

# Chaperone-mediated folding and assembly of myosin in striated muscle

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## Summary

**De novo folding and assembly of striated muscle myosin was analyzed by expressing a GFP-tagged embryonic myosin heavy chain (GFP-myosin) in post-mitotic C2C12 myocytes using replication defective adenoviruses. In the early stages of muscle differentiation, the GFP-myosin accumulates in bright globular foci and short filamentous structures that are later replaced by brightly fluorescent myofibrils. Time-lapse microscopy shows that the intermediates are dynamic and are present in elongating and fusing myocytes and in multinucleated myotubes. Immunostaining reveals the co-localization of the molecular chaperones Hsc70 and Hsp90 with the GFP-myosin in the intermediates, but not in the mature myofibrils. Uninfected cells have similar intermediates suggesting a common pathway for myosin maturation. Two conformation-sensitive antibodies that bind the unfolded**

**motor domain and the coiled-coil conformation of the rod demonstrate that in the intermediates, the myosin rod is folded but the motor domain is not folded. Electron microscopy reveals that the intermediates contain loose filament bundles surrounded by a protein rich matrix. Geldanamycin, a specific inhibitor of Hsp90, reversibly blocks myofibril assembly and triggers accumulation of myosin folding intermediates. We conclude that multimeric complexes of nascent myosin filaments associated with Hsc70 and Hsp90 are intermediates in the folding and assembly pathway of muscle myosin.**

Movies available on-line

Key words: Myosin, Muscle, Folding, Molecular chaperone, GFP

## Introduction

Myosins form a large family of actin-based motor proteins that have evolved to perform a wide variety of motility based cellular functions. There are eighteen known classes of this family of molecular motors reflecting the specialization of the member proteins (Berg et al., 2001). All myosin classes share a conserved catalytic domain of ~750 residues that contains the actin and ATP binding sites. The core of the catalytic domain is a compact structure consisting of a seven-stranded mostly parallel  $\beta$  sheet flanked by three  $\alpha$  helices on each side (Rayment et al., 1993). Additional N- and C-terminal extensions give rise to class-specific differences in function and distribution of myosin superfamily members.

The class II striated muscle myosin group is unique amongst all other classes of this large superfamily in a number of important ways. First, the striated myosin II family members, including skeletal and cardiac muscle myosin, are expressed at levels that far exceed all other myosin classes. Second, striated muscle myosin is incorporated into the highly organized sarcomeric units of the specialized cytoskeleton of skeletal and cardiac muscle. These two unique characteristics have contributed greatly to our current understanding of the mechanism of action of this fascinating family of molecular motors through the systematic analysis of the abundant and readily isolated striated muscle myosin (Geeves and Holmes, 1999).

Considering its abundance, it is perhaps paradoxical that

striated muscle myosin has been exceedingly difficult to produce as a recombinant protein in heterologous expression systems. The successful production of recombinant striated muscle myosin has been limited to expression in muscle systems (Chow et al., 2002; Kinose et al., 1996; Swank et al., 2000). The difficulty in the expression of striated muscle myosin in non-muscle cells has been attributed recently to a folding defect, suggesting that folding and assembly of components unique to muscle are needed (Barral et al., 2002; Chow et al., 2002; Price et al., 2002; Srikakulam and Winkelmann, 1999).

Although an extensive body of work has focused on myofibrillogenesis and the structure and composition of the sarcomere, very little is known about the fate of newly synthesized myosin or the mechanism of incorporation into the sarcomere. In a reticulocyte lysate expression system the folding of a skeletal muscle heavy meromyosin subfragment (HMM) is mediated by the eukaryotic chaperones including the cytosolic chaperonin, CCT (Srikakulam and Winkelmann, 1999). This system supports folding and dimerization of the rod and light chain binding to the myosin heavy chain. The folding of the motor domain is slow but can be improved by addition of a muscle cell extract suggesting a role for muscle-specific factors in the folding pathway of the motor domain. This hypothesis is confirmed by analysis of a chimeric protein containing only the striated myosin II motor domain fused to GFP (Chow et al., 2002). Efficient folding of the small motor

domain::GFP chimera is possible only when it is expressed in muscle cells.

We have developed a novel approach to follow the fate of newly synthesized myosin in differentiating muscle cells. A replication-defective adenovirus was used to induce synchronous expression of GFP-myosin in post mitotic C2C12 cells, and the synthesis and incorporation of the GFP-myosin into the contractile cytoskeleton of the cultured muscle cells was analyzed. We found that newly synthesized myosin forms a complex with Hsp90 (heat shock protein 90) and Hsc70 (constitutively expressed heat shock related protein 70). This complex contains myosin molecules with partially folded motor domains, assembled into nascent myosin filaments. The molecular chaperones appear to participate in the initial folding of myosin and remain associated with partially folded intermediates to chaperone folding and promote myofibril assembly.

## Materials and Methods

### Replication-defective adenovirus containing a GFP-myosin gene

The chicken skeletal muscle myosin heavy chain (MHC) is encoded by an embryonic chicken myosin cDNA that has been epitope tagged as previously described (Kinose et al., 1996; Molina et al., 1987). The myosin sequence was cloned to the 3'-end of the coding sequence of a thermal stable, fast folding and fluorescence-enhanced variant of green fluorescent protein (GFP) (Siemering et al., 1996) creating a 7 kb cDNA encoding an N-terminal GFP::myosin heavy chain chimeric protein (GFP-myosin). The cDNA of the GFP-myosin was cloned between an enhanced CMV promoter and a SV40 polyadenylation signal in the AdEasy shuttle vector pCMVShuttle for construction of the replication-deficient adenovirus AdGFP-MHC (He et al., 1998). The details of construction of GFP::MHC chimeric cDNA and the production and amplification of the recombinant adenovirus AdGFP-MHC using the AdEasy vector system are described elsewhere (Wang et al., 2003).

### Cell culture and adenovirus infections

Maintenance and passaging of the mouse myogenic cell line, C2C12 (CRL 1772; American Type Culture Collection, Rockville, MD), has been described previously (Chow et al., 2002; Kinose et al., 1996). For immunofluorescence microscopy, 35 or 60 mm plastic dishes were coated with 10 µg/ml of mouse EHS cell laminin (Colognato et al., 1999) and washed extensively with sterile phosphate-buffered saline (PBS) prior to plating cells. The cells were seeded at an initial density of  $7.5 \times 10^4$  cells/cm<sup>2</sup>. Cells were plated on 60 mm permanox tissue culture dishes (Nalge Nunc, Naperville, IL) for analysis by electron microscopy. Cells at 60-70% confluence were induced to differentiate by shifting to fusion medium (10% horse serum, 1% fetal bovine serum, and 89% Dulbecco's modified Eagle's medium). Cells withdrew from the cell cycle and 24-36 hours later were infected with AdGFP-MHC ( $\sim 1-3 \times 10^9$  PFU/ml) at a multiplicity of infection of 750-1000. Virus was administered in 0.5 ml and 1.0 ml of fusion medium for 35 mm and 60 mm dishes, respectively. Two hours after infection the dishes were supplemented with fresh medium, and incubation was continued at 37°C, and 5% CO<sub>2</sub> for an additional 18-48 hours. Some C2C12 cells were treated with 130 pM geldanamycin (Life Technologies Inc., Gaithersburg, MD) in fusion medium.

