Cardiac dysfunction and heart failure are associated with abnormalities in the subcellular distribution and amounts of oligomeric muscle LIM protein

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Boateng SY, Belin RJ, Geenen DL, Margulies KB, Martin JL, Hoshijima M, de Tombe PP, Russell B. Cardiac dysfunction and heart failure are associated with abnormalities in the subcellular distribution and amounts of oligomeric muscle LIM protein. Am J Physiol Heart Circ Physiol 292: H259–H269, 2007. First published September 8, 2006; doi:10.1152/ajpheart.00766.2006.—Prolonged hemodynamic overload results in cardiac hypertrophy and failure with detrimental changes in myocardial gene expression and morphology. Cysteine-rich protein 3 or muscle LIM protein (MLP) is thought to be a mechanosensor in cardiac myocytes. Therefore, the subcellular location of MLP may have functional implications in health and disease. Our hypothesis is that MLP becomes mislocalized after prolonged overload, resulting in impaired mechanosensing in cardiac myocytes. Using the techniques of biochemical subcellular fractionation and immunocytochemistry, we found MLP exhibits oligomerization in the membrane and cytoskeleton of cultured cardiac rat neonatal myocytes. Nuclear MLP was always monomeric. MLP translocated to the nucleus in response to 10% cyclic stretch at 1 Hz for 48 h. This was associated with a threefold increase in S6 ribosomal protein (P < 0.01; n = 3 cultures). Adenoviral overexpression of MLP also resulted in a twofold increase in S6 protein, suggesting that MLP can activate ribosomal protein synthesis in the nucleus. In ventricles from aortic-banded and myocardially infarcted rat hearts, nuclear MLP increased by twofold (P < 0.01; n = 7) along with a significant decrease in the nonnuclear oligomeric fraction. The ratio of nuclear to nonnuclear MLP increased threefold in both groups (P < 0.01; n = 7). In failing human hearts, there was almost a complete loss of oligomeric MLP. Using a flag-tagged adenoviral MLP, we demonstrate that the COOH terminus is required for oligomerization and that this is a precursor to stretch sensing and subsequent nuclear translocation. Therefore, reduced oligomeric MLP in the costamere and cytoskeleton may contribute to impaired mechanosensing in heart failure.

hypertrophy; mechanosensing; cytoskeleton; nucleocyttoplasmic shuttling

Many proteins shuttle from subcellular compartments to the nucleus and may act as molecular sensors (4). In myocytes, some of these nucleocytoplasmic proteins are located in costameric focal adhesions and the cytoskeleton where mechanical forces are transmitted. One such protein, muscle LIM protein (MLP) or cysteine-rich protein 3, contains two zinc finger LIM domains. MLP is known to interact with many proteins of the cytoskeleton, including α-actinin and the titin-binding protein telethonin (34). MLP is thought to be part of a mechanosensing mechanism in myocytes, and this is likely to occur through two main mechanisms. First, the LIM domains enable the protein to interact with a variety of other cellular proteins, many of which are associated with known signaling pathways. The potential role of MLP as a sensor of mechanical activity through its interactions with other proteins has been thoroughly reviewed recently (18). Second, the protein contains a nuclear localization signal, and its translocation to the nucleus may enable it to act as a transcription factor, directly regulating gene expression (10, 23). Mutations in MLP lead to cardiomyopathy in humans, and deletion of the protein in transgenic mice leads to a number of cytoskeletal and functional abnormalities (14, 22, 29).

The subcellular location of MLP and its response to mechanical activity may have important functional implications for the cardiac myocyte. However, such studies have been limited by the lack of methods that accurately fractionate the major cellular compartments. Cell lysis before differential centrifugation contaminates these distinct subcellular compartments. We have used a detergent-based cell fractionation method using differential solubility to avoid the disruption of cell integrity (30).

