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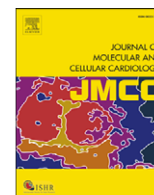

Original article

# Myocyte remodeling in response to hypertrophic stimuli requires nucleocytoplasmic shuttling of muscle LIM protein

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CSRP3 or muscle LIM protein (MLP) is a nucleocytoplasmic shuttling protein and a mechanosensor in cardiac myocytes. MLP regulation and function was studied in cultured neonatal rat myocytes treated with pharmacological or mechanical stimuli. Either verapamil or BDM decreased nuclear MLP while phenylephrine and cyclic strain increased it. These results suggest that myocyte contractility regulates MLP subcellular localization. When RNA polymerase II was inhibited with α-amanitin, nuclear MLP was reduced by 30%. However, when both RNA polymerase I and II were inhibited with actinomycin D, there was a 90% decrease in nuclear MLP suggesting that its nuclear translocation is regulated by both nuclear and nucleolar transcriptional activity. Using cell permeable synthetic peptides containing the putative nuclear localization signal (NLS) of MLP, nuclear import of the protein in cultured rat neonatal myocytes was inhibited. The NLS of MLP also localizes to the nucleolus. Inhibition of nuclear translocation prevented the increased protein accumulation in response to phenylephrine. Furthermore, cyclic strain of myocytes after prior NLS treatment to remove nuclear MLP resulted in disarrayed sarcomeres. Increased protein synthesis and brain natriuretic peptide expression were also prevented suggesting that MLP is required for remodeling of the myofilaments and gene expression. These findings suggest that nucleocytoplasmic shuttling MLP plays an important role in the regulation of the myocyte remodeling and hypertrophy and is required for adaptation to hypertrophic stimuli.

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## 1. Introduction

Cells are constantly remodeling through a process of mRNA translation and protein turnover. This remodeling occurs in response to local changes and requires the activity of molecular sensors. There is growing evidence that a number of cytoplasmic, plasma membrane and cytoskeletal proteins shuttle constantly in and out of the nucleus in response to various stimuli [1]. These nuclear-cytoplasmic cycling proteins can act as messengers or transcription factors, allowing the nuclear machinery to respond rapidly to environmental changes.

In myocytes, the Z-disc within the sarcomere harbors a number of these shuttling proteins including CSRP3 or muscle LIM protein (MLP). MLP is a member of a family of proteins containing one or more LIM domains, that mediate specific protein–protein interactions [2]. MLP interacts with multiple proteins within the Z-disc, including α-actinin and the titin-binding protein telethonin (T-cap) [3]. We have recently shown that MLP accumulates in the nucleus in response to human heart failure and in two animal models of cardiac disease [4]. In cultured neonatal myocytes we also showed that MLP translocated to the nucleolus in response to cyclic mechanical strain, thereby activating ribosomal protein synthesis.

MLP has at least two potential mechanisms of action in myocytes; through its structural role and interaction with other signaling molecules in the cytoplasm, and/or through its role as a transcription factor in the nucleus [5]. The protein is thought to shuttle to the nucleus via a putative 6 amino acid nuclear localization signal (NLS). Using synthetic cell permeable peptides containing this NLS it is possible to competitively inhibit the nuclear translocation of the endogenous protein. One such membrane permeable peptide is the fibroblast growth factor (FGF) membrane translocating peptide sequence (AAVALLPAVLLALLAP). This peptide has been used to import the NLS of the transcription factor NFκB, thereby blocking nuclear translocation and the subsequent activation of T-cells [6].

Various unsuccessful attempts have been made to determine the cellular function of MLP through mutagenesis and adenoviral transfection in cardiac myocytes [7,8]. We have previously shown that this is likely related to interference with MLP oligomerisation which is required for its function as a mechanosensor thus

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abrogating this approach for mechanistic studies [4]. Therefore, we use a new strategy by inhibiting MLP nuclear translocation, which takes advantage of the putative MLP-NLS sequence along with the cell permeable domain. We first show that treatment of cultured rat neonatal myocytes with this NLS peptide prevents MLP nuclear translocation confirming that this sequence is functionally effective. Inhibition of MLP nuclear translocation prevents the increased protein accumulation associated with phenylephrine treatment and cyclic strain in cardiac myocytes. When myocytes were cyclically strained in the absence of nuclear MLP, the sarcomeres were in disarray suggesting that cycling MLP or a factor associated with it is required for maintenance and remodeling of the myofilaments. These data suggest that MLP has a broad range of functions in cardiac myocytes and is required for adaptation to hypertrophic stimuli.

## 2. Materials and methods

### *2.1. Cell culture*

Myocytes were isolated from the cardiac ventricles of 1–2 day old Sprague Dawley rats by sequential collagenase digestion, as previously described [9]. Briefly, cells were pre-plated to reduce non-myocyte cell contamination and then plated (1 million cells/cm$^2$) on fibronectin (25 μL/mL) coated silicone Petri dishes in PC1 medium (BioWhittaker, Walkersville, MD) for 24 h and transferred to a DMEM:M199 serum free medium. All drug treatments were done 24 h after being placed in DMEM:M199 medium. To examine MLP expression in response to inhibition of transcription, cells were treated with 12.5 mg/L actinomycin D for 10 h, a concentration which inhibits both polymerase I and RNA polymerase II. These conditions were based on previous work [10]. A concentration of 1 μM α-amanitin was used to inhibit RNA polymerase II for 24 h. Some groups of cells were treated with 7.5 mM BDM, 10 μM verapamil or phenylephrine for either 24 or 48 h in DMEM:M199 medium. For inhibition of MLP nuclear translocation, synthetic peptides were used containing the fibroblast growth factor (FGF) membrane translocating peptide sequence (AAVALLPAVLLALLAP) and the NLS of MLP. The control peptide did not have the additional NLS. Cells were treated with these synthetic peptides for either 24 or 48 h at a concentration ranging from 20 to 100 μM depending on the experiment.