### Native proteins and binding assays

Monoclonal antibodies 12C5.3 and 10F12.3 react specifically with

chicken skeletal muscle myosin and were prepared and characterized as previously described (Winkelmann et al., 1995; Winkelmann et al., 1993). The mAbs F18 and F59 react with striated muscle myosin heavy chains (Miller et al., 1989). Monovalent Fab fragments were prepared by digestion of the purified IgG with papain (Winkelmann and Lowey, 1986). Actin and myosin were prepared from adult White Leghorn chicken pectoralis muscle and myosin S1 (subfragment 1) was prepared by papain digestion (Winkelmann et al., 1993; Winkelmann and Lowey, 1986). The binding of monovalent Fab antibody fragments of mAbs F18 and F59 to either myosin S1 in a rigor complex with F-actin (acto-S1) ([S1]=0.125 µM) or to synthetic myosin filaments ([myosin]=0.10 µM) was determined by co-sedimentation at 300,000 g for 20 minutes. The supernatant and pellet fractions were analyzed by SDS-PAGE (Laemmli, 1970) and free and bound Fab were quantitated by laser densitometry.

### Immunofluorescence microscopy

Cells were processed for immunofluorescence 24-96 hours post induction. All steps were performed on ice with cold buffers. Cells were rinsed with PBS and fixed by incubation in 4% paraformaldehyde in PBS for 15 minutes then washed three times with PBS. Cells were permeabilized with 1% Triton X-100 in PBS for 5 minutes, rinsed with PBS, and blocked with 1% BSA/PBS. For immunolabeling, the blocked cells were incubated with primary antibodies at 2 µg/µl for 1 hour to overnight. The antibodies used included: anti-Hsc70 mAb (1B5), anti-Hsp90 mAb (2D12), anti-TCP-1 polyclonal subunit specific antibodies (Stressgen, Victoria, Canada), and anti-myosin mAbs F59 and 10F12.3. The immunolabeled species were visualized with secondary antibodies conjugated to tetramethyl rhodamine isothiocyanate (TRITC). The anti-Hsc70, and anti-Hsp90 antibodies were both rat mAbs and were detected with TRITC-conjugated rabbit anti-rat IgG. The mouse anti-myosin mAbs were detected with TRITC-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO). The secondary antibodies were diluted 1:600 in 1% BSA, 0.05% Tween in PBS. Excessive secondary antibody was discarded and the dishes washed extensively with PBS before sealing the surface with a glass coverslip and FITC guard mounting medium (Molecular Probes, Eugene, OR). Some samples were fixed with cold 100% methanol rather than paraformaldehyde and stained with mAb F59. In some experiments FITC-conjugated F59 was used at 2 µg/ml together with 5 µg/ml DAPI (Sigma, St Louis, MO).

Digital images were collected on an Olympus IX70 inverted fluorescence microscope (Olympus America Inc., Melville, NY) with a MicroMax cooled CCD camera (Roper Scientific, Princeton, NJ) using IpLab image analysis software (Scanalytics Inc., Fairfax, VA). Confocal images were collected on an Olympus IX81 with a CARV Nipkow disc confocal unit (Atto Biosciences, Rockville, MD) and SensiCam QE camera (Cooke Corp., Auburn Hills, MI). For confocal microscopy, a glass coverslip was sealed over the cell layer and images were collected with a 60× 1.2 NA water immersion objective. The time-lapse experiments were done with a PDMI-2 microincubator and perfusion pump (Harvard Apparatus Inc., Holliston, MA) with the cells plated on laminin-coated 35 mm dishes.

### Electron microscopy

Cells growing on 60 mm permanox dishes were fixed with 2.5% paraformaldehyde, and 2.5% glutaraldehyde. Areas rich in folding intermediates were selected and marked on the dishes. The samples were dehydrated through a graded alcohol series and embedded in epon and sectioned (Birk et al., 1988; Moncman et al., 1993). Gold sections were cut with a diamond knife, picked up on formvar-coated grids and stained with 2% uranyl acetate followed by 1% phosphotungstic acid, pH 3.2. Sections were examined and photographed using a JEOL 1200EX transmission electron microscope operated at 80 kV.

### Co-immunoprecipitation of S1 with Hsp90 and Hsc70

The S30 fraction was prepared essentially as described previously (Srikakulam and Winkelmann, 1999). The Papain MgS1 subfragment of pectoralis muscle myosin was dissolved in S30 buffer supplemented with 6.5 M guanidine hydrochloride (GuHCl). The denatured myosin S1 was then dialyzed against S30 buffer without GuHCl and the denatured protein precipitate was recovered by centrifugation and resuspended in S30 buffer containing 6.5 M GuHCl at ~5 mg/ml. Aliquots of denatured S1 were diluted 10 fold into either S30 fraction or S30 buffer. In a separate reaction, S30 fraction and 6.5 M GuHCl were combined in the same ratio as the reaction containing the denatured myosin S1. The reactions were incubated at room temperature for 30 minutes. Formaldehyde-fixed *Staphylococcus aureus* (Immuno-Precipitin; Life Technologies Inc., Gaithersburg, MD) was washed and blocked with 1% BSA/PBS for 15 minutes. 100  $\mu$ l aliquots of the blocked *S. aureus* cells were incubated with an equal volume of rabbit anti-mouse IgG at 2  $\mu$ g/ $\mu$ l, for 1 hour on ice. The Immuno-Precipitin was washed twice with S30 buffer and incubated for an additional hour with either 100  $\mu$ l of anti-S1 mAb 12C5.3 at 1 mg/ml (12C5.3-precipitin), or PBS (non-immune-precipitin). The 12C5.3-precipitin, and non-immune-precipitin were washed twice with S30 buffer. The reaction containing denatured S1 and S30 fraction was divided into two aliquots and incubated with either 12C5.3-precipitin, or the non-immune-precipitin for 40 minutes at room temperature. All the other reactions were incubated with 12C5.3-precipitin. The precipitin pellets were collected by centrifugation, washed extensively and resuspended in SDS gel loading buffer. The samples were resolved on 10% SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with rat monoclonal anti-Hsc70 IgG at 2.5  $\mu$ g/ml, rabbit polyclonal anti-Hsp90 $\alpha$  IgG (Lab Vision Corp., Fremont, CA) at 5  $\mu$ g/ml and mouse anti-myosin mAb 12C5.3 at 1  $\mu$ g/ml.