In this study, we have shown that, in cultured neonatal myocytes, MLP translocates to the nucleus and nucleolus after 48 h of cyclic stretch at 10% maximum strain. This translocation is associated with a threefold increase in the S6 ribosomal protein, a protein involved in protein synthesis. Adenoviral overexpression of MLP in cultured myocytes also increased S6 protein twofold, suggesting that MLP can directly activate ribosomal protein synthesis within the nucleolus. In two different rodent models of cardiac dysfunction, we analyzed the localization of MLP in the cytosolic, membrane, nuclear, and cytoskeletal compartments. We show for the first time that MLP exhibits oligomerization by forming dimers, trimers, and tetramers in myocytes in addition to its known 20-kDa mono-
meric form. In failing human hearts, during myocardial infarction, and in aortic banding in rat hearts, MLP accumulates in the nucleus. This results in a significant decline in the oligomeric nonnuclear MLP fraction. These data indicate that loss of nonnuclear MLP may be part of a “declining sensor” phenomenon in myocytes, following long-term stimulation and stress on the heart. We propose that this loss of mechanical sensing can contribute to maladaptation and failure.

MATERIALS AND METHODS

Cell culture. Myocytes were isolated from the cardiac ventricles of 1- to 2-day-old Sprague-Dawley rats by sequential collagenase digestion, as previously described (6). Primary heart cultures were obtained from neonatal rats according to Institutional Animal Care and Use Committee and National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publication 85-23, Rev. 1985). Briefly, cells were preplated to reduce nonmyocyte cell contamination and then plated (1 million cells/cm²) on fibronectin (25 μg/ml)–coated silicone Flexcell sixwell dishes in PC1 medium (BioWhittaker, Walkersville, MD) for 24 h and transferred to a DMEM-M199 serum-free medium.

Adenoviral constructs and myocyte infection. Mouse MLP was amplified by RT-PCR and verified by sequencing. A flag-tag sequence (DYKDDDDK) was fused to the COOH terminus before subcloning into shuttle vector pΔE1sp1A (Microbix Biosystems, Ontario, Canada) with a cytomegalovirus promoter and SV40 poly(A) to generate replication-deficient recombinant adenovirus type 5 as previously described (27). Adenovirus was amplified and titered via standard methods (26).

Application of extrinsic mechanical load. Cyclic mechanical deformation of cultured neonatal rat ventricular myocytes was produced with a Flexcell strain unit (model FX-4000; Flexcell International, McKeesport, PA). In this study, we used a sinusoidal stretch at 10% maximum strain and 1 Hz for 48 h. The cyclic stretch was performed without the posts.

Cellular composition and subcellular fractionation. For subcellular fractionation of myocytes, the ProteoExtract subcellular proteome kit from Calbiochem was used. This method uses a previously described detergent-based protocol (30). Cellular proteins were sequentially extracted into four compartments: cytosolic, membrane/organelles, nuclei, and cytoskeleton. Digitonin-EDTA is used to remove the cytosol. Triton-EDTA is used to remove the membrane-organelle fraction. Tween 20-deoxycholate-benzonase removes the nuclei. Fi-
nally, SDS is used to remove the cytoskeleton. Cells were briefly washed three 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 accuracy of the fractionation method was verified with antibodies to well-documented subcellular distribution markers.

**Western blotting for analysis of proteins.** 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 BSA 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). For non-denaturing conditions, samples were not treated with heat or reducing agents. For denaturing conditions, however, 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 COOH terminus), phalloidin (Molecular Probes), anti-β1-integrin (Research Diagnostics), connexin 43 (Research Diagnostics), 70-kDa heat shock protein (Research Diagnostics), RNA polymerase II (Abcam), S6 ribosomal protein (Cell Signaling), fibrillarin (Abcam), and myosin heavy chain for the myofibrillar-cytoskeletal fractions (MF20; Iowa Hybridoma Bank). Horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies were used to visualize proteins by enhanced chemiluminesence (Amer- sham). 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 (8).

**Immunochemistry and image analysis.** After the various experimental protocols, cells for immunocytochemical staining were fixed in 4% paraformaldehyde for 3 min and then in 100% methanol at −20°C for 1 min. Fixed cells were immunostained with antibodies as described previously (5). Rhodamine and Alexa fluor-conjugated secondary antibodies (Molecular Probes) were used to visualize the specific proteins. Fluorescently labeled cells were then viewed with a Zeiss model LSM 510 laser scanning confocal microscope.

