Dynamics of myosin replacement in skeletal muscle cells

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Submitted 12 June 2015; accepted in final form 6 September 2015

Ojima K, Ichimura E, Yasukawa Y, Wakamatsu JI, Nishimura T. Dynamics of myosin replacement in skeletal muscle cells. Am J Physiol Cell Physiol 309: C669–C679, 2015. First published September 16, 2015; doi:10.1152/ajpcell.00170.2015.—Highly organized thick filaments in skeletal muscle cells are formed from ~300 myosin molecules. Each thick-filament-associated myosin molecule is thought to be constantly exchanged. However, the mechanism of myosin replacement remains unclear, as does the source of myosin for substitution. Here, we investigated the dynamics of myosin exchange in the myofibrils of cultured myotubes by fluorescent recovery after photobleaching and found that myofibrillar myosin is actively replaced with an exchange half-life of ~3 h. Myosin replacement was not disrupted by the absence of the microtubule system or by actomyosin interactions, suggesting that known cytoskeletal systems are dispensable for myosin substitution. Intriguingly, myosin replacement was independent of myosin binding protein C, which links myosin molecules together to form thick filaments. This implies that an individual myosin molecule rather than a thick filament functions as an exchange unit. Furthermore, the myosin substitution rate was decreased by the inhibition of protein synthesis, suggesting that newly synthesized myosin, as well as preexisting cytosolic myosin, contributes to myosin replacement in myofibrils. Notably, incorporation and release of myosin occurred simultaneously in myofibrils, but rapid myosin release from myofibrils was observed without protein synthesis. Collectively, our results indicate that myosin shuttles between myofibrils and the nonmyofibrillar cytosol to maintain a dynamic equilibrium in skeletal muscle cells.

fluorescence recovery after photobleaching; myosin; myofibril; skeletal muscle; thick filament

THE MYOSINS COMPRISE A LARGE superfamily of motor proteins that play fundamental roles in various cellular functions, including cytokinesis, endocytosis, vesicle transportation, stress fiber contractility, and so on (8). A total of 40 myosin genes are found in the human, which encode 12 distinct classes of myosin (3, 8). The conventional class II myosin is defined as muscle-isolated myosin and generates contractile forces via interactions with actin-containing filaments in muscles. A single myosin molecule consists of dimerized myosin heavy chains (Myh)s and two pairs of myosin light chains, termed the regulatory and essential light chains (58). Myosin can be proteolytically divided into three major pieces: subfragment 1 (S1), subfragment 2 (S2), and light meromyosin (LMM). S1 is also referred to as the globular head and exhibits actin-binding capability and ATPase activity.

The myosin rod contains the S2 and LMM fragments. Both S2 and LMM are composed of α-helical coiled-coil structures. LMM is essential for the formation of myosin filaments or paracrystals in in vitro test tube assays (51) and also for the formation of thick filaments in muscle cells (6, 39). The self-assembling property of LMM is attributed to the presence of the assembly competence domain, which is, in turn, made up of 28 amino acid residues at the LMM COOH-terminus (45, 49, 52). Extensive study on skeletal muscle myosin reveals that LMM also determines myosin solubility. Myosin was originally extracted from skeletal muscle tissues by using salt solutions with a high ionic strength (e.g., 0.6 M KCl), but myosin is scarcely solubilized by solutions at a physiological ionic strength (42). Due to these solubility properties, LMM-containing myosin fragments aggregate and/or form filaments in vitro test tube assays employing solutions at physiological ionic strength (2, 35). Although myosin forms thick filaments in skeletal muscle cells, it remains unclear how myosin exists in the nonmyofibrillar cytoplasm, which likewise is a solution of physiological ionic strength.

A bipolar thick filament in skeletal muscle myofibrils contains ~300 myosin molecules (5, 31). In developing skeletal muscle cells, the thick filaments assemble independently of the thin filaments (20, 38). Precursors of thin-filament/Z-band, I-Z-I bodies, are irregularly shaped aggregates, and the longitudinally aligned I-Z-I bodies are termed stress fiberlike structures (SFLSs) (1). These SFLSs interact with preformed 1.6-μm bipolar thick filaments to form nonstriated myofibrils that are often observed in the growth tips of myotubes (20, 38). Alternatively, SFLSs are transformed, with thick filaments being added gradually. Even myosin is gradually incorporated into SFLSs to form nonstriated myofibrils (17, 57). Finally, the I-Z-I bodies are reconfigured into mature square-lattice Z bands, and the thick filaments are translocated between the Z bands to form definitive striated myofibrils (9, 20, 38). During sarcomere formation, the size of the thick filament is kept constant, while I-Z-I bodies rearrange their shape and size. Myosin molecules are packed together into thick filaments by interactions with myosin binding protein C (Mybpc; found at multiple sites along the filament) and myomesin (Myom; located at the center of the thick filament, in the M line). The thick filaments retain their location in the mature sarcomere through direct interactions with the giant elastic protein, termed connectin/titin, and also through indirect interactions with connectin/titin via Mybpc and Myom (15, 21, 28).