## Results

### Myosin transits through intermediates before assembling in myofibrils

A replication-defective adenovirus containing a GFP::myosin chimeric gene driven by a CMV promoter has been developed and used to infect post mitotic C2C12 myocytes in culture (Wang et al., 2003). This cell line is an established model for studying the expression of muscle-specific genes and the assembly of muscle proteins into striated myofibrils (McMahon et al., 1994). The adenovirus vector permits the simultaneous infection of a large population of fusing C2C12 myocytes and the GFP tag facilitates the direct observation of the fate of the newly synthesized GFP-myosin.

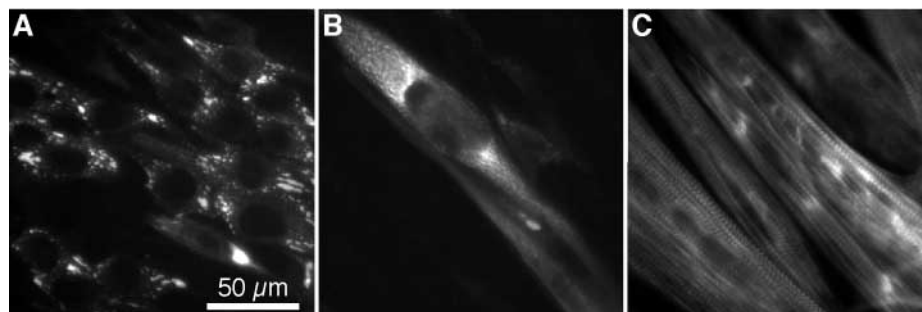
The GFP-myosin is readily detected in live cells within 12-18 hour of infection by fluorescence microscopy, and the accumulation and assembly of the GFP-myosin in these cells can be followed over the course of 4-6 days in culture. In the early stages of expression, about 18-24 hours post-infection, the GFP-myosin is found as brightly fluorescent globular foci (Fig. 1A), and as short, disordered filamentous structures (Fig. 1B). As differentiation proceeds and myotubes emerge, the early forms are replaced by

striated myofibrils. By 72 hours post-infection, most of the GFP-myosin is found assembled into striated myofibrils (Fig. 1C). At this later stage of expression and assembly, the GFP-myosin is readily extracted and purified with the embryonic striated muscle myosin of the C2C12 myotubes. The GFP-myosin associates with the endogenous myosin light chains and forms functional motor molecules capable of moving actin filaments in vitro at rates characteristic of an embryonic muscle myosin (Wang et al., 2003).

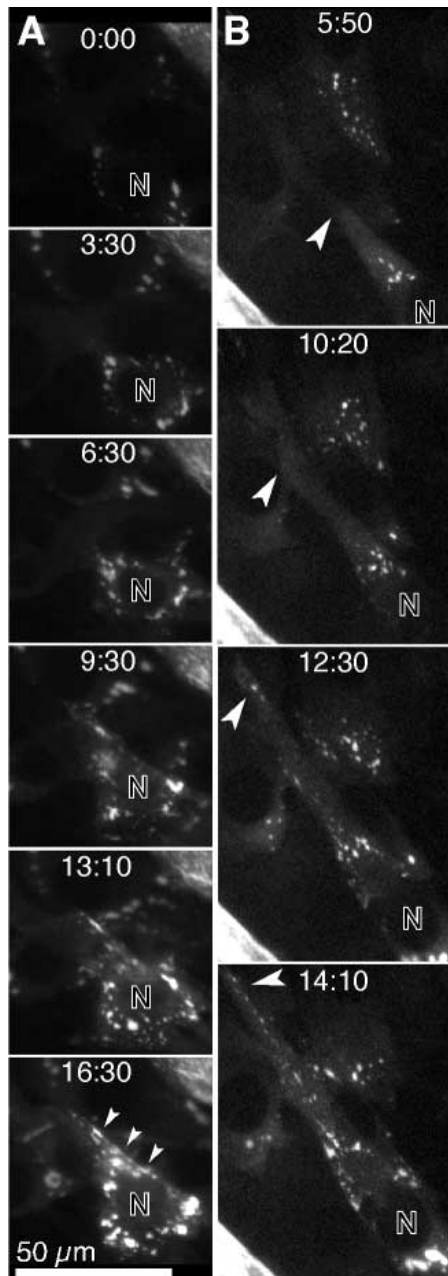
In live cells imaged by time-lapse digital fluorescence microscopy (Fig. 2), the GFP-myosin emerges as bright spots typically at the periphery of the cells (Movie 1, <http://dev.biologists.org/supplemental>). The GFP-myosin is present in post-mitotic fusing cells and is actively re-distributed or transported within cells as they elongate (Fig. 2B). The early fluorescent structures undergo many shape changes, including coalescing to form larger bodies before redistributing throughout the cytoplasm and stretching into long, thin, filamentous threads as the cells elongate and fuse (Fig. 2A). In rare dividing cells, the fluorescent protein is distributed between the daughter cells. The abundance of these fluorescent structures in early stages of infection, and their active re-distribution and shape changes suggest that newly synthesized myosin accumulates into these structures before it is reorganized and assembled into sarcomeres. These structures may represent folding and assembly intermediates in the myosin maturation pathway.

### Early myosin intermediates co-localize with molecular chaperones

If the globular and filamentous structures described above are folding intermediates, then they are likely to be associated with molecular chaperones: e.g. Hsp70/Hsc70, Hsp90 or the cytoplasmic chaperonin, CCT. We screened C2C12 cells by immunofluorescence with antibodies specific for Hsc70, Hsp70, the Hsp90 $\alpha$  and  $\beta$  subunits and the CCT subunits (TCP-1 $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\theta$  and  $\zeta$ ). The chaperones Hsp90, Hsp70 and Hsc70 are abundant proteins in myoblasts and myotubes and immunofluorescent staining with antibodies against these



**Fig. 1.** Fluorescence microscopy of postmitotic C2C12 myocytes, infected with recombinant adenovirus, AdGFP-MHC, reveals the distribution of the expressed GFP-myosin. (A,B) GFP-myosin expression is readily apparent within 18-24 hours of infection. The GFP-myosin first appears in either fluorescent globular foci (A) or short filamentous structures (B). These globular and filamentous myosin intermediates are found distributed throughout the cytoplasm of young multi-nucleated syncytia as well as mono-nucleated myocytes. (C) As the differentiation of the C2C12 cells proceeds, large multinucleated myotubes develop (72-120 hours post infection) and the distribution of the GFP-myosin shifts to a pattern characteristic of striated myofibrils.