**Transverse aortic banding procedure.** As described previously (31), rats were initially anesthetized with methoxyflurane inhaled in a closed chamber and intubated with an 18-gauge angiocath needle. Surgical anesthesia was maintained by 1.5% isoflurane delivered through a vaporizer with a mixture of 95% oxygen-5% carbon dioxide connected in series to a rodent ventilator with the stroke volume set at 2–3 ml/min and a respiration rate of 80 per minute. A thoracotomy

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**Fig. 2.** Detergent-based subcellular fractionation of cultured rat neonatal myocytes. A–E: cardiac myocytes were stained for actin (green) with phalloidin, connexin 43 (red), and nuclei with 4',6-diamidino-2-phenylindole (DAPI) (blue). Arrows show former positions of nuclei. F–I: cardiac myocytes stained with MLP (red) at various stages of the subcellular fractionation process. After removal of the cytoplasm, MLP staining appears more striated. Removal of the membranes leaves nuclear MLP and cytoskeletal MLP intact. Finally, removal of the nuclei leaves just cytoskeletal MLP. J–M: same cells stained for actin with phalloidin and DAPI for nuclei.
was then performed in rats by a left lateral incision and transaction of the first rib and retraction of the thymus. The transverse aorta was isolated, and extruded PE-50 tubing was tied around the aorta between the right and left carotid artery bifurcations with 5-0 silk suture. The tubing was then removed, leaving the suture in place and creating a 40% coarctation and an effective pressure gradient across the stenosis. After the stenosis, the thoracotomy was closed in three layers with 6-0 silk sutures, and the chest cavity was evacuated. Animals were then given buprenorphine [0.2 mg/kg; injected into the thoracotomy site (50 µl) at the time of surgery]. Animals were killed with end-stage heart failure 6–7 mo postsurgery.

**Coronary ligation procedure.** As described previously (13), rats were initially anesthetized with methoxyflurane inhaled in a closed chamber and intubated with an 18-gauge angiocath needle. Surgical anesthesia was maintained by 1.5% isoflurane delivered through a vaporizer with a mixture of 95% oxygen-5% carbon dioxide connected in series to a rodent ventilator with the stroke volume set at 2–3 ml/min and a respiration rate of 80 breaths/min. A left thoracotomy was performed to expose the heart, and the pericardium was then ruptured. The heart was exteriorized, and the left coronary artery was ligated 5 mm from the ostium with 7-0 silk suture to produce a myocardial infarction. A chest tube was placed in the thoracic cavity, and the thoracotomy was closed in three layers (intercostal muscles, pectoral muscles, and skin) followed by evacuation of the chest cavity and removal of the tube. Animals were allowed to recover in a heated cage before being returned to the animal facility. They were monitored twice daily for normal respiration after surgery or if they appeared moribund, they were euthanized. Sham-operated rats underwent the same procedure as described above except the left coronary artery was not ligated. Animals were killed with end-stage heart failure 6–7 mo postsurgery.

**Hemodynamic analysis.** For hemodynamic analysis, the right carotid artery was dissected free from surrounding tissue and a Millar ultraminiature pressure transducer with conductance electrodes (Millar Instruments; SPR-838, 2 F) was inserted into the artery to measure baseline arterial pressure. The catheter was then advanced into the left ventricle, and baseline recordings of heart rate, left ventricular systolic-diastolic pressure, maximum rates of pressure rise and fall ( ± dP/dt), and tension time index were recorded. Baseline left ventricular pressure-volume loops were also recorded in the rat at steady state and during inferior vena cava occlusion. To occlude the inferior vena cava near the junction of the right atrium, we accessed the thoracic cavity through a midline incision over the xiphoid process and entered through the diaphragm. The inferior vena cava was isolated and occluded while simultaneously measuring pressure-volume loops.

