Original Article

Mechanism of Incorporation of Newly Synthesized Myosin Alkali Light Chain into Myofibril

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ABSTRACT

Objectives: The present work was designed to study the incorporation of newly synthesized myosin alkali light chain (MLC) molecules into myofibris.

Materials and Methods: cDNA of fast skeletal muscle type of MLC tagged with green fluorescence protein (LC3f-GFP) was transfected into cultured chicken cardiomyocytes, and the assembly of expressed LC3f-GFP was observed in living cells under a fluorescence microscope equipped with a cooled CCD camera.

Results: At 14-16 hours after transfection, LC3f-GFP was diffusely distributed in the cytoplasm of cardiomyocytes. In some cells, however, intense fluorescence spots of LC3f-GFP were found along myofibrils with a periodically of 1.2 µm. Confocal microscopy of such cells, stained with rhodamine-labeled phalloidin, revealed the fluorescence spots of LC3f-GFP localized at both ends of A-bond. When these cells were further incubated, LC3f-GFP came to be localized at all levels of the A-bands by 26 hours after transfection.

Conclusion: These results indicate that myosin filaments are not replaced with newly synthesized myosin molecules at once along their length, but molecules in filaments are replaced individually from their ends.

Keywords: Myosin light chain, isoform, epitope-tag, cDNA transfection, immunofluorescence confocal microscopy

INTRODUCTION

Recently, rapid advances in the principles and basic tools of molecular biology have helped to unveil the structure and regulatory mechanism of genes. Intra cellular fine structures and their molecular characteristics have also been clarified by various morphological techniques. However, the mechanism and dynamics of formation of functional structures from proteins, synthesized from genetic information, have not received much attention.

Myofibril is formed by regular arrangement of various proteins. It is therefore an ideal model structure for studying the above mentioned theme. Myosin is a major contractile protein found in all muscle cells and in most non muscle cells as well [1]. It is represented by a multigene super family, composed of 12 different classes [2]. The myosin II class (conventional myosin), is the best studied class of myosin and represents myosins from the three muscle types, skeletal, cardiac and smooth muscle, as well as non muscle isoforms. Conventional myosin is a hexamer composed of two pairs of myosin heavy chain (MyHC) essential or alkali light chain (LC) and regulatory light chain[3]. A molecule of MyHC consists of a globular head joined to a long rod-like tail, and one copy of each of the light chains associates with MyHC near the head-rod junction, also known as myosin neck [4, 5]. At the myosin neck, the light chains are arranged in tandem with the LC more proximal to the head and wrap around an 8.5 nm long, hydrophobic α-helix that extends from the C-terminus of the globular head of MyHC [6, 7]. A long alpha-helix in the myosin head constitutes a lever arm together with the light chains. This is important for the efficient motility of myosin [8]. The light chains are thought to provide rigidity to the neck, and thereby to enforce the power stroke generated during actomyosin interaction [9, 10, 11]. Further, phosphorylation of the regulatory light chain triggers the activation of the contractile apparatus of vertebrate smooth muscle and nonmuscle cells [12,13] and plays a modulatory role in striated muscle by altering the calcium sensitivity of force production [14,15]. Similarly, phosphorylation of MLC is a critical biochemical determinant of cellular contraction. It facilitates the aqueous outflow in trabecular meshwork cells [16]. Saszi et al [17] have brought evidence to suggest that myosin based contractility plays an important role in the regulation of epithelial function, particularly paracellular permeability. This appears to be correlated by the report of Russo et al [18], who noted that small epithelial wounds heal by purse-string contraction of an actomyosin ring that is also regulated by myosin light chain. Extensive variation exists in MLC isoform expression in mammalian skeletal muscles fibers. There

are distinct patterns among different species and among the muscles with in an individual species [19].

The contribution of the regulatory light chain to myosin's enzymatic activity ahs been well studied. Evidence suggests that myosin II is activated by regulatory light chain (RLC) phosphorylation. Inactivation of myosin results from RLC dephosphorylation by MLC phosphates containing a myosin phosphatase targeting submit MYPT1 [20]. The role of LC is less well understood except for its mechanical functions. Accumulating evidence suggests however, that distinct isoforms of LC are presented in different muscles and in nonmuscle cells. These isoforms may therefore be associated with different contractile properties of these tissues [21]. This is corroborated by the well established fact that mutations in specific structural elements of the motor protein myosin are directly linked to debilitating diseases involving malfunctioning striated muscle cells [22]. The present study was undertaken to elucidate the mechanism of incorporation of newly synthesized myosin alkali light chain into myofibrils.

MATERIALS AND METHODS

Construction of LC Expression Plasmids.

Full length cDNA clones encoding the chicken LC3f [23] were used in this study. The epitope-tag encoding the 11 carboxy-terminal amino acids of vesicular stomatits virus (VSV) glycoprotein [24] was introduced into the 3' end of the coding sequence of these cDNAs. The cDNA were then subcloned into the eukaryotic expression vector pSCT, as described by Soldati and Perriard [25].

Construction of GFP-tagged (LC3f-GFP)

The 3' end of the coding sequence of the VSV-tagged LC3f cDNA was modified by PCR to remove stop codon and to introduce the ***BamHI*** site. The PCR product was subcloned into the GFP expression plasmin pEGFP-N1 (Clontech lab. Inc.) using the ***BanHI*** site in the multiple cloning sites of the plasmid.