**Fig. 2.** Time-lapse fluorescent microscopy reveals the accumulation and dynamics of GFP-myosin in post-mitotic C2C12 myocytes. Fluorescent images of two different cells (A,B) from the same field are shown at various times after starting observation. Cells were infected 24 hours before the start of the time-lapse sequence. (A) The GFP-myosin is first detected near the periphery of the cell in globular intermediates (the position of the nucleus, N, is indicated). The fluorescence intensity and number of globules increases with time and the globules move about the cytoplasm as this mono-nucleate myocyte elongates. In this example, the globular intermediate is pulled along with the elongating cell, and stretches into a 25–30  $\mu\text{m}$  long fiber that is labeled with arrowheads in the 16 hour 30 minutes frame. (B) Another cell elongates (arrowhead marks the end of the cell) and with the elongation there is a redistribution of the globular intermediates to the very tip of the cytoplasmic extension. Cells were maintained at 34°C with a heated microscope stage and perfused with heated media. A time-lapse movie of this experiment can be found in Supplemental Data (<http://jcs.biologists.org/supplemental>).

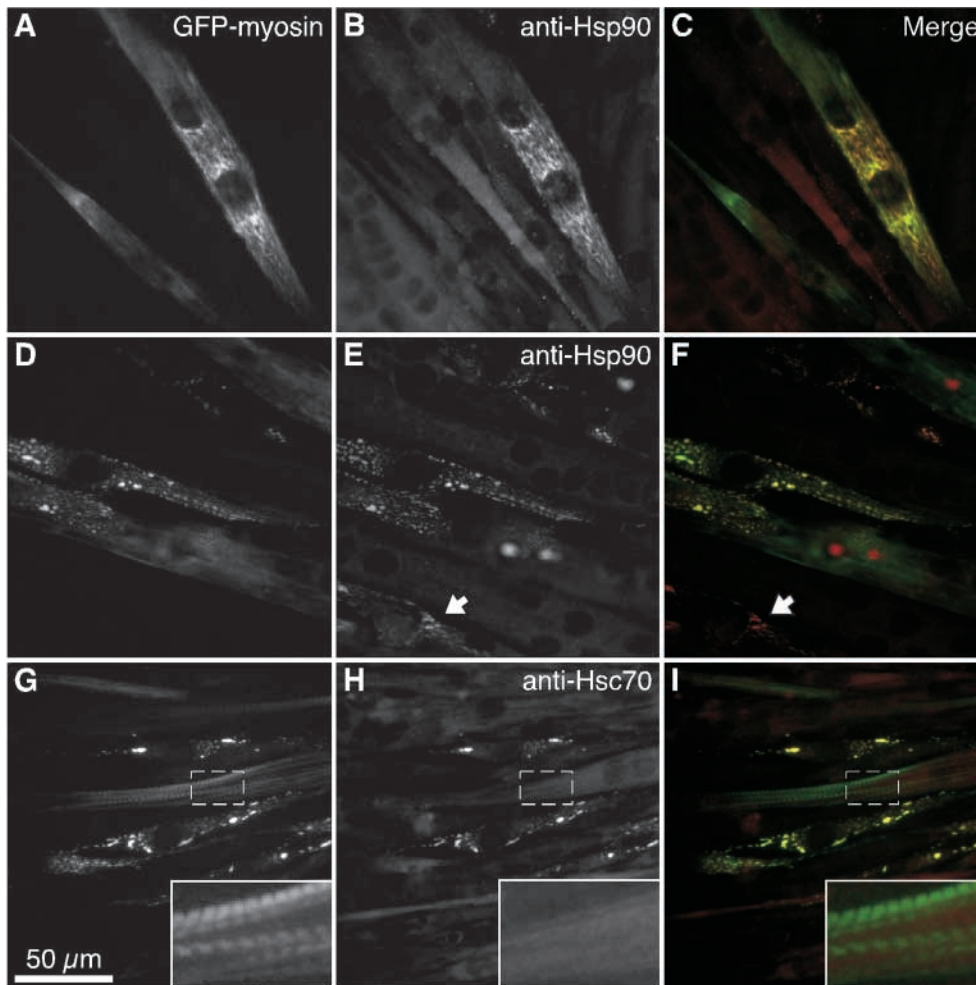
chaperones yielded diffuse cytoplasmic staining and brightly stained structures that were both stronger than the no-primary antibody control (data not shown). Unfortunately, none of the CCT subunit antibodies that were tested provided immunofluorescent staining that was significantly different from the no-primary antibody controls so we were unable to draw any conclusion about the localization of the cytosolic chaperonins in muscle cells.

The immunofluorescence screening of AdGFP-MHC-infected C2C12 cells revealed co-localization of both Hsp90 and Hsc70 with these myosin intermediates. Both Hsp90 (Fig. 3A–F) and Hsc70 (Fig. 3G–I) co-localize with GFP-myosin in the globular intermediates as well as the short filaments, but neither of these molecular chaperones co-localize with the GFP-myosin that has already assembled into striated myofibrils. Since molecular chaperones selectively bind non-native conformations of proteins, the co-localization of Hsp90 and Hsc70 with GFP-myosin in the intermediate structures but not the mature myofibrils, suggests they are myosin folding or maturation intermediates. With the levels of virus that were used, uninfected cells are usually present in many of the microscopic fields. The anti-Hsp90 (Fig. 3E,F) and anti-Hsc70 antibodies react with structures similar to the GFP-myosin folding intermediates in some uninfected cells.

#### Chaperone-associated myosin has partially unfolded heads

The conformation of myosin in the molecular chaperone-associated intermediates was probed with a conformation sensitive antibody. The mAb F59 reacts with an epitope that has been mapped to residues 211–231 of most members of the vertebrate striated muscle myosin family (Miller et al., 1989). These residues are buried within the 3D structure of the myosin motor domain (Rayment et al., 1993) and are not accessible in the native protein. Anti-myosin mAb, F59, reacts strongly with the SDS-denatured C2C12 myosin and the GFP-myosin isolated from infected C2C12 cells (Fig. 4A,B). In contrast, the F59 epitope is not accessible for antibody binding in native myosin filaments or native myosin S1 bound to actin (Fig. 4C). Binding of an F59 Fab fragment to synthetic myosin filaments and to acto-S1 was quantitated in a sedimentation assay and compared to another anti-myosin mAb F18. The epitope for mAb F18 maps to an exposed sequence (residues 65–80) on myosin II motor domain (Miller et al., 1989; Winkelmann et al., 1993). There is negligible binding of F59 to either the myosin filaments or the acto-S1 complex in this assay compared to the saturated binding of the F18 Fab fragment. The F59 epitope is readily recognized on the denatured protein but inaccessible in the native myosin motor domain. Therefore, this antibody is a useful probe for investigating the conformation of myosin motor domain in the folding intermediates.