Skeletal muscle cells are highly capable of altering their sizes, leading to hypertrophy and atrophy of the muscle mass. Under conditions of hypertrophy and atrophy, individual skeletal muscle fibers must regulate the number of constituent myofibrils. Even during retention of muscle mass, the number of myofibrils is constantly maintained, but each myofibrillar protein must be exchanged. Nevertheless, we do not fully understand how myofibrillar protein components in each sarcomere are remodeled by newly synthesized or presynthesized...
proteins in living muscle cells, without any functional damage to the muscle.

Here, we focused on the replacement of enhanced green fluorescence protein (eGFP)-tagged Myh in the myofibrils of cultured skeletal muscle cells by using fluorescence recovery after photobleaching (FRAP) to reveal myosin substitution dynamics. Our data show that myosin is actively exchanged in myofibrils, and that myosin incorporation into and release from myofibrils occur simultaneously. Furthermore, myosin substitution dynamics are affected by the amount of preexisting myosin in the non-myofibrillar cytosol, as well as the amount of newly synthesized myosin. Taken together, our findings shed new light on the dynamic equilibrium of myosin between myofibrils and the non-myofibrillar cytosol.

MATERIALS AND METHODS

Cell culture and transfection. Experimental animals were cared for as outlined in the Guide for the Care and Use of Experimental Animals (Animal Care Committees of the Hokkaido University and the NARO Institute of Livestock and Grassland Science), which the committee accepted. Primary chick skeletal muscle cells were isolated from day 11 chick embryonic pectoral muscles and cultured on Matrigel-coated (BD Bioscience) Labtech chamber slides (Nalge Nunc International) for immunofluorescence staining, or on collagen-coated glass bottom dishes (Matsunami Glass Ind.) for FRAP experiments. The growth medium [10% chick embryo extract and 10% horse serum in minimum essential medium (all from Life Technologies)] was shifted to differentiation medium (1.5% chick embryo extract and 5% horse serum in minimum essential medium) to induce muscle differentiation on the next day of transfection. Growth and differentiation media were supplemented with 100 U/ml penicillin and 0.1 mg/ml streptomycin (Life Technologies). Transfection was performed by using LipoFectamine LTX and Plus reagents on the day after cell plating (Life Technologies).

To block the polymerization of microtubules, nocodazole (Sigma) was added to the culture medium at a final concentration of 2 μM (34). The actomyosin interaction was inhibited by treating the cells with N-benzyl-p-toluene sulphonamide (BTS; Tokyo Chemical Industry) at a final concentration of 30 μM (26), and protein synthesis was prevented by treating the cells with cycloheximide (CX; Wako Pure Chemical Industries) at a final concentration of 10 μM (14). Chemical agents were added to the medium at 1 h (nocodazole, BTS, and CX) or 10 h (CX) before the initiation of experiments and, in the case of the FRAP assays, maintained in the medium throughout the experiments. Dimethyl sulfoxide (DMSO) was added to the medium as vehicle controls.

cDNA constructs. Full-length mouse Myh3 cDNA was cloned by a polymerase chain reaction-based method, as described previously (39). In brief, cDNA corresponding to mouse Myh3 (45–5868 in NM_001099635) contained in the pcDNA3.1/N-FLAG vector was subcloned into a pcEGFP vector (Clontech) or a humanized monomeric Kikume green-red 1 vector (KikGR1; Medical and Biological Laboratories). The cDNAs corresponding to mouse Mybp1 (228–3611 in NM_001252372) and mouse Myom3 (111–4430 in NM_001085509) were cloned from first-strand cDNA, which was prepared from mouse myotube mRNA on day 8 of culture, and then subcloned into a pmCherry vector (Clontech). Polymerase chain reaction primers for Mybp1 and Myom3 were as follows: Mybp1, 5′-tttgattct ATGC-CAGAACCCACTAAG-3′ and 5′-tttgattct TTACGACCTGTGCT-GCCC-3′; and Myom3, 5′-tttgattct ATGACTCTGCCC-CAGCCC-3′ and 5′-tttgattct TTACTCTTTCTTCAGCTCC-3′. All generated fluorescent proteins were tagged at the NH2-terminus of the target proteins, and all constructs were verified by sequencing with a 3730 DNA Analyzer (Applied Biosystems).