**Statistics.** For the experiments described here, at least three separate primary cultures were averaged. Each culture used ~30 neonatal hearts. All values are means ± SE. All values of significance were calculated by the appropriate comparisons: one-way ANOVA or the Student’s unpaired t-test. Differences among means were considered significant at *P* < 0.05. Data were analyzed by GraphPad and SigmaStat statistical software.

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**Fig. 3. Oligomerization of MLP.** A: equal proportions of the subcellular fractions were separated by Western blotting and probed with an MLP antibody. MLP appears as multiple bands of differing molecular masses. The membrane fraction showed 2 bands: one just below 46 kDa and a second just below 66 kDa. These are interpreted to represent dimeric and trimeric forms of MLP, respectively. The nuclear fraction showed only a 20-kDa band, interpreted to represent monomeric MLP. Although equal amounts of the cytoskeleton fraction showed little or no MLP bands, a 6-fold loading of the cytoskeletal fraction showed a protein band at ~80 kDa. This is interpreted to represent a tetrameric form of MLP. There are also a couple additional faint bands in this lane, which are most likely a nonspecific reaction of the MLP antibody due to the lane being overloaded 6-fold. B: the nitrocellulose blot was then stripped and reprobed several times for proteins whose subcellular distribution is well documented. Constitutive heat shock protein 70 (HSP70) was found in the cytoplasmic and membrane fractions. β1-Integrin was only found in the membrane fraction. RNA polymerase II (Pol2) was only found in the nuclear fraction, whereas myosin heavy chain was found in the cytoskeletal fractions only. C: Western blot of MLP from unfraccionated cultured neonatal rat myocytes processed with nondenaturing conditions. All of the MLP oligomers observed from the fractionation in A can also be seen in lanes 1 and 2 of C. Lane 3 shows the nuclear fraction in which only the 20-kDa monomer is present.

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RESULTS

MLP and mechanical stretch in neonatal myocytes. MLP is thought to be a mechanosensor in cardiac myocytes; however, the response of the protein to mechanical stimulation has never been examined. To determine how MLP responds to mechanical stimulation, cells were stretched cyclically at 10% maximum strain for 48 h at 1 Hz. Figure 1, A and B, shows that endogenous MLP is expressed in both the cytoplasm and nucleus in spontaneously beating neonatal myocytes. At 10% strain, MLP translocates to a distinct region within the nucleus. In Fig. 1, C–E (coating with MLP and fibrillin), a nucleolar protein showed that the translocation of MLP is to the nucleolus after 10% cyclic strain. Figure 1F shows examples of Western blots of MLP, S6 ribosomal protein, and the amido black-stained nitrocellulose membrane. The stained membrane is used to provide an additional standardization of protein loading. This method has been previously described in detail (8). Figure 1G shows that quantified total MLP was unchanged after 10% cyclic strain for 48 h. Figure 1H shows quantitation of S6 ribosomal protein, used as a measure of ribosomal protein synthesis. This protein showed a threefold increase following 10% cyclic strain. This demonstrates that the translocation of MLP to the nucleolus occurs during a time of increased ribosomal protein synthesis.

Subcellular distribution of MLP in cultured neonatal myocytes. With the use of a detergent-based method for cell fractionation, cultured cells can separate into four distinct subcellular compartments by sequential extraction. We validated this method in neonatal rat ventricular myocyte cultures at each stage of the fractionation process using immunocytochemistry with phalloidin for actin (cytoskeleton) and connexin 43 (membrane gap junction) and 4′,6-diamidino-2-phenylindole for nuclei (Fig. 2, A–E). The fractions were collected in the order of cytosolic, membranes/organelles, nuclei, and then cytoskeleton. These results show that detergent-based subcellular fractionation can be successfully used to isolate the main compartments of cultured myocytes while maintaining cell attachment and integrity.

Neonatal cardiac myocytes were immunostained for MLP, actin, and nuclei at various points in the fractionation process (Fig. 2, F–I). Note that nonmyocytes do not stain for MLP, indicating specificity. MLP is found in several cellular compartments: membrane, nuclei, and cytoskeleton. After the cytoplasm and membrane fractions had been removed, myocytes were stained with MLP (red) and the Z-disk protein α-actinin (green) to determine where cytoskeletal MLP is located.