C2C12 cells infected with AdGFP-MHC were fixed with cold paraformaldehyde, a mild fixative, or with cold methanol prior to immunostaining with mAb F59. The GFP-myosin is observed in both the folding intermediates and in striated myofibrils in infected cells; however, the mAb F59 reacts only with the myosin folding intermediates in the paraformaldehyde-fixed cells and not with the striated myofibrils (Fig. 5A–C). Methanol fixation induces partial denaturation of cellular proteins, and the myosin motor domain



**Fig. 3.** Immunofluorescence microscopy of C2C12 cells expressing GFP-myosin (green channel in A,D,G) after fixation and staining (red channel) with either an Hsp90 antibody (B,E) or an Hsc70 antibody (H). In cells expressing GFP-myosin the filamentous and globular early intermediates co-localize with Hsp90, and Hsc70. The GFP-myosin assembled in striated myofibrils does not co-localize with either Hsp90 or Hsc70 (inset is an enlargement of the boxed regions in G-I showing a small region of a striated myofibril). Most uninfected cells show diffuse staining of Hsp90 and Hsc70 in the cytosol. In addition, anti-Hsp90 and anti-Hsc70 staining reveals structures similar to the early myosin intermediates in an uninfected population of cells that lack GFP-myosin (e.g. the cell marked by an arrow in E and F). In the color merge panels (C,F,I) the structures in which Hsp90 and Hsc70 (red) co-localize with GFP-myosin (green) are yellow.

is particularly sensitive to alcohol denaturation (Winkelmann et al., 1993). Hence, after methanol fixation all myosin containing structures, including myofibrils, are labeled with mAb F59 (Fig. 5D-F). We conclude that with mild fixation, which preserves the native myosin conformation, F59 specifically stains the globular and filamentous myosin folding intermediates indicating that the motor domain is partially unfolded in the chaperone-associated intermediates.

The occurrence of the chaperone-associated myosin intermediates is not restricted to adenovirus-infected C2C12 cells expressing GFP-myosin. Both the filamentous and the globular myosin intermediates are found in uninfected myocytes stained with F59 and anti-Hsp90 (Fig. 5G-I) or with F59 and anti-Hsc70 (data not shown). The colocalization of Hsp90 and Hsc70 with F59-labeled myosin in uninfected cells shows that myosin intermediates occur in the myosin maturation pathway during differentiation of muscle cells and are not induced by virus infection or by over-expression of GFP-myosin.

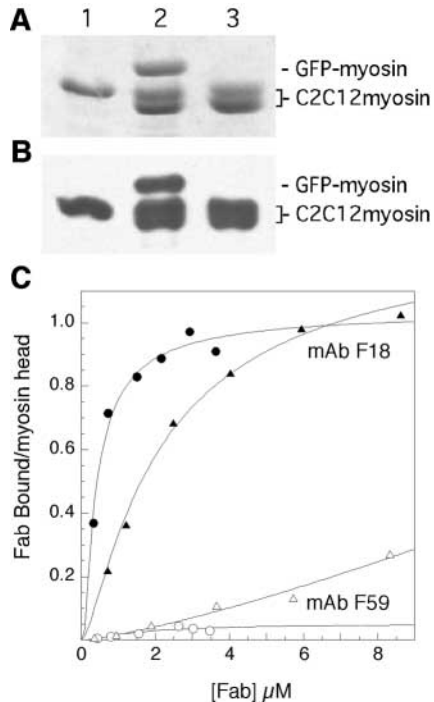
#### Folding intermediates arise early in the muscle differentiation pathway

The timing of the appearance of the folding intermediates in differentiating myocytes was determined in uninfected cells.

The fraction of the myocyte nuclei that are associated with F59<sup>+</sup> and Hsp90<sup>+</sup> myosin containing intermediates was scored 48-96 hour after inducing differentiation (Table 1). To get an accurate count of nuclei and to establish an association between nuclei and the cytoplasmic folding intermediates, 3D confocal volume datasets were used for scoring the cells. At 48 hours post-induction 14% of the nuclei were associated with cells that stained positive for myosin folding intermediates (F59<sup>+</sup>/Hsp90<sup>+</sup>). The fraction of nuclei associated with F59<sup>+</sup>/Hsp90<sup>+</sup> cells decreases dramatically to <4% by 72-96 hours post-induction. Although the cells fuse and change shape dramatically during this period, the number of nuclei/field remains constant ( $P < 0.01$ ) indicating that the cells are no longer dividing, and there is no evidence for apoptotic cell death. We conclude that most of the folding intermediates found in the cells at 48 hours must partition by 72 hours into folded protein that no longer stains F59<sup>+</sup> and Hsp90<sup>+</sup>.

The same conclusion was reached using forced expression of GFP-myosin. Post-mitotic myocytes were infected for 2.5 hours with AdGFP-MHC virus 24 hour after induction, excess virus was washed away and then the appearance of folding intermediates monitored (GFP-myosin<sup>+</sup>/Hsp90<sup>+</sup>). We also could score nuclei associated with native GFP-myosin in this experiment based on the appearance of striated myofibrils (Table 1). The results show that accumulation of the folding

intermediates peaks about 24 hour post-infection (48 hour post-induction) and decreases with time after that. The appearance of folded GFP-myosin assembled in myofibrils correlates with the disappearance of the Hsp90<sup>+</sup> folding intermediates, suggesting a partitioning of the intermediates into native myosin.



**Fig. 4.** Characterization of the conformation-sensitive anti-myosin mAb F59. (A) Stained 6% SDS-PAGE pattern of purified chicken pectoralis myosin (1), myosin from infected (2), and uninfected (3) C2C12 cells. The expressed GFP-myosin and the endogenous C2C12 myosin are resolved and migrate at 250 kDa and 220 kDa respectively. (B) Western blot of the same samples probed with anti-S1 mAb F59. F59 reacts with all three striated muscle myosin types. (C) Native myosin binding assay with anti-myosin mAbs F59 and F18. Synthetic myosin filaments (circles) or a rigor complex of actin-S1 (triangles) were incubated with Fab fragments of F59 (open symbols) and F18 (filled symbols). The free and bound Fab fragments were separated by sedimentation of the myosin-containing filaments and quantitative SDS-PAGE and densitometry. The smooth curve is the fit to a standard binding isotherm (Winklemann and Lowey, 1986).

### Myosin maturation complexes contain nascent thick filaments

Dimerization of the coiled-coil myosin rod domain is an early event in HMM folding and precedes motor domain folding (Srikakulam and Winkelmann, 1999). The mAb, 10F12.3, immunoprecipitates nascent HMM from translation reactions only under native conditions (Srikakulam and Winkelmann, 1999). This antibody recognizes an epitope corresponding to the coiled-coil conformation of residues 1137-1147 within the S2 subfragment of chicken striated muscle myosin. The striated myofibrils and myosin folding intermediates in C2C12 cells expressing GFP-myosin are both stained with anti-S2 (10F12.3) indicating that the rod domain has formed a coiled-coil dimer (Fig. 6A,B).