Cells Cultures:

Culture of 7 day's old chicken cardiomyocytes was prepared as described previously [26]. Cells dissociated by trypsinization were seeded at a concentration of 2 x 105 cells in 1. 5 ml of culture medium in 35 mm dishes. The medium consisted of 75% potassium-free balanced solution (116mM NaCl, 0.8mM MgSO4, 0.9 mM CaCl2, 26.2 mM NaHCO3, 5.5 mM dextrose, pH 7.3), 20% Medium 199 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), 4% horse serum (Life Technologies, Inc., Grand Island, NY, USA) and 1 % penicillin-streptomycin mixture. Cells were maintained at 37 ^{o}C in an atmosphere of 5 % CO2 in air at saturation humidity.

DNA Transfection:

DNA of LC constructs was prepared by the clear lysate technique and banded on CsCl gradients. Cells were allowed to grow for 20 – 24 hours before transfection of LC constructs. For transfection of chicken cardiomyocytes, LipofectAMINE (life technologies, Inc.) was used. Vector DNA (2µg) and 10µl of lipofectAMINE (2mg/ml in membrane filtered water) were mixed in 200µl of Opti-MEM 1 (Life Technologies, Inc.) and incubated for 40 minutes to allow DNA-liposome complexes to form. Just prior to the transfection the cells were rinsed twice with culture medium, and then the solution was replaced by tha DNA – liposome complex solution. After 14-16 hours of incubation, the cells were rinsed with the culture medium.

Microscopy of LC3f-GFP:

Cells expressing LC3f-GFP were observed under a Zeiss Axiovert 100TV equipped with a cooled CCD camera (PXL 1400, photometrics, Tuscon, AZ, USA) using a Zeiss Plan-Neofluar (100x/1.3) objective lens. Images were assembled using IPLab spectrum software (Signal Analytics, Vienna, VA, USA). Cells transfected with LC3f-GFP were rinsed briefly with phosphate buffered saline (PBS) and then fixed with 3% paraformaldehyde in PBS for 10-15 minutes. After a brief rinse with PBS and culture medium, the cells were stained with rhodamine-labeled phalloidin, and examined under a Zeiss LSM 410 invert confocal laser scanning microscope equipped with helium/neon and argon/krypton lasers using a Zeiss plan Apochromat (63x/1.4) objective lens. Projection views were generated from sets of consecutive optical sections taken through the whole depth of cells at intervals of 0.7µm using of Zeiss LSM 410 software.

RESULTS

In cells where only GFP was expressed it was diffusely distributed throughout the cytoplasm. Prolonged incubation did not affect the distribution within the cells.

Incorporation of LC3f-GFP into Myofibrils

In order to study how newly formed LC molecules are incorporated into myofibrils, LC3f-GFP was expressed in cultured chicken cardiomyocytes, in the present study and its dynamics was monitored in living cells.

When only GFP was expressed, it was always diffusely distributed throughout the cytoplasm. Even after prolonged incubation of cells, it was still not incorporated into myofibrils. When LC3f-GFP was transfected, it was diffusely distributed in the cytoplasm of cardiomyocytes at 14-16 hours after transfection. At higher magnification, intense fluorescence bands of

LC3f-GFP were found along myofibrils with a periodicity of 1.3µm. The width of each band was approximately 0.4 µm, and the distance from the edge of one band to the edge of the neighboring band was 1.6 um which was equivalent to the width of the A-band. When these cells were further incubated, fluorescence of LC3f-GFP in the cytoplasm was reduced and its band patterns became more obvious after 26 hours of transfection. Each fluorescence band had broadened to 0.7µm, but the distance between the edges of two adjacent bands was unchanged.

When cells were fixed and stained with rhodamine-labeled phalloidin at early stages of LC3f-GFP expression, periodically aligned fluorescence spots (or bands) of LC3f-GFP were localized around the center of phalloidin-positive actin bands.

DISCUSSION

Dynamics of myoprotein assembly onto myofibrils has been studied by using microinjection of fluorescently labeled proteins like actin [27, 28, 29], α actinin [30, 31], and myosin [32, 33]. Green fluorescence protein (GFP) has emerged as a strong reporter molecule for monitoring gene expression [34], protein dynamics [38] and protein-protein interaction [35, 36, 37, 38]. Since intracellularly expressed GFP is visible under a fluorescence microscope without fixation or staining of the cells. It has been demonstrated that GFP fused to the C-terminus of the LC does not interfere with the localization and function of the LC [39].

In the present study, it has been observed that LC3f-GFP expressed in chicken embryonic cardiomyocytes was initially diffusely distributed in the cytoplasm and/or arranged in periodic bands along myofibrils at early staged, and then localized at all levels of the A-bands later. Because GFP itself could not be incorporated into myofibrils, it seems reasonable to suggest that changes of the LC3f-GFP localization might reflect the dynamics of LC3f in the cells.

Confocal microscopy of fixed cells stained with rhodamine-phalloidin at early stages revealed that LC3f-GFP was localized around the center of actin bands. This position corresponds to the Z-line, or to the ends of A-bands. It is well known that contraction of myofibrils usually occurs during fixation of muscle cells [36]. Judging from these results and the data obtained by measurement of the LC3f-GFP bands, the position of the periodic LC3f-GFP bands observed in living cells at early stages seems to correspond to the end of the A-band. Therefore, the present results indicate that myosin filaments are not replaced with newly synthesized myosin molecules all at once along their entire length. Molecules in the filaments are not replaced individually from their ends. This is consistent with the fact that there is greater exchange of new MyHC at the ends than in the central region of the hick filament [40]. However, it still remains to be resolved whether nascent LC molecules can directly replace molecules in the thick filaments or associate with newly synthesized MyHC molecules prior to their assembly into thick filaments.

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