For a biochemical analysis of MLP, subcellular fractions of cardiac myocytes were analyzed by Western blotting after 4 days of culture using proteins with known subcellular localization for verification (Fig. 3A). We used antibodies to the constitutive 70-kDa heat shock protein for cytosol/membrane, β1-integrin for membranes, RNA polymerase II for nuclei, and myosin heavy chain for the myofibrillar-cytoskeletal fractions (MF20). MLP antibody was also tested on noncardiac samples, such as liver and kidney, and showed no reactivity (data not shown).

No MLP could be detected in the cytosolic fraction. In the membrane fraction, two bands of ~45 and 66 kDa were found and interpreted as dimeric and trimeric forms of MLP, respectively. These were not found in any other fractions. In the

Table 1. Summary of physiological and morphological data

<table>
<thead>
<tr>
<th>Group</th>
<th>Heart rate, beats/min</th>
<th>LVSP, mmHg</th>
<th>LVEDP, mmHg</th>
<th>LVDP, mmHg</th>
<th>dP/dt, mmHg/s</th>
<th>RVW-to-Body wt Ratio</th>
<th>LV-to-Body wt Ratio</th>
<th>WW-to-DW Ratio</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>166 ± 9</td>
<td>95 ± 2</td>
<td>−5 ± 2</td>
<td>3 ± 1</td>
<td>5 ± 1</td>
<td>0.12 ± 0.02</td>
<td>0.13 ± 0.02</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>142 ± 11</td>
<td>100 ± 2</td>
<td>−10 ± 2</td>
<td>4 ± 1</td>
<td>2 ± 1</td>
<td>0.11 ± 0.02</td>
<td>0.12 ± 0.02</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>Aortic banding</td>
<td>142 ± 11</td>
<td>100 ± 2</td>
<td>−10 ± 2</td>
<td>4 ± 1</td>
<td>2 ± 1</td>
<td>0.11 ± 0.02</td>
<td>0.12 ± 0.02</td>
<td>0.03 ± 0.01</td>
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Values are means ± SE. LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; LVDP, left ventricular-developed pressure; dP/dt, Pressure rise and fall. *Control vs. myocardial infarction, P < 0.001; ‡Control vs. aortic banding, P < 0.05; §Control vs. myocardial infarction, P > 0.05; †Control vs. myocardial infarction, P < 0.05; ‡Control vs. aortic banding, P > 0.05.
nuclear fraction, there was strong MLP staining for a band of ~20 kDa, interpreted as the monomeric form. Although equal loading failed to detect the MLP in the cytoskeletal fraction, sixfold loading revealed a band of ~80 kDa, interpreted as the tetrameric form. This may suggest a difference in the sensitivity of the antibody for the native and denatured forms of MLP detected by immunonchemistry and Western blotting, respectively. These data show that the fractionation methodology can be used to detect MLP within myocytes. The findings indicate that MLP is an oligomeric protein forming monomers, dimers, trimers, and tetramers, each located in specific compartments of cultured neonatal myocytes.

To determine whether these oligomers were present in unfractionated myocytes, whole cell lysates were run under non-denaturing conditions, in the absence of reducing agents and heat (Fig. 3C). All oligomers determined by fractionation could also be observed from whole cell lysates. Thus MLP oligomers are not an artifact of subcellular fractionation and can be measured directly under these new conditions.

**MLP subcellular distribution in models of cardiac disease.**

To determine the MLP subcellular distribution in models of cardiac disease, we studied aortic-banded and myocardially infarcted hearts. A summary of the physiological and morphological data from these animals is shown in Table 1. In one group, rats were operated to produce myocardial infarction, a treatment that induced a predominantly systolic dysfunction, as determined by decreased left ventricular developed pressure. In the second, group cardiac hypertrophy was induced by constriction of the ascending aorta. This treatment induced a predominantly diastolic dysfunction, as determined by the significantly raised left ventricular end-diastolic pressure. However, both models represent models of end-stage heart failure.

