) [10], the Ca^{2+}/calmodulin-dependent

Abbreviations: AID, autoinhibitory domain; ANF, atrial natriuretic factor; CaN, calcineurin; CaNAβca, constitutively active mutant of CaNAβ; CsA, cyclosporine A; GFP, green fluorescent protein; GST, glutathione-*S*-transferase; mAKAPβ, muscle A-kinase anchoring protein; NE, norepinephrine; NFATc, nuclear factor of activated T-cell; PE, phenylephrine; PKA, protein kinase A; RyR2, ryanodine receptor.

* Corresponding author. Tel.: +1 305 243 7863; fax: +1 305 243 3906.
E-mail address: mkapiloff@med.miami.edu (M.S. Kapiloff).

[1] These authors contributed equally to this manuscript.

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protein phosphatase calcineurin Aβ (CaN, PP2B) [11], protein phosphatase 2A [12], hypoxia-inducible factor 1α (HIF1α) and ubiquitin E3-ligases involved in HIF1α regulation [13], myopodin [14], the ryanodine receptor Ca^{2+}-release channel (RyR2) [12,15] and the sodium/calcium exchanger NCX1 [16]. Due to the association of these various enzymes and ion channels with mAKAPβ in the cardiac myocyte, we have proposed that mAKAPβ complexes are important for the regulation of pathologic myocyte remodeling in response to upstream cAMP, calcium, and MAPK signals and hypoxic stress [13,17]. In support of this hypothesis, mAKAPβ expression in myocytes is required for the full induction of neonatal myocyte hypertrophy in vitro by adrenergic and cytokine agonists [10,11].

CaN is a pleiotropic Ca^{2+}/calmodulin-dependent serine/threonine phosphatase composed of a catalytic A-subunit and a regulatory B-subunit [18]. There are three mammalian A-subunits, of which Aα and Aβ are expressed ubiquitously and Aγ is restricted to testes. Aα and Aβ have been studied by genetic deletion and are not functionally redundant. For example, only the CaNAβ isoform is important for the induction of pathologic cardiac hypertrophy and the survival of myocytes after ischemia [19,20]. Important calcineurin substrates in vivo include four of the five members of the nuclear factor of activated T-cell transcription factor family (NFATc 1–4). In addition to forming heterodimers with other transcription factors, NFATc can bind directly to CaN through conserved PxIxIT and LxVP motifs [21]. CaN binding facilitates dephosphorylation of the N-terminal NFATc regulatory domain, inducing NFATc nuclear translocation from the cytoplasm. Accordingly, NFATc isoforms serve important roles in cardiac development and myocyte hypertrophy [22].

Previously, we showed that CaNAβ is associated with mAKAPβ in cardiac myocytes [11]. However, it remains unclear how scaffolding by this relatively low abundant protein contributes to CaN signaling. In this study, we characterize the direct binding of CaNAβ to mAKAPβ. Moreover, we provide evidence that recruitment of CaNAβ and NFATc3 to mAKAPβ complexes is important for the transduction of hypertrophic signaling.

2. Materials and methods

2.1. Antibodies and antiserum

Commercially available antibodies were as follows: rabbit and mouse anti-Flag (Sigma), anti-S tag (Novagen), anti-His tag (Santa Cruz), mouse anti-HA tag (Sigma), mouse anti-myc tag (monoclonal 4A6, Millipore), rabbit anti-CaNAβ (Santa Cruz), mouse anti-NFATc1 (BD Biosciences), rabbit anti-NFATc3 (Santa Cruz), rabbit anti-CaNAβ (Santa Cruz), mouse anti-α-actinin (monoclonal EA-53, Sigma), rabbit anti-rat atrial natriuretic factor (ANF; US Biological), horseradish peroxidase (HRP)-conjugated donkey secondary antibodies (Jackson ImmunoResearch) and Alexa dye-conjugated donkey secondary antibodies (Molecular Probes). HRP-conjugated VO145, OR010 (Covance), VO56, and VO54 rabbit and 720 (Covance) mouse anti-mAKAP antibodies were as previously described [5–7].