This conclusion was confirmed by electron microscopy. Electron micrographs of regions of cells rich in myosin folding intermediates reveal loose, filament bundles embedded within a protein rich matrix (Fig. 6C). These structures are identical to the myosin filament bundles observed by immunoelectron microscopy in Cos 7 cells expressing this striated muscle myosin (Moncman et al., 1993). The filaments are ~1 μm long and are arranged in loose parallel or cross-hatched arrays. They are surrounded by a lightly staining protein matrix that is devoid of polysomes suggesting that this is a post-translational cytoplasmic domain. Thus, the large protein matrices correspond to the myosin maturation complexes and contain short myosin filaments arranged in loose bundles.

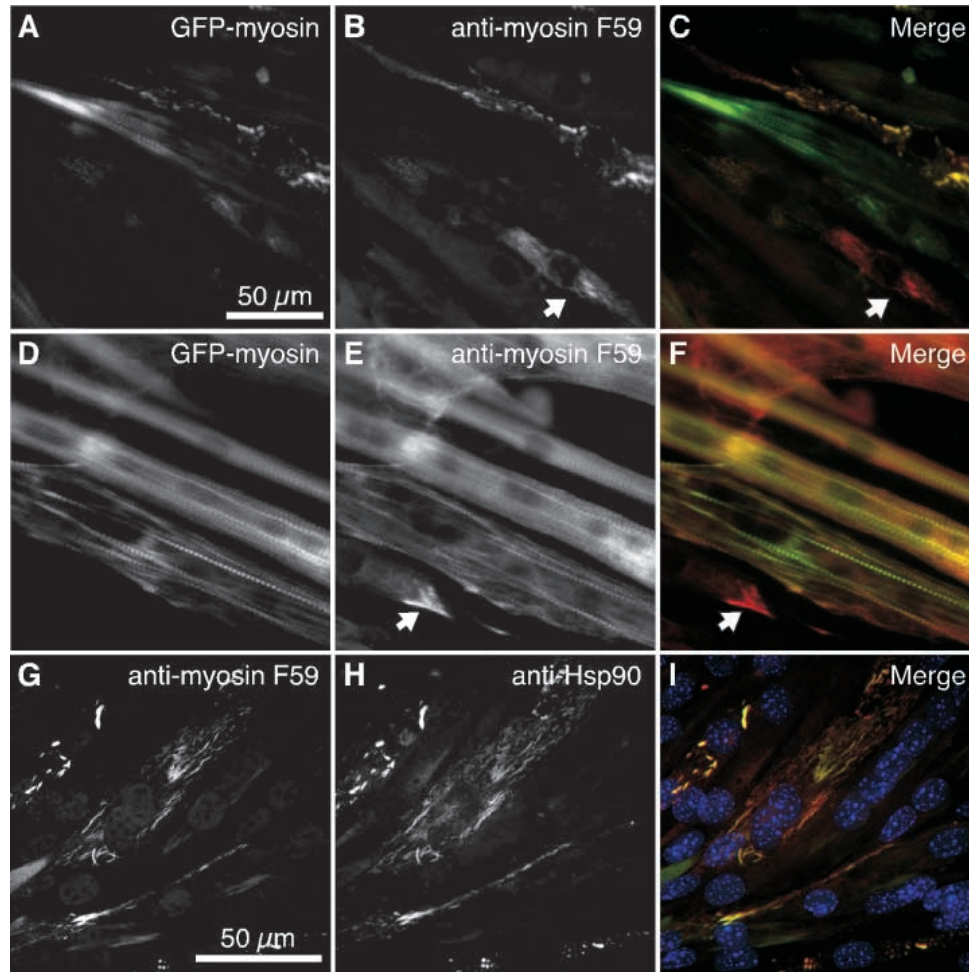
### Myosin can be trapped by Hsp90 in folding intermediates

The results show that at least in some cells myosin transits through folding or maturation intermediates before it is released for assembly; however, does all the striated muscle myosin transit through this pathway? Geldanamycin is an ansamycin antibiotic that specifically binds in the Hsp90 ATPase pocket and can trap substrates in chaperone complexes by blocking ATP-dependent dissociation of Hsp90 (Prodromou et al., 1997; Stebbins et al., 1997; Young and Hartl, 2000). Post-mitotic C2C12 myocytes 30 hour after initiating differentiation were treated with geldanamycin for 22 hours, then the medium was changed and the cells were allowed to recover for an additional 40 hours. Cells were fixed 22 hours after drug treatment and 40 hours after drug wash-out, then

**Table 1. Distribution of myosin folding intermediates in C2C12 myocytes**

Category	Hours after shift to fusion media		
	48 hours	72 hours	92-96 hour*
Uninfected cells			
Folding intermediates (F59 <sup>+</sup> /Hsp90 <sup>+</sup> )	14.0±2.4%	3.8±1.9%	3.1±1.7%
Infected cells (GFP-myosin <sup>+</sup> )			
Folding intermediates (Hsp90 <sup>+</sup> )	23.9±5.3%	8.7±4.0%	6.4±2.2%
Native myosin (Hsp90 <sup>-</sup> )	2.0±2.4%	24.8±8.2%	22.0±8.9%
Total infected	25.9±5.1%	33.4±5.6%	27.5±11.8%

The distribution of myosin folding intermediates in uninfected C2C12 myocytes was scored 48-96 hours after inducing differentiation. Plates were fixed with paraformaldehyde and stained with mAbs F59 and anti-Hsp90 and with DAPI. Three-color confocal volume datasets (172×128 μm×6-10 μm) were recorded and scored for the fraction of nuclei that were associated with cells containing F59<sup>+</sup> and Hsp90<sup>+</sup> folding intermediates. Similarly, 24 hours post-induction, cells were infected for 2.5 hours with AdGFP-MHC. Excess virus was washed away and cells were fixed and stained with anti-Hsp90 and with DAPI at 48, 72 and \*92 hour post-induction. The nuclei were scored for association with folding intermediates (Hsp90<sup>+</sup>) or native GFP-myosin assembled into myofibrils (Hsp90<sup>-</sup>). The average number of nuclei/field did not change ( $P < 0.01$ ) from 48-96 hours for either experiment, and there was no evidence of apoptosis in the scored fields based on nuclear staining with DAPI.