![Figures A to J](image)

**Fig. 4.** Analysis of monomeric and oligomeric MLP in adult rat models of myocardial disease. A: Western blot of MLP from control adult rat hearts treated under non-denaturing conditions. All monomeric and oligomeric forms of MLP can be seen in these hearts. B: typical gel from control, aortic-banded, and myocardial-infarcted (MI) rat hearts. MLP shows 4 main bands. Note that the gel has been cut to allow the differences between the control and diseased hearts to be better visualized. In the aortic-banded animals, there is a reduction in the ~40-kDa (oligomer) and a subsequent increase in the 20-kDa (monomeric) form of MLP. C: quantification of the 20-kDa monomer (nuclear MLP), which is increased in aortic-banded rat hearts. D: quantification of cytoplasmic MLP (a sum of all the oligomers, bands 2-4), which is significantly decreased in the aortic-banded hearts. E: ratio of nuclear to cytoplasmic MLP, which is significantly increased after aortic banding. F: total MLP (measured separately under denaturing conditions) in these hearts, which is significantly increased after aortic banding. G-J: similar analyses of MLP in rat hearts after myocardial infarction. G: quantification of the 20-kDa nuclear monomer, which is significantly increased after myocardial infarction. H: quantification of cytoplasmic MLP (a sum of all the oligomers), which is significantly decreased in the diseased hearts. I: ratio of nuclear to cytoplasmic MLP, which is significantly increased in MI. J: total MLP, which is also significantly increased after MI.

*Controls vs. aortic banded, P < 0.05. **Controls vs. aortic banded, P < 0.01. #Controls vs. MI, P < 0.05. ##Controls vs. MI, P < 0.01.
After MLP oligomers were identified, non-denaturing conditions (no heat or reducing agents) were used in Western immunoblot analysis of hearts from control rats, aortic-banded rats, and rats with myocardial infarction (Fig. 4). MLP bands identical to those observed in the cultured myocytes were found and assumed to be monomeric, dimeric, trimeric, and tetrameric forms. MLP appears mostly as 20- and 40-kDa bands, with much smaller amounts at 60 and 80 kDa. Figure 4B shows nonsaturated blots of MLP, showing all four forms following aortic constriction and myocardial infarction. In aortic-banded hearts, there was a decline in the dimeric form (corresponding to membrane bound in cultured myocytes) and an increase in the monomeric MLP (the nuclear form) compared with sham controls. Quantification shows a doubling of the nuclear MLP and a concomitant decrease in the dimeric nonnuclear form compared with control animals. The ratio of nuclear to nonnuclear MLP showed a threefold increase in the failing hearts. These results indicate that hypertrophy does not prevent MLP oligomerization and render it nonfunctional. Compared with the endogenous protein, overexpressed, flagged adenoviral MLP did not locate to the nucleus (Fig. 6, A–C). This experiment was also repeated in mouse myocytes to ensure that the effects were not due to species differences, and

COOH terminus of MLP is required for oligomerization and stretch sensing. To determine the functional role of nuclear MLP and its oligomeric forms, neonatal rat myocytes were infected with adenovirus expressing mouse MLP with or without a COOH-terminal flag. We hypothesized that the flag would prevent MLP oligomerization and render it nonfunctional. Compared with the endogenous protein, overexpressed, flagged adenoviral MLP did not locate to the nucleus (Fig. 6, A–C). This experiment was also repeated in mouse myocytes to ensure that the effects were not due to species differences, and

Various Western blotting conditions (Fig. 5). Samples were run under various non-denaturing and denaturing conditions (no reducing agents or heat). The four main bands of MLP could be seen in normal hearts but were barely discernable in the failing hearts. The tissue was also analyzed under reducing conditions (with β-mercaptoethanol) and/or heat. When heart samples were treated only with β-mercaptoethanol and without heat, the trimeric and tetrameric forms of the protein were no longer visible, although a faint dimeric MLP band was still discernable. When the same tissue was treated with β-mercaptoethanol and heat, the oligomeric forms of the protein were no longer visible with only the monomer remaining. These results indicate that, when samples are prepared from whole tissue, oligomeric forms of MLP are not resistant to denaturing conditions. Denaturing conditions produce only monomeric MLP, explaining why oligomeric MLP has not been previously reported. This new methodology also allows changes in subcellular MLP to be measured in whole human heart tissue without biochemical fractionation. Total MLP was also measured by denaturing conditions in normal and failing human hearts (Fig. 5C). Total MLP was increased twofold in the failing hearts.

