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Liver Kinase B1 complex acts as a novel modifier of myofilament function and localizes to the Z-disk in cardiac myocytes☆

Samantha M. Behunin [a, b], Marissa A. Lopez-Pier [b, c], Rachel M. Mayfield [b, d], Christiane A. Danilo [a, b], Yulia Lipovka [b, d], Camille Birch [b, c], Sarah Lehman [a, b], Jil C. Tardiff [b, e], Carol C. Gregorio [b, e], John P. Konhilas [a, b, *]

[a] Department of Physiology, University of Arizona, Tucson, AZ, USA
[b] Sarver Molecular Cardiovascular Research Program, University of Arizona, Tucson, AZ, USA
[c] Department of Biomedical Engineering, University of Arizona, Tucson, AZ, USA
[d] Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ, USA
[e] Department of Cellular and Molecular Medicine, University of Arizona, Tucson, AZ, USA

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ABSTRACT

Contractile perturbations downstream of Ca^{2+} binding to troponin C, the so-called sarcomere-controlled mechanisms, represent the earliest indicators of energy remodeling in the diseased heart [1]. Central to cellular energy "sensing" is the adenosine monophosphate-activated kinase (AMPK) pathway, which is known to directly target myofilament proteins and alter contractility [2–6]. We previously showed that the upstream AMPK kinase, LKB1/MO25/STRAD, impacts myofilament function independently of AMPK [5]. Therefore, we hypothesized that the LKB1 complex associated with myofilament proteins and that alterations in energy signaling modulated targeting or localization of the LKB1 complex to the myofilament. Using an integrated strategy of myofilament mechanics, immunoblot analysis, co-immunoprecipitation, mass spectroscopy, and immunofluorescence, we showed that 1) LKB1 and MO25 associated with myofibrillar proteins, 2) cellular energy stress re-distributed AMPK/LKB1 complex proteins within the sarcomere, and 3) the LKB1 complex localized to the Z-Disk and interacted with cytoskeletal and energy-regulating proteins, including vinculin and ATP Synthase (Complex V). These data represent a novel role for LKB1 complex proteins in myofilament function and myocellular "energy" sensing in the heart.

1. Introduction

The cardiac myocyte undergoes extensive metabolic and energetic remodeling during the progression of heart disease. Contractile perturbations downstream of Ca^{2+} binding to troponin C, the so-called sarcomere-controlled mechanisms, may represent the earliest indicators of this remodeling [1]. Accordingly, the *dynamics* of cardiac contraction and relaxation during cardiovascular disease (CVD) are governed by downstream mechanisms, particularly the *kinetics* and *energetics* of the cross-bridge cycle [7]. Energy disturbance from CVD initiates cellular signaling cascades that become integrated with cross-bridge kinetics at the level of the contractile proteins, or *myofilament* [1,8].

The failing heart has long been characterized as energy starved [9–11] and central to this energetic remodeling is an alteration in the production, use and delivery of adenosine triphosphate (ATP). In fact, disturbances in the creatine kinase(CK)/adenylate kinase(AK) phosphotransfer system are observed early in CVD and are stronger predictors of heart failure mortality than functional status [12]. Given the physical barriers to rapid diffusion within the myocyte, physical association of CK, AK, and other key enzymes in the phosphotransferase system optimizes efficient transfer of phosphoryl groups to adenosine diphosphate (ADP) [13]. These phosphotransfer components exist in discrete microdomains and are localized to sarcomeric myofibrils acting as hubs for energy "sensing" [14,15]. More importantly, these phosphotransferase enzymes display remarkable plasticity in function and

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* Corresponding author. University of Arizona, Department of Physiology, Sarver Molecular Cardiovascular Research Program, Medical Research Building, Room 320, Tucson, AZ 85721-0241, USA. Tel.: +1 (520) 626 6578; fax: +1 (520) 626 7600.
E-mail address: konhilas@arizona.edu (J.P. Konhilas).

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compartmentation during energy stress [16]. Perturbations in the balance of ATP supply and demand commonly occur during CVD. During this imbalance, AK amplifies the amount of adenosine monophosphate (AMP) within these microdomains while it attempts to preserve ATP levels for contraction [17].

Central to AMP sensing is the adenosine monophosphate-activated kinase (AMPK) pathway, which also displays remarkable plasticity during CVD. AMPK is a phylogenetically conserved heterotrimeric complex consisting of a catalytic α subunit and regulatory β and γ subunits [18]. An increase in myocellular AMP, as occurs with cardiac disease, allosterically activates AMPK and permits phosphorylation of the α catalytic subunit at Thr^{172} by the upstream Liver Kinase B1 (LKB1) kinase complex [19–21]. LKB1 acts in concert with MO25 (mouse protein 25) and STRAD (ste-related adaptor protein) to phosphorylate AMPK potentiating its activity and promoting ATP producing pathways while inhibiting ATP consuming pathways [20,21]. In addition, AMPK targets Ser^{150} of Troponin I ($p\text{-}cTnI_{ser150}$) and subsequently increases myofilament sensitivity to Ca^{2+} [2–6].