FRAP assays and photoconversion experiments. At 7 days postdifferentiation, skeletal muscle cells transfected with an expression vector encoding an eGFP + Myh3 fusion protein were subjected to FRAP analysis with a TCS-S5 confocal Laser Scanning Microscope (Leica). Doubly transfected skeletal muscle cells carrying the eGFP + Myh3 expression vector and a vector encoding either a mCherry + Myom3 or mCherry + Mybp1 fusion protein were also used for FRAP assays. The regions of interest were bleached for 30 s, and the FRAP data were recorded at 1-h intervals by using Leica Confocal Software. The relative fluorescence intensity was calculated as the ratio of the fluorescence intensity of the bleached area to the fluorescence intensity of the unbleached area at each time point. For the calculations, a curve-fitting method was used to extract the mobile fraction (Fr) and the half-life (t1/2) from the FRAP curve, as previously reported (55). ImageJ 1.46r (National Institutes of Health) was used to fit the data to the following equation: FI = Fr [1 – e(t/t1/2)] + c, where FI is the relative fluorescence intensity of the bleached area at time t, Fr is of the exponential process with rate constant b, and c is relative fluorescence intensity of the bleached area at time 0.

KikGR1 is green fluorescent protein that can be irreversibly converted to a red color following irradiation with ultraviolet (UV) light (53). KikGR1 + Myh3 fusion protein was utilized for a green-to-red photoconversion analysis with a TCS-S5 Confocal Microscope. The regions of interest were photoactivated after UV irradiation, and images were recorded at 1-h intervals. The relative green fluorescence intensity was calculated as the ratio of the green fluorescence intensity at each time point relative to that of the green fluorescence intensity pre-photoconversion. To correct these intensities, the relative green fluorescence intensity pre-photoconversion was defined as 1, and the relative green fluorescence intensity just after photoconversion was defined as 0. The relative red fluorescence intensity was similarly calculated as the ratio of the red fluorescence intensity at each time point relative to that of the red fluorescence intensity immediately after photoconversion. To correct these intensities, the relative red fluorescence intensity just after photoconversion was defined as 1, and the relative red fluorescence intensity just after photoconversion in the non-photoconverted area was defined as 0. The Fr and t1/2 for photoconversion were calculated as described above. All FRAP and photoconversion analyses were carried out by using a microscope stage top incubator (Tokai Hit) to control the temperature, humidity, and CO2 concentration.

Immunofluorescence staining. The staining protocol has been described in detail by Ojima et al. (40). The antibodies used in the immunofluorescence assays were as follows: primary mouse anti-sarcomeric α-actinin antibody (1:1,000 dilution; clone GA5; Sigma), primary mouse anti-Myh antibody (1:100 dilution; clone F59; Developmental Studies Hybridoma Bank, University of Iowa) (33), primary mouse anti-Myom antibody (1:50 dilution; clone B4; Developmental Studies Hybridoma Bank, University of Iowa) (16), and Alexa-555-conjugated secondary antibodies (Life Technologies). Specimens were stored with mounting media (Vector Laboratories), and subsequently analyzed by using a LSM 700 Confocal Laser Scanning Microscope (Carl Zeiss,) equipped with a Plan-Apochromat ×63 (numerical aperture 1.4) lens. Images were manipulated by using Zen 2012 imaging software (Carl Zeiss.).

Preparation of cytosolic myosin. The cytosolic fraction was isolated as described by Isaacs and Fulton (23), with slight modifications. Briefly, the cytosolic fraction from cultured skeletal muscle cells was collected as follows. First, day 6–7 cultured chick muscle cells were immersed in lysis buffer [10 mM Tris-HCl (pH 7.5), 0.15 M CsCl, 1 mM EDTA-Cs, and 0.5% (vol/vol) Triton X-100] containing protease inhibitors (28 μM E64, 1.5 μM aprotonin, 50 μM leupeptin, 40 μM bestatin, 0.7 μM calpastatin, and 2 mM phenylmethylsulfonyl fluoride) for 30 min at 4°C. The lysate was centrifuged at 1,500 g for 10 min to remove cellular debris. The supernatant fraction was further centrifuged at 100,000 g for 1 h. The protein concentration of the centrifuged supernatant was
quantified with a Bradford protein assay kit (Bio-Rad Laboratories). Specimens were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then stained with SyproRuby (Bio-Rad Laboratories). Gels were scanned by using an Ettan DIGE Imager (GE Healthcare UK).

**Immunoblot analysis.** Specimens were subjected to SDS-PAGE, as described above, followed by immunoblot analysis. After gels were transferred to Immobilon-P transfer membranes (Millipore), the membranes were blocked with 5% nonfat skim milk (Nacalai Tesque) and incubated with anti-myosin antibody (1:1,000 dilution; clone MF20; Developmental Studies Hybridoma Bank, University of Iowa) (48), anti-GFP antibody (1:2,000 dilution; clone GF090R; Nacalai Tesque), anti-GAPDH antibody (1:2,000 dilution; clone 3H12, Medical and Biological Laboratories) or anti-red fluorescence protein antibody cocktail (1:1,000 dilution; mixture of clones IG9 and 3B5; Medical and Biological Laboratories). Next, the membranes were incubated with peroxidase-conjugated secondary antibodies (1:10 dilution; Nichirei), and the immunoreactive bands were visualized by using the Pierce enhanced chemiluminescence-Plus Western Blotting Substrate (Thermo Fisher Scientific). Band intensities were measured with Image-J1.46r (National Institutes of Health).