**H265**

**Fig. 5.** MLP from nonfailing and failing human hearts examined under different electrophoretic conditions. A: samples run under non-denaturing conditions (no reducing agents or heat). Lanes 1 and 2, nonfailing hearts; lanes 3 and 4, failing hearts. The failing hearts have little oligomeric MLP compared with the nonfailing. B: effect of the reducing agent β-mercaptoethanol and heat on the same tissue samples. In lanes 1–4, the 4 samples have been treated only with β-mercaptoethanol and without heat. In lanes 5–8, the same samples have been treated with β-mercaptoethanol and heat. C: quantitation of total MLP in failing and nonfailing human hearts measured under denaturing conditions. Results are from 4 nonfailing and 7 failing hearts. There is a significant increase in total MLP in failing human hearts. *Failing vs. nonfailing, P < 0.05.
the same results were observed (data not shown). The flagged MLP was also insensitive to mechanical stimulation and remained extranuclear even after 48 h of cyclic stretch at 10% maximum strain. Figure 6L shows total MLP from control neonatal rat myocytes and from those infected with either flagged or nonflagged viral MLP. Total MLP is significantly increased with infection from both viruses. The nonflagged MLP also resulted in more than a twofold increase in S6 ribosomal protein, but no change was observed with the flagged protein. This demonstrates that MLP can directly activate ribosomal protein synthesis. The COOH-terminal flag appears to render MLP nonfunctional. Subcellular fractionation of myocytes infected with the tagged viral MLP showed that the protein was only expressed in the cytosolic fraction and did not oligomerize. Thus the COOH terminus of MLP is required for oligomerization and for mechanosensing.

**DISCUSSION**

Mutations in human MLP or its deletion in transgenic mice result in cardiomyopathy (14, 22, 25, 29). However, the regulation of the intracellular distribution of the protein in myocytes has never been examined. In this paper, we report novel findings that provide insight into the mechanism of the hypertrophic and remodeling processes in the heart. Using an improved method for subcellular fractionation in which cell integrity is preserved, we show the subcellular distribution of MLP in myocytes, mainly in the nucleus, membrane, and cytoskeleton.

In addition to the 20-kDa monomeric form, MLP protein exhibits oligomerization forming dimers, trimers, and tetramers. The monomeric form of MLP is only found in the nucleus of cultured myocytes, whereas all nonnuclear forms are oligomeric. Using this new information, we have measured the nuclear distribution of MLP in aortic-banded and myocardially infarcted rat hearts. These diseased hearts show a significant accumulation of monomeric MLP in the nucleus and a concomitant decrease in the oligomeric forms in the other cell compartments. There is also a compensatory increase in total MLP in these hearts. Both of these animal models represent models of heart failure. Previously, in a model of right ventricular heart failure, it was suggested that nuclear MLP may be increased; however, only the monomeric form of MLP was measured (10). An important finding in our study is that the increase in nuclear MLP is associated with a concomitant decline in the amounts of the protein elsewhere in the cell.