2.2. Expression vectors

The rat mAKAP siRNA and control siRNA expression plasmids and the mammalian expression plasmids (pCDNA3.1 (−) mychis vector, Invitrogen) for wildtype rat mAKAPα and mAKAPβ are as previously described [11,12]. Full-length deleted forms of rat mAKAPβ were generated by site-directed mutagenesis using the Quickchange method (Stratagene) and sense and antisense oligonucleotides to the following sequences: del 1301–1400 — GAGGACAGCCCACTGGGATGCAGCCAATG; del 1401–1500 — CCGGACCCCAAATGTATTTTGTAAAAAGTCCTGC; del 1501–1600 — CCCCTTCTTGGTGGTTTTTATAAGACAATGAGGATCTC. A Flag-tagged full-length NFATc3 expression vector was constructed using a cDNA provided by Dr. Neil Clipstone. HA-tagged CaNAβ expression vectors were constructed using a cDNA obtained by PCR using mouse brain cDNA. Adenovirus that express the various proteins were generated using the Adeno-X Tet-Off System (Clontech) [12]. All bacterial expression vectors were constructed by subcloning relevant PCR products into the pET30 (Novagen) or pGEX4T parent vectors (Pharmacia). All plasmid constructs were verified by sequencing, and details of the various constructions are available upon request. pET15 bacterial expression vectors for PKA catalytic subunit and GSK-3β were the gifts of Dr. Susan Taylor and Dr. Peter Roach, respectively. pET15-CaNAβB and pBB131 vectors were the gift of Jun O. Liu [23].

2.3. Ventricular myocyte culture

Ventricular myocytes (over 90% free of fibroblasts) were prepared from 2- to 3-day old Sprague–Dawley rats, as previously described [6]. The cells were plated in Dulbecco's Modified Eagle medium (DMEM) with 17% Media 199, 1% penicillin/streptomycin solution (P/S, Gibco-BRL), 10% horse serum and 5% fetal bovine serum (FBS) at 32,000 and 125,000 per cm^2 for immunocytochemical and biochemical experiments, respectively. After overnight in plating medium, the myocytes were maintained in culture for up to 1 week in maintenance medium (79% DMEM, 20% Media 199, and 1% P/S) supplemented with 50 μM phenylephrine before use. For adenoviral-based expression, the myocytes were infected with adenovirus (MOI = 15–100) using the Adeno-X Tet-Off System (Clontech). For plasmid-based expression, the myocytes were transfected with Transfast (Promega) as suggested by the manufacturer. Transfection efficiencies were typically between 1% and 5%.

Myocyte immunocytochemistry and morphometrics was performed as previously described by digital wide-field fluorescent microscopy using IPLab 4.0 software (BD Biosciences) [11]. All data are expressed as mean ± s.e.m. Each n represents the results of experiments using separate primary cultures. Within each experiment, ~25 cells were measured for each condition for both morphometric and ANF expression studies. ANOVA was calculated as a single factor with $\alpha = 0.05$; individual p-values were calculated using two-tailed, paired Student's t-tests.

For NFAT reporter assays, neonatal myocytes (120,000/cm^2) were transfected with Transfast and cultured for 48 h before analysis using the Dual Luciferase Reporter Assay System (Promega) and a Berthold Centro X luminometer. The NFAT-firefly luciferase reporter vector containing nine NFAT binding sites 5′ to the -164 αMHC minimal promoter was a gift of Jeffrey Molkentin [24]. The -164 αMHC minimal promoter was inserted into pRL-null (Promega) to provide the control renilla luciferase vector.

2.4. Other cell culture

HEK293 and COS-7 cells were maintained in DMEM with 10% FBS and 1% P/S. These cells were transiently transfected with Lipofectamine 2000 (Invitrogen) or infected with adenovirus and Adeno-X Tet-Off virus (Clontech) as suggested by the manufacturers.

2.5. Co-immunoprecipitation

For immunoprecipitation, tissues were homogenized using a Polytron or cells were lysed in IP buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton-X-100, 1 mM DTT) plus an inhibitor cocktail (1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM benzamidine, 1 mM AEBSF, 50 mM NaF, 1 mM sodium orthovanadate). Soluble proteins were separated by centrifugation at 20, 000 × g for 10 min. Antibody and 10 μl pre-washed protein-A or protein-G agarose beads (50% slurry, Upstate) or 10 μl HA or Flag antibody-conjugated sepharose beads (Sigma) were added to extracts and incubated overnight with rocking at 4 °C. Beads were washed three times for 5 min at 4 °C with IP buffer. Bound proteins were size-