**RESULTS**

The fusion protein, eGFP + Myh3, is actively replaced in myofibrils. A single thick filament in each sarcomere of striated muscles contains ~300 myosin molecules (5, 31). We queried whether the myosin molecules in thick filaments are replaced in cultured skeletal muscle cells. To do this, eGFP-tagged Myh3 was exogenously expressed in cultured chick skeletal muscle cells. Consequently, the eGFP + Myh3 fusion protein was precisely incorporated into the A bands or thick filaments where eGFP signals were located between sarcomeric actinin-positive Z bands (Fig. 1, A–F, J, and K). Myom, a myosin-associated protein, was located in the M-lines of the eGFP-positive thick filaments (Fig. 1, G–I and L), indicating that exogenously expressed eGFP + Myh3 can properly form thick filaments. We also verified the molecular sizes of the exog-

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**Fig. 1.** Localization of endogenous myofibrillar proteins and exogenously expressed enhanced green fluorescence protein (eGFP) + myosin heavy chain 3 (Myh3). A–F: day 7-cultured myotubes were stained with anti-sarcomeric actinin antibody (A), anti-Myh antibody (D), and anti-myomesin (Myom) antibody (G). Images of exogenously expressed eGFP + Myh3 in B, E, and H were superimposed onto the images in A, D, and G to yield the images in C, F, and I, respectively. Sarcomeric α-actinin-positive Z bands are indicated by the white arrowheads in A and C. Central bare zones that were negative for Myh signals are denoted by the white arrowheads in D and F. Myom signals detected in the M-lines are indicated by the white arrowheads in G and I. Central bare zones of eGFP-positive thick filaments are indicated by the black arrowheads in B, C, E, F, H, and I. Bars, 5 μm. J–L: representative line-scan profiles for the fluorescence images are shown to demonstrate the variation of fluorescence intensity for each marker along the myofibrils. The x- and y-axes signify the length of the myofibrils (μm) and the fluorescence intensity (arbitrary units), respectively. M–O: expression levels of exogenous eGFP + Myh3 fusion proteins and endogenous Myh in cultured muscle cells were evaluated by immunoblotting. Exogenously expressed eGFP + Myh3 (white arrowhead) and eGFP (arrow) were detected with anti-GFP antibody (M), while endogenous Myh was detected with anti-Myh antibody (arrowhead; N). O: anti-GAPDH antibody was used to show loading controls.
Table 1. Mobile fractions and t₁/₂ values of eGFP + Myh3 in cultured myotubes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mobile Fraction, %</th>
<th>t₁/₂, h</th>
<th>n</th>
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<tbody>
<tr>
<td>Control</td>
<td>50.21 ± 16.90</td>
<td>2.97 ± 0.40</td>
<td>7</td>
</tr>
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<td>Striated myofibrils</td>
<td></td>
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<tr>
<td>Nocodazole</td>
<td>33.85 ± 5.11</td>
<td>2.31 ± 0.79</td>
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<tr>
<td>BTS</td>
<td>47.39 ± 6.09</td>
<td>3.38 ± 0.71</td>
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<tr>
<td>Control</td>
<td>83.47 ± 3.99</td>
<td>0.43 ± 0.11</td>
<td>5</td>
</tr>
<tr>
<td>Nonstriated myofibrils</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nocodazole</td>
<td>75.31 ± 7.14</td>
<td>0.49 ± 0.10</td>
<td>4</td>
</tr>
<tr>
<td>BTS</td>
<td>89.55 ± 10.03</td>
<td>0.45 ± 0.22</td>
<td>3</td>
</tr>
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Values are means ± SE; n, no. of experiments. Mobile fractions and half-life (t₁/₂) values are used as indicators of enhanced green fluorescence protein (eGFP) + myosin heavy chain 3 (Myh3) mobility and eGFP + Myh3 recovery speed. In each treatment, significant differences between the mobile fractions and the t₁/₂ were observed for striated vs. nonstriated myofibrils (P < 0.05). Control, vehicle control cells; BTS, N-benzyl-p-toluene sulphonamide.


Fig. 2. Fluorescence recovery after photobleaching (FRAP) of eGFP + Myh3 in cultured myotubes. A and B: representative time course images of eGFP + Myh3 fluorescence recovery in striated myofibrils (A) and nonstriated myofibrils (B). Rectangles indicate the bleached areas. Insets in A show recovery of eGFP–stratified signals along myofibrils detected by line-mode scan in the bleached areas. The length and the intensity of the fluorescence are shown on the x-axis (μm) and the y-axis (arbitrary units), respectively. Bars, 10 μm. C: time course of the relative fluorescence recovery data. Fluorescence signals were measured at the indicated time points. Reference images were acquired before photobleaching (prebleaching). The signals were calculated as the ratio of the eGFP intensity at the indicated time point to that of the reference image, and exponential curves were fitted to the data (see MATERIALS AND METHODS). Note that the half-life (t₁/₂) values were significantly different between striated myofibrils and nonstriated myofibrils (Table 1). Striated myofibrils, n = 6; nonstriated myofibrils, n = 5. D and E: cells were treated with 2 μM nocodazole (D) or 30 μM N-benzyl-p-toluene sulphonamide (E) for 1 h, and FRAP analysis was then conducted in the presence of each reagent. The data were calculated as the ratio of the eGFP intensity at the indicated time points to that of the reference image, and exponential curves were fitted to the data. D: striated myofibrils, n = 4; nonstriated myofibrils, n = 4. E: striated myofibrils, n = 3; nonstriated myofibrils, n = 3. Values are means ± SE.