Evidence exists to support the idea that AMP binding to AMPK initiates assembly of an activating complex that brings LKB1/MO25/STRAD into close association with AMPK allowing phosphorylation of Thr^{172} on AMPKα by LKB1/MO25/STRAD [22]. Once the components of the upstream kinase complex are bound together LKB1/MO25/STRAD becomes constitutively active [23]. Therefore, controlling when and where the subunits of the LKB1/MO25/STRAD complex are expressed is key to regulating its function. Indeed, there have been some studies in adipocytes and skeletal muscle that suggest posttranslational modification of LKB1 leads to alterations in subcellular localization [24,25]. Therefore, the AMPK/LKB1 signaling pathway is subject to regulation by not only AMP pools but also to alterations in the subcellular association of AMPK and LKB1/MO25/STRAD. This mechanism of regulation is similar to the phosphotransferase CK system, which is a paradigm of subcellular localized enzyme organization [16,26]. For this reason, we propose that AMPK/LKB1 complex proteins act as a nodal point for sensing changes in CK and AK activity through changes in the ATP pool and directly tuning myofilament function to the energetic demand through post-translational modifications.

We recently presented data illustrating how differing molar ratios of AMPK and LKB1/MO25/STRAD impact myofilament function. In addition, we provided the first evidence that the upstream LKB1/MO25/STRAD complex had the ability to modify contractile function independently of AMPK [5]. For these reasons we hypothesized that the LKB1 complex associates with myofilament proteins and that alterations in energy signaling modulate targeting or localization of the LKB1 complex to the myofilament. To that end we have found that 1) myofibrillar proteins retained or bound LKB1 and MO25; 2) cardiovascular energy stress re-distributed LKB1 and MO25 localization within myofibrillar proteins; 3) LKB1 complex proteins robustly localized at the Z-Disk.

2. Materials and methods

2.1. Animal subjects

Animal models included male Sprague-Dawley rats, aged two months; four month old male R403Q transgenic mice; and four month old male c57/bl6 mice. All experiments were performed using protocols that adhered to guidelines and approved by the Institutional Animal Care and Use Committee at the University of Arizona and to NIH guidelines for care and use of laboratory animals.

2.2. Isolation and perfusion with 5-aminoimidazole-4-carboxamide ribonucleotide

Rats were anesthetized with isoflurane and their hearts rapidly excised. Control hearts were then retrogradely perfused through the aortic stump with a Krebs-Henseleit solution (NaCl 118.5 mmol/L, KCl 5 mmol/L, $MgSO_4$ 1.2 mmol/L, NaH_2PO_4 2 mmol/L, D-(+)-glucose 10 mmol/L, $NaHCO_3$ 25 mmol/L, $CaCl_2$ 0.2 mmol/L, and 2,3-Butanedione monoxime (20 mmol/L)) [27]. For separate 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) treatment, a modified Krebs-Henseleit solution containing 2 mM AICAR was used for perfusion. All hearts were retrogradely perfused for 30 min. Following perfusion, hearts were flash frozen and stored for proteomic analysis at −80 °C.

2.3. Trabecular isolation and treatment with LKB1/MO25/STRAD

Sprague-Dawley rats were anesthetized with isoflurane and their hearts rapidly excised. Hearts were then retrogradely perfused with a modified Krebs-Henseleit solution. Thin, even, free standing trabeculae were isolated from the right ventricular wall, as well as left ventricular papillaries. Following isolation, trabeculae and cut papillaries were transferred to an ice-cold relax solution (Na_2ATP 5.95 mmol/L, $MgCl_2$ 6.41 mmol/L, EGTA10 mmol/L, K^+Propionate 50.25 mmol/L, phosphocreatine 10 mmol/L, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) 100 mmol/L, phenylmethylsulfonyl fluoride (PMSF) 0.1 mmol/L, dithiothreitol (DTT) 1 mmol/L, 50 U/mL creatine phosphokinase, and protease inhibitor cocktail (Sigma) 4 μL cocktail/ml solution) containing 1% Trition X-100 for overnight demembranation at 4 °C [2,27]. Following demembranation, 50 nM of active recombinant LKB1/Mo25/STRAD (Millipore) was added to demembranated trabeculae for 30 min at 30 °C. After incubation, fibers were washed (15 min; repeated three times) with standard relaxing buffer on ice. The Ca^{2+}-Force relationship was then measured for each fiber, followed by flash freezing and storage at −80 °C. The treatment/incubation protocol is summarized in Fig. 1.

2.4. Experimental apparatus and protocol

The experimental apparatus for mechanical measurements of cardiac trabeculae was similar to that described previously [27]. The fiber was attached to the apparatus via aluminum T-clips to stainless steel hooks that extended from a high-speed servomotor (Aurora Scientific model 315C) and a modified silicon strain gauge force transducer (model AE801, Kronex, CA), both of which were attached to X-Y-Z manipulators mounted on a temperature controlled stage (15 ± 0.1C). Force was digitally converted with an A/D converter and custom software (LabVIEW, National Instruments; Austin, Texas) for off-line analysis. Ca^{2+}-sensitivity of tension development as a function of sarcomere length was determined by activating the muscle during a series of pre-activating-activating-relaxation cycles using a range of free [Ca^{2+}] in the activating solutions (Table 1) selected in random order [27].

Sarcomere length (SL) was set at 2.2 μm determined from the first order He-Ne laser light diffraction band monitored by a 2048 pixel high speed linear CCD sensor (Dexela Ltd., London, UK) and adjusted to maintain constant SL throughout contraction. Fibers were allowed to reach steady state tension and then rapidly slackened by 20% of total fiber length. The difference between steady state tension and slacked tension determined total tension. Active tension at each [Ca^{2+}] is the difference between total tension and relaxed, passive tension. For all experiments, fibers that did not retain 85% of initial maximal tension or a detectable diffraction pattern were discarded. Given these stringent criteria, yields for

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