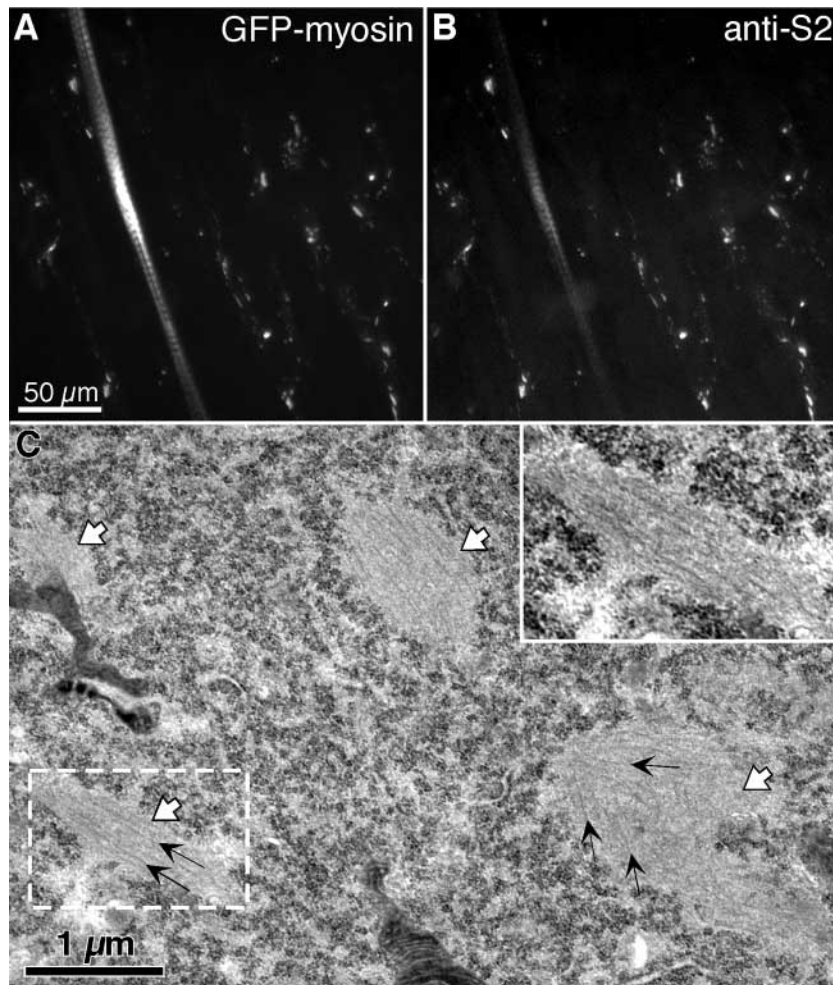


**Fig. 5.** The myosin folding intermediates exhibit partially folded motor domains. C2C12 cells expressing GFP-myosin were fixed either in 4% paraformaldehyde (A-C,G-I), or 100% methanol (D-F), and labeled with anti-myosin mAb, F59. (A) In paraformaldehyde-fixed cells the GFP-myosin is found in striated myofibrils and in folding intermediates (green). (B) The mAb F59 labels the intermediates, but not the mature myofibrils of infected or uninfected myotubes (red). (C) The color merged image shows a green myotube surrounded by yellow and red labeled cells containing the myosin folding intermediate. The arrow in B and C marks an uninfected cell that contains F59-stained folding intermediates. Methanol fixation partially denatures cellular proteins and exposes the F59 epitope in the striated myofibrils. (D) The GFP-myosin is assembled predominantly in striated myofibrils in the myotubes of this 96-hour culture. (E) F59 uniformly labels all the myosin-containing structures in the methanol fixed sample, resulting in a complete overlap with the GFP-myosin as seen in the color merged image (F). The arrow in E and F mark an uninfected cell that contains an F59 stained folding intermediate. (G-I) Uninfected C2C12 myocytes, 48 hour after transfer to differentiation medium, were paraformaldehyde fixed and stained with mAb F59 (green), anti-Hsp90 (red) and DAPI (blue). The filamentous and globular folding intermediates stain with both F59 and anti-Hsp90 in this projection of a 3D confocal microscopy volume dataset. The magnification in A-F is the same.

stained with F59 (Fig. 7). Using methanol fixation so that F59 stains all the muscle myosin, unfolded and folded, the untreated control cells show normal differentiation with much of the myosin already assembled into striated myofibrils at 52 hours post-induction (Fig. 7A). In contrast, the cells treated with geldanamycin for 22 hour are filled with myosin trapped in the filamentous and globular folding intermediates (Fig. 7B). The association of partially folded myosin with Hsp90 in these complexes was confirmed by paraformaldehyde fixation of the 22 hour drug-treated cells and staining with F59 and anti-Hsp90 (Fig. 7C,D). Hsp90 and Hsc70 (data not shown) are both found associated with the trapped myosin intermediates. The large increase in partially folded myosin co-localizing with

Hsp90 and Hsc70 triggered by inhibiting substrate release from Hsp90 indicates that all newly synthesized myosin transits through these myosin maturation complexes.

The effect of geldanamycin can be reversed by washing out the drug, leading to a resumption of myofibril assembly in the recovering cells (Fig. 7E,F). The extent of recovery after 40 hours suggests that the trapped myosin has partitioned into the newly formed myofibrils. Surprisingly, fusion and elongation of the myotubes do not appear to be disrupted by temporarily inhibiting Hsp90 activity. However, if geldanamycin is not removed and incubation with the drug is continued for ~60 hours, the cells do not recover and instead detach from the surface and die (data not shown).



**Fig. 6.** The rod myosin domain is folded and supports filament formation. (A) The GFP-myosin in infected C2C12 cells fixed with paraformaldehyde. (B) mAb 10F12.3 selectively binds the native coiled-coil conformation of S2 region of the rod domain and labels the GFP-myosin in the folding intermediates as well as in the striated myofibrils. (C) Areas of infected C2C12 cells containing the myosin maturation intermediates were marked during processing for electron microscopy and sections of these regions examined. The electron micrograph of an area of one cell reveals unusual cytoplasmic structures that contain loosely packed fibrils enclosed in a protein dense matrix that is devoid of polysomes (white arrows). Individual filaments in these structures are just discernable (black arrows). These intermediates range from 1-9  $\mu\text{m}$  in length, and 0.5-3.5  $\mu\text{m}$  in diameter and contain filamentous material organized in loose parallel or cross-hatched patterns. The boxed region is enlarged in the upper right corner. The filaments are best seen when viewed at a glancing angle.

Hsp90 and Hsc70 associate with a denatured myosin S1. Newly synthesized myosin in the chaperone-associated maturation intermediates has partially folded motor domains suggesting the participation of the chaperones in motor domain folding. To test this hypothesis, we looked for an association of Hsp90 and Hsc70 with an unfolded myosin motor domain by co-immunoprecipitation assays. Native myosin S1 was denatured and diluted into an S30 cytoplasmic fraction from C2C12 cells. This fraction contains a rich complement of molecular chaperones and was shown to promote myosin folding (Srikakulam and Winkelmann, 1999). The myosin S1 was immunoadsorbed from this mixture with an anti-S1 mAb. The immunoprecipitate was then assayed for Hsp90 and Hsc70 by SDS-PAGE and immunoblotting (Fig. 8). Both molecular chaperones, Hsp90 and Hsc70, were captured in the immunoprecipitate of the denatured myosin S1. These chaperones are absent in reactions lacking either the anti-S1 mAb or the myosin S1, suggesting that the association of Hsp90 and Hsc70 with non-native myosin S1 is specific.