To investigate the dynamics of eGFP + Myh3 in living myotubes formed by skeletal muscle cells, we determined the exchange rate of eGFP + Myh3 in myofibrils by using the FRAP technique. The relative fluorescence intensity of Myh3 in both striated and nonstriated myotubes was similar to that in vehicle control myotubes (Fig. 2E). Therefore, the myosin ATPase activity or the actin-myosin interaction is not necessary for the replacement of eGFP + Myh3 in myofibrils (Table 1).

eGFP + Myh3 replacement is independent of thick-filament-associated protein substitution. Thick filaments mainly consist of myosin, but thick-filament-associated proteins (e.g., Mybpc and Myom) are also necessary for thick-filament formation (16, 36). We investigated whether myosin-associated proteins affect the substitution of eGFP + Myh3 in striated myofibrils by utilizing mCherry + Mybpc1 and mCherry + Myom3 in thick-filament incorporation experiments. As a result, exogenously expressed mCherry + Mybpc1 and mCherry + Myom3 were precisely localized to thick filaments in cultured myotubes and coexpressed with eGFP + Myh3 (Fig. 3, A–F). The molecular sizes of mCherry + Mybpc1 and mCherry + Myom3 were examined by immunoblot analysis and revealed protein bands of the expected sizes (Fig. 3G). Furthermore, simultaneous FRAP scanning of eGFP and mCherry signals showed that the fluorescence intensity value of the mCherry + Mybpc1/Mf was significantly lower than that of the coexpressed eGFP + Myh3/Mf, suggesting an independent substitution of eGFP + Myh3 and mCherry + Mybpc1 (Fig. 3H; Table 2). Our FRAP data also showed that the t₁/₂ of the mCherry + Mybpc1 and mCherry + Myom3 tended to be shorter than the t₁/₂ of eGFP + Myh3, although no statistically significant differences were observed (Fig. 3I; Table 2). Thus the eGFP + Myh3 substitution did not depend on replacement of thick-filament-associated proteins in striated myofibrils.

Myosin replacement is affected by the inhibition of protein synthesis. We have shown that myosin in striated myofibrils is dynamically exchanged, but the source of myosin for replacement remains unclear. To examine whether newly synthesized myosin is required for myosin replacement in striated myofibrils, cultured skeletal muscle cells were incubated with 10 μM CX to block de novo protein synthesis, followed by FRAP analysis. CX was added to the medium at 1 h (CX1h) or 10 h (CX10h).
before FRAP analysis and maintained throughout the experiments. The fluorescence intensity value of the eGFP/H11001 Myh3 Mf in CX10h-treated myotubes was decreased to 47% of the value in the vehicle control group, but fluorescence recovery of eGFP/H11001 Myh3 was still observed in the presence of CX (Fig. 4A; Table 3). These results suggest that myosin in myofibrils is exchanged not only by nascent myosin, but likewise by a preexisting pool of myosin reserved in the myotubes.

As newly synthesized myosin is preferentially associated with the cytoskeletal network (23), we evaluated whether preexisting myosin is reserved in the nonmyofibrillar cytosolic fraction of the myotubes, where myosin generally forms aggregates due to the physiological ionic strength of the cytoplasm (42). The nonmyofibrillar cytosolic fraction was carefully isolated from skeletal muscle cells by using a Triton X-100-containing buffer without mechanical homogenization. This fraction was defined as the supernatant remaining after ultracentrifugation at 100,000 g for 60 min and contained myosin, as assessed by immunoblot analysis with a skeletal muscle-specific anti-myosin antibody (MF20) (Fig. 4C).

Next, the muscle cells were treated with 10 μM CX for 12 h to determine whether blockade of de novo protein synthesis decreases nonmyofibrillar cytosolic protein and myosin content. Protein concentrations in control (DMSO vehicle treated) and CX-treated cells were 15.06 ± 0.25 and 11.93 ± 0.63 mg/ml (means ± SE), respectively. Thus cytosolic protein

Table 2. Mobile fractions and t1/2 values of eGFP + Myh3- and mCherry tagged myosin-associated proteins in doubly transfected myotubes