In a previous study, it was suggested that MLP may dimerize (1), but the full extent of its oligomerization and their subcellular distribution had not been realized. Some MLP oligomers may provide a structural role in the myofilaments as in skeletal muscle (3), whereas others may be part of a mechanosensing mechanism. The reduction in MLP outside the nucleus in disease could have important implications for mechanosensing because forces are transmitted at the costamere. Our data here suggest that the loss of nonnuclear MLP may be part of a “declining sensor” phenomenon, as illustrated in Fig. 7. This is consistent with previous suggestions that impaired myocyte function may be initiated by a loss of mechanosensing (11). Continuous long-term stimulation may eventually result in decreased mechanosensing because messengers like MLP become confined to the nucleus. Because both the membrane and cytoskeleton can experience and transmit cellular forces, a reduction in MLP in those locations would be detrimental. Such desensitization is a biological phenomenon also seen in other systems such as the downregulation of β-adrenergic receptors after prolonged catecholamine stimulation (15) and insulin resistance that develops after prolonged high dietary...
carbohydrate consumption (21). This hypothesis may also help explain the "transition to failure," in which prolonged compensatory hypertrophy eventually leads to failure. The transition to failure may occur when the decline of sensing proteins in the cytoskeleton and membrane regions become limiting. The increase in total cellular MLP may occur as the cell attempts to restore the reduced fraction outside the nucleus. However, even with this increase, there is still a significant reduction in the nonnuclear pool of MLP in diseased hearts. The increase in total cardiac MLP in disease has been recently shown to be associated with reduced ejection fraction (33). In failing human hearts, the oligomeric forms of MLP was almost completely absent. The differences in the distribution and amounts of oligomeric MLP between the two animal models and the explanted human hearts may reflect differences in the severity of heart failure. It could also suggest that the two animal models do not completely recapitulate the nonspecific and inherited forms of human heart failure. It is probable that MLP is one of a number of proteins that cycle to the nucleus in response to mechanical activity, relaying information to the nucleus. We propose that the changes to MLP may be indicative of a general phenomenon within myocytes in response to prolonged overload. The effects on MLP appear to be similar regardless of the cause of cardiac dysfunction.

Our data surprisingly showed that there was very little MLP in the cytosol of cultured neonatal myocytes. All nonnuclear MLP was oligomeric, bound within the membrane, or in the cytoskeleton. A number of laboratories have reported that overexpression of MLP-green fluorescence protein fusions in culture results in significant myocyte death (2, 17). Ectopic expression of MLP produces large amounts of the protein in the cytosol where it is not usually found. Our results show that the COOH terminus of MLP is necessary for oligomerization because the flagged protein remained monomeric and did not translocate to the nucleus when cells were stretched. The NH2 terminus of MLP has been shown to interact with the titin-binding protein telethonin as part of the mechanosensing mechanism in myocytes (22). However, because the COOH-terminal-blocked MLP remained monomeric and cytosolic, it is likely that oligomerization of the protein precedes its incorporation into the myofilaments. This would also suggest that the loss of oligomeric MLP following cardiac disease would result in the loss of mechanosensing.

The translocation of MLP to the nucleolus was associated with a large increase in S6 ribosomal protein after 10% cyclic stretch, and adenovirally overexpressed native MLP increased this synthesis. This indicates that MLP can activate ribosomal protein synthesis in the nucleolus. The synthesis of additional ribosomes is known to be an essential requirement for myocyte hypertrophy by a previously unknown mechanism (16). MLP may act as a direct link between mechanical activity and subsequent protein synthesis, in part through activation of ribosome transcription in the nucleolus. The synthesis of ribosomes is a complex process (12), and how MLP activates this process within the nucleolus remains to be elucidated.

In summary, we have shown for the first time that MLP translocates to the nucleolus in response to cyclic stretch where it activates ribosomal protein synthesis. MLP exhibits oligomerization, a process that involves the COOH terminus of the protein. Oligomerization also appears to be a prerequisite to mechanosensing. In failing human hearts, infarcted rat hearts, and aortic-banded rat hearts, MLP accumulates in the nucleus as a monomer with a concomitant decline in the cytoplasmic oligomeric forms. Based on these localization studies, we suggest that a significant decline in peripheral oligomeric MLP may contribute to the pathogenesis of heart failure. These results indicate that the reduction in nonnuclear MLP may be part of a "declining sensor" phenomenon within the heart after prolonged stress. We suggest that long-term stimulation and stress on the heart leads to a downregulation of mechanosensing due to nuclear accumulation of nucleocytoplasmic shuttling proteins like MLP. Therefore, reduced oligomeric MLP in the costamere and cytoskeleton may contribute to impaired mechanosensing in heart failure.

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