## Discussion

### Forced expression of GFP-myosin reveals an assembly pathway

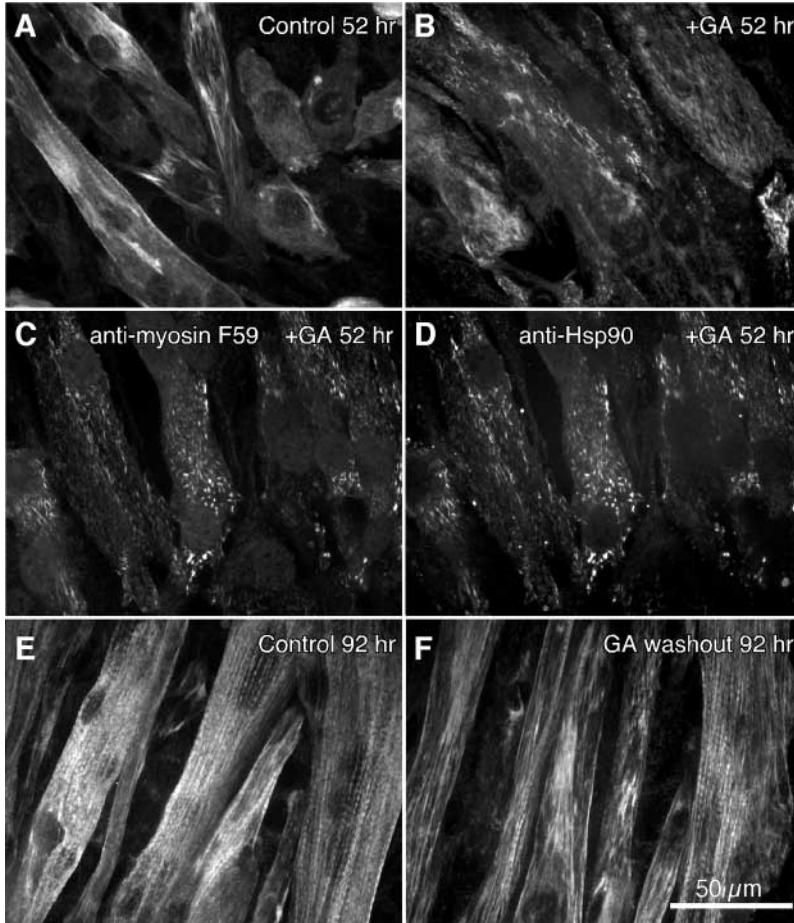
The assembly of myosin in striated muscle entails events at three levels: the folding of the myosin molecule, the assembly

of myosin filaments, and the incorporation of the filaments into the striated myofibril. We have developed an approach for examining the very earliest events in this process and for following the maturation of myosin through all stages of myofibrillogenesis. Using a replication defective adenovirus to deliver a GFP::myosin heavy chain chimeric gene to differentiating C2C12 myocytes, we can turn-on the synthesis of the GFP-myosin and analyze its interactions in a population of cells, or track its path through a single cell. This approach has revealed folding intermediates that precede the assembly of myosin into striated myofibrils. These intermediates associate with the general molecular chaperones Hsc70 and Hsp90. The myosin molecules in these intermediates appear to have dimerized rod domains that assemble into small filaments; however, they lack a completely folded motor domain. These structures appear to represent an intermediate in the myosin maturation and assembly pathway.

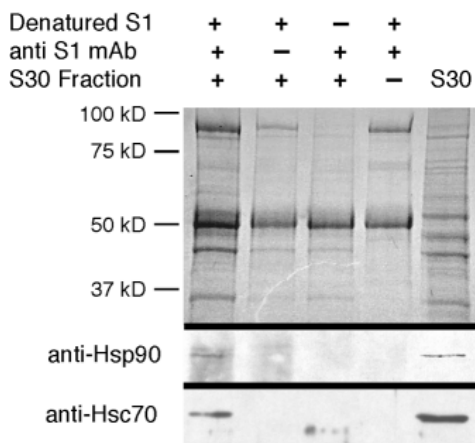
### Striated muscle myosin folding

The model in Fig. 9 summarizes a proposed pathway for myosin folding in striated muscle cells. The first step in the pathway is the association of the newly synthesized myosin heavy chain with molecular chaperones. The analysis of the folding of a HMM co-expressed with myosin light chains in a cell-free system has shown that the light chains bind prior to





**Fig. 7.** Geldanamycin treatment of C2C12 myocytes triggers accumulation of myosin maturation intermediates. (A) Control, untreated C2C12 myocytes fixed with cold methanol 52 hour after induction of differentiation and stained with mAb F59. Under these conditions, F59 stains all the muscle myosin, folded and unfolded. (B) Accumulation of myosin trapped in maturation intermediates in myocytes that were treated for 22 hour with geldanamycin (GA) is revealed after methanol fixation and F59 staining. Myosin maturation intermediates accumulate in all myosin-expressing cells. (C,D) Paraformaldehyde fixation of cells treated for 22 hour with geldanamycin reveals the association of the partially folded myosin (F59 staining) with Hsp90 (anti-Hsp90 staining). (E) Control C2C12 myocytes 92 hour post-induction fixed with cold methanol and stained with mAb F59. The large, well differentiated myotubes are filled with striated myofibrils. (F) Geldanamycin-treated cells 40 hour after washing out the drug show reversal of drug effects and recovery of the myofibril formation. These cells were also fixed with methanol and stained with F59 to detect both the folded and unfolded myosin.

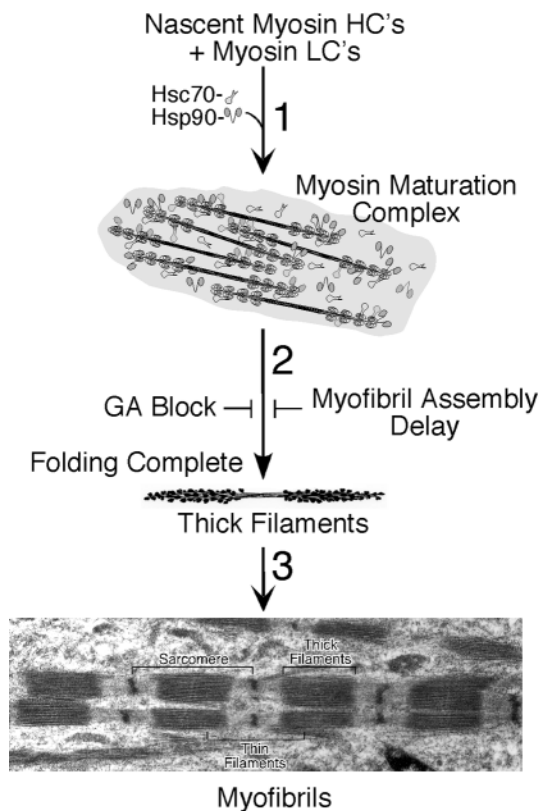


**Fig. 8.** Association of Hsp90 and Hsc70 with denatured myosin S1. The myosin S1 proteolytic fragment of pectoralis muscle myosin was denatured with 6.5 M guanidine HCl