<table>
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<tr>
<th>Combination</th>
<th>Mobile Fraction, %</th>
<th>t1/2, h</th>
<th>n</th>
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<tr>
<td>eGFP + Myh3</td>
<td>48.32 ± 9.61*</td>
<td>3.77 ± 1.44</td>
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</tr>
<tr>
<td>mCherry + Mybpc1</td>
<td>80.50 ± 4.75*</td>
<td>1.29 ± 0.51</td>
<td>4</td>
</tr>
<tr>
<td>eGFP + Myh3</td>
<td>74.10 ± 7.06</td>
<td>3.90 ± 0.75</td>
<td>3</td>
</tr>
<tr>
<td>mCherry + Myom3</td>
<td>82.88 ± 9.38</td>
<td>1.95 ± 0.06</td>
<td>3</td>
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Values are means ± SE; n, no. of experiments. Mybpc1, myosin binding protein C1; Myom3, myomesin 3. *P < 0.05 (P = 0.0209).
content was largely reduced in CX-treated myotubes (Fig. 4B). Furthermore, the intensity of the Myh polypeptide was significantly lower in the CX-treated myotubes than in the control DMSO-treated myotubes (Fig. 4C and D).

\( \text{eGFP} + \text{Myh3 is rapidly released from striated myofibrils in CX10h-treated myotubes.} \)

To further evaluate the effects of cytosolic Myh amount on the release of Myh from striated myofibrils, we utilized the photoconvertible fluorescent protein, KikGR1. The color of this protein irreversibly shifts from green to red following exposure to UV light (53). Green-colored thick filaments were observed in KikGR1 + Myh3-expressing myotubes before the UV-triggered photoconversion (Fig. 5A). After photoconversion, red-colored thick filaments appeared. The relative fluorescence intensity of the red fluorescent protein decreased over the 10-h course of the experiment, while that of the green fluorescent protein increased.

Monitoring KikGR1 + Myh3 signals showed that the incorporation of myosin into myofibrils and the releasing of myosin from myofibrils occur concurrently (Fig. 5B).

Finally, the fluorescence intensity of KikGR1 + Myh3 in CX10h myotubes was monitored after photoconversion. Different shuttling rates of KikGR1 + Myh3 were observed between the myofibrils and the cytosol in CX10h myotubes (Table 4; Fig. 5B). The \( t_{1/2} \) of the red fluorescence in the CX10h myotubes was drastically decreased relative to the \( t_{1/2} \) of the control, indicating that the liberation of myosin from myofibrils was more rapid than the insertion of myosin into myofibrils. These results indicate that release of KikGR1 + Myh3 in myofibrils was facilitated by a reduction of cytosolic myosin content in CX10h-treated myotubes.

**DISCUSSION**

**Dynamic exchange of myosin in myofibrils.** The sarcomere is a highly organized structure that appears to be rigidly constructed when observed under an electron microscope. However, the current FRAP data reveal active replacement of eGFP + Myh3 in the myofibrils of cultured myotubes, implying that the sarcomere is actually dynamic in nature. The \( t_{1/2} \) of eGFP + Myh3 in striated myofibrils is ~3 h by FRAP analysis. Classical studies of myosin turnover rates employed radioactive isotopes to calculate the \( t_{1/2} \) of myosin as ~6 days in cultured myotubes (46). Differences in the \( t_{1/2} \) values obtained by FRAP and radioisotope labeling protocols are probably caused by method-selective concepts of the \( t_{1/2} \) of proteins. The myosin \( t_{1/2} \) calculated by FRAP analysis reflects the ratio of replaced myosin to preexisting myosin in myofibrils (i.e., the exchange rate), while the myosin \( t_{1/2} \) quantified by radioisotope labeling reflects the entire life span of the myosin molecule (i.e., from translation to degradation). Given that the myosin \( t_{1/2} \) by radioisotope labeling is substantially longer than the myosin exchange rate in myofibrils, myosin frequently shuttle between myofibrils and the nonmyofibrillar cytosolic pool in muscle cells.

The \( t_{1/2} \) of myosin, as determined by our FRAP analysis, is longer than the \( t_{1/2} \) values of other myofibrillar proteins. This is particularly the case for thin-filament and Z-band components, whose \( t_{1/2} \) values have been reported as minutes (30, 54, 55). However, our data are largely consistent with previous findings showing a slow recovery of fluorescence-labeled myosin through the use of microinjection and FRAP techniques (25). Furthermore, an extensive FRAP-based study by da Silva

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<th>Treatment</th>
<th>Mobile Fraction, %</th>
<th>( t_{1/2}, ) h</th>
<th>( n )</th>
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<tbody>
<tr>
<td>Control</td>
<td>39.77 ± 2.61*†</td>
<td>4.48 ± 0.95</td>
<td>9</td>
</tr>
<tr>
<td>CX1h</td>
<td>27.62 ± 2.33*</td>
<td>2.99 ± 0.59</td>
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<tr>
<td>CX10h</td>
<td>18.89 ± 1.76*†</td>
<td>5.51 ± 1.25</td>
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</table>

Values are means ± SE; \( n \), no. of experiments. Cells were preincubated with 10 \( \mu M \) cycloheximide (CX) for 1 h (CX1h) or 10 h (CX10h). Fluorescence recovery after photobleaching analyses were then carried out in the presence of CX. *\( p < 0.01 \) (\( P = 0.0032 \)) and †\( p < 0.01 \) (\( P = 0.00001 \)).
Lopes and colleagues (7) demonstrated a \( t_{1/2} \) of 2.1 h for GFP knock-in connectin/titin in cardiomyocytes. The comparatively rapid displacement of thin-filament and Z-band components might be partly due to their small molecular sizes relative to myosin (\( \sim 500 \text{kDa} \)) and connectin/titin (\( \sim 3,000 \text{kDa} \)) (32, 56). Indeed, protein diffusion in the myocytoplasm is largely dependent on molecular size, with smaller molecules showing faster diffusion (41). Furthermore, the amount of nonmyofibrillar myosin present in the cytoplasm may affect its \( t_{1/2} \) in FRAP assays (see below). Table 4. Mobile fractions and \( t_{1/2} \) values of KikGR1 + Myh3 in cultured myotubes

<table>
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<tr>
<th>Treatment</th>
<th>Mobile Fraction, %</th>
<th>( t_{1/2}, \text{h} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>84.16 ± 8.00</td>
<td>2.73 ± 0.38</td>
</tr>
<tr>
<td>CX10h</td>
<td>60.7 ± 6.63</td>
<td>1.77 ± 0.43</td>
</tr>
<tr>
<td>Red</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>83.24 ± 3.76</td>
<td>3.26 ± 0.50*</td>
</tr>
<tr>
<td>CX10h</td>
<td>82.23 ± 4.21</td>
<td>0.53 ± 0.11*</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n = 6 \) experiments for vehicle control and 5 experiments for CX10h. \( *P < 0.01 \) (\( P = 0.0012 \)).

Mechanism of myosin replacement in myofibrils. Thick filaments maintain their locations in sarcomeres by interacting with connectin/titin through Mybpc at multiple binding sites on thick filaments (29). Mybpc helps to stabilize the thick-filament structure through interactions with the myosin LMM (12). Despite conformational complexities of thick filaments, myosin is dynamically replaced in myofibrils without disintegration of thick filaments or sarcomeric structures. When myosin is substituted, the question can be asked, what is the exchange unit for myosin in the myofibrils: individual thick filament(s) or individual myosin molecule(s)? Here, we observed independent substitutions of eGFP + Myh3 and mCherry + Mybpc1 during FRAP measurements in doubly transfected myotubes that expressed eGFP + Myh3 and mCherry + Mybpc1. This result suggests that an individual myosin molecule and/or a myosin oligomer is exchanged instead of a thick filament as a replacement unit in myofibrils. Furthermore, eGFP fluorescence failed to show uniform recovery along thick filaments, as assessed by FRAP in line-scan mode (Fig. 2A). The nonuniform recovery of eGFP also supports the idea that individual myosin molecules serve as myofibrillar replacement units. In addition, nonmyofibrillar floating...
thick filaments are not observed among myofibrils via electron microscopy, as far as we know. While individual myosin molecules are probably exchanged between myofibrils and the nonmyofibrillar cytosol, we cannot yet determine whether a single myosin molecule and/or a short myosin filament functions as a replacement unit.

How can myosin be exchanged between myofibrils and the nonmyofibrillar cytosolic pool? Given our results that myosin replacement in myofibrils is not affected by the absence of a microtubule-based transport system, by actomyosin interactions, or by the overexpression of thick-filament-associated proteins, we propose that myosin itself, and in particular, the LMM domain, targets substitution sites in myofibrillar thick filaments. For example, biochemical data demonstrated that purified myosin and LMM form filaments or paracrystals in in vitro test tube assays (22, 43, 51). Furthermore, our laboratory previously showed that LMM is the minimum required domain for incorporation into thick filaments in cultured myotubes, while S1 is dispensable (39). Moreover, mutations in LMM caused poor incorporation into thick filaments, indicating the importance of this domain for proper targeting to thick filaments (59). Thus, myosin exchange between myofibrils and nonmyofibrillar cytosol may be largely dependent on LMM.

Cytosolic pool of myosin in muscle cells. Our current findings suggest that myofibrillar myosin can be substituted by preexisting myosin in the cytosol surrounding the myofibrils. What, then, is the source of the nonmyofibrillar cytosolic myosin? Our CX10h FRAP data indicate that, although newly synthesized myosin contributes to myosin replacement, myosin is still exchangeable without supplying nascent myosin. Therefore, we hypothesize that most cytosolic myosin is in fact derived from recycled myosin. This idea is also supported by evidence showing that nascent myosin polypeptides immediately associate with the cytoskeleton following their translation (23, 24, 27). In contrast to myosin, replacement of other myofibrillar proteins is not affected by CX-mediated inhibition of protein synthesis (7, 55), which might be explained by the following two phenomena. First, embryonic muscle cells contain relatively large cytosolic pools of myofibrillar proteins other than myosin (47), and, second, muscle cells do not have the capacity to reserve myosin in the cytoplasm due to the propensity of myosin molecules to aggregate in solutions (e.g., cytosol) at physiological ionic strength (42).

In this study, incorporation and dissociation of myosin occurred simultaneously in the myofibrils of KikGR1 + Myh3-expressing myotubes. Interestingly, the myosin dissociation rate in CX10h-treated myotubes was significantly faster than that in control myotubes, although a significant difference was not observed in the myosin incorporation rates between CX10h-treated and control myotubes. These results suggest a dynamic equilibrium of myosin between myofibrils and nonmyofibrillar cytosol. In other words, an overflow of released myosin may partially reflect a condition of muscle atrophy, in which the excess myosin is released from myofibrils due to a lack of newly synthesized myosin in the cytoplasm. The KikGR1 + Myh3 experiments may indicate a decreased number of thick filaments in the myofibrils, or a decreased number of myosin molecules in the thick filaments. The latter case implies an incomprehensible phenomenon, where the number of myosin molecules in a thick filament might decrease. Further experiments are required to explore this possibility.

Of note, cytosolic myosin may have functions other than provision of a myosin pool for reuse in myofibrils. During myofibrillar protein turnover, damaged cytosolic Myh undergoes muscle RING finger (Murf) 1- and/or Murf3-dependent degradation (4, 11). Although Murf1 ubiquitinates myosin-associated proteins (i.e., Mybpc and myosin light chains) even in myofibrils, Murf1 and Murf3 only recognize Myh in the cytoplasm of skeletal muscle cells (4, 11). Therefore, nonmyofibrillar cytosolic myosin may be selected either for the recycling pathway to replace myosin in thick filaments, or for the degradation pathway via the proteasome system.

Myofibrillar proteins other than myosin, including sarcomeric α-actinin, cypher, and actin, are also exchanged between myofibrils and the cytoplasmic pool (30, 55). As indicated above, however, the manner in which myosin exists in the cytosol is of particular interest because various in vitro biochemical studies demonstrated that myosin forms aggregates or filaments under such conditions, and high ionic strength solutions (e.g., 0.6 M KCl) are necessary to solubilize myosin (42). We do not fully understand how myosin can exist in the cytoplasm without aggregation. If myosin molecules are predominantly incorporated into thick filaments immediately after their translation from mRNA (23), this would explain the successful myofibrillar integration of newly synthesized myosin without aggregation, but not the protection of recycled myosin in the cytosol from aggregation. Gasda and colleagues (13) recently proposed that the myosin chaperone, UNC45, contributes to the precise alignment of myosin head domains to form thick filaments and sarcomeres in Caenorhabditis elegans. Because myosin forms a complex with UNC45 in the cytosol (10, 50), UNC45 oligomers might possibly capture...
myosin molecules or short myosin filaments to permit their distribution in the cytosol without aggregation. 

Other than myosin chaperones, the l-histidine amino acid residue also reportedly induces solubilization of myosin in low ionic strength solutions in in vitro test tube assays (18, 19). Apparently, the myosin tail region is loosened by the presence of l-histidine, and myosin is subsequently dispersed in solutions of physiological ionic strength without aggregation. Therefore, the microenvironment afforded by amino acid residue distribution in the cytosol might help to preclude myosin accretion, although further studies are necessary to support this hypothesis.

Conclusions. Figure 6 provides a model of myosin dynamics in skeletal muscle cells. Incorporation of myosin into, and dissociation of myosin from, thick filaments occurs simultaneously in these cells. Myosin release from myofibrils yields the nonmyofibrillar fraction in the cytoplasm, where cytosolic myosin is prevented from forming aggregates under conditions of physiological ionic strength by an unknown mechanism. Nonmyofibrillar cytosolic myosin is chosen for degradation or recycling via specific pathways. Damaged cytosolic myosin is degraded by the Murf1/Murf3 proteasome system (4, 11), while preexisting cytosolic myosin is reused for substitution of myosin in myofibrils, although de novo synthesized myosin is preferentially incorporated into the myofibrils (23). Alternatively, it is possible that some proportion of myosin could be exchanging directly between neighboring/adjacent myofibrils and bypassing the cytosol. Collectively, our present findings not only demonstrate the dynamic exchange of myosin in myofibrils, but also shed light on the significance of nonmyofibrillar cytosolic myosin, which aggregates in solutions of physiological ionic strength in in vitro test tube assays, but not in skeletal muscle cells.

ACKNOWLEDGMENTS
We are deeply grateful for enthusiastic discussions with Professor H. Lee Sweeney (University of Pennsylvania) and the late Professor Emeritus Howard Holtzer (University of Pennsylvania).

GRANTS
This work was supported in part by a Japan Society for the Promotion of Science (JSPS) KAKENHI grant (grant 22580301) to K. Ojima, and by a Uehara foundation grant to K. Ojima.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