### *2.2. Cellular composition and subcellular fractionation*

For subcellular fractionation of myocytes, the ProteoExtract Subcellular Proteome Kit from Calbiochem was used. This method uses a detergent-based protocol[11] and has been previously described [4]. Briefly, cellular proteins were sequentially extracted into four compartments: cytosolic, membrane/organelles, nuclear and cytoskeleton. Digitonin/EDTA is used to remove the cytosol. Triton/EDTA is used to remove the membrane/organelle fraction. Tween/deoxycholate/benzonase removes the nuclei. Finally SDS is used to remove the cytoskeleton. Cells were briefly washed 3 times in PBS between each extraction fraction to prevent cross-contamination. After each fraction, cells were observed by microscopy to ensure that they were still attached to the dish. Cell integrity is maintained throughout the fractionation process. The fractions were then frozen at −80 °C prior to analysis.

### *2.3. Western blotting for analysis of protein expression*

Neonatal rat ventricular myocytes were rinsed with warm PBS and then scraped from the silicone membranes or dishes in lysis buffer containing 1% SDS and protease inhibitor cocktail (Sigma). The Bradford method was used to determine total protein using crystalline bovine serum albumin as standard. For whole heart protein analysis, tissue was ground in liquid nitrogen and added to lysis buffer containing 1% SDS, 50 mM NaF and protease inhibitor cocktail (Sigma). Samples were treated with β-mercaptoethanol and heated to 100 °C for 5 min. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Hybond C, Amersham). Blots containing either whole cell lysates or fractionated cells were probed for anti-MLP (produced by Invitrogen using the last 14 amino acids from the carboxy terminus), phalloidin (Molecular Probes), fibrillarin (Abcam), histone 2B (Abcam), α-sarcomeric actinin (Abcam), α-sarcomeric actin (Abcam), desmin (BD Transduction Laboratories), Clock (Abcam) and myosin MF20 (Hybridoma Bank, Iowa). Horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Research Diagnostics Inc) were used to visualize proteins by enhanced chemiluminescence (ECL, Amersham). The bands corresponding to the various proteins were quantified by laser densitometry. Protein bands were further standardized to total protein loading by using the amido black-stained nitrocellulose membrane as described previously [12].

### *2.4. Quantitative real-time-PCR*

Real-time quantitative polymerase chain reaction (RT-PCR) was performed using a LightCycler thermocycler (Roche Diagnostics) as previously described [13]. Briefly, total RNA was isolated from cell culture using TRIzol (Invitrogen) reagent. The RNA amplification kit with SYBR Green 1 (Roche Molecular Biochemical) used 100 ng in a one-step RT-PCR reaction. The reaction conditions for RT were 55 °C for 15 min, which was followed by a four-step PCR amplification with signal acquisition at 80–89 °C for 2 s (depending on the melting temperature for each PCR product, determined by the melting curve analysis) for 40 cycles. The RT-PCR reaction was quantified using in vitro transcribed mRNA standards, prepared for each gene, that were run alongside to develop a standard curve. The second derivative maximum (log linear phase) for each amplification curve was determined and plotted against the standard curve to calculate the amount of product [13]. Primers were: Brain natriuretic peptide (BNP): (F) 5′-GCTGGAGCTGATAAGAGAAA and (R) 5′-GGAATTTC-GAAGTCTCTCCT and normalized against ribosomal protein L7: (F) 5′-GAAGCTCATCTATGAGAAGGC and (R) 5′-CAGACGGAGCAGCTGCAG-CAC to ensure equal loading.

### *2.5. Immuno-cytochemistry and image analysis*

After the various experimental protocols, cells for immuno-cytochemical staining were fixed in 4% paraformaldehyde for 3 min and then 100% methanol at −20 °C for 1 min. Fixed cells were immunostained with antibodies as described previously [14]. Rhodamine and Alexa Fluor-conjugated secondary antibodies (Molecular Probes) were used to visualize the specific proteins. Fluorescently-labeled cells were then viewed using a Zeiss Model LSM 510 laser scanning confocal microscope.

### *2.6. Biaxial mechanical strain of cardiac myocytes*

Cyclic mechanical deformation of cultured neonatal rat ventricular myocytes was produced with a Flexcell Strain Unit (Model FX-4000, Flexcell International, McKeesport, PA). A sinusoidal cyclic strain at 10% maximum strain and 1 Hz for 48 h was used without the posts for biaxial strain.

### *2.7. Fabrication of uniaxial scaffolds and cyclic strain*

Neonatal myocytes were polarized on textured substrates and exposed to cyclic uniaxial strain (Flexcell) in either the transverse axis or in alignment with cells. Textured substrates for cell culture were fabricated as previously described [15]. Briefly, micron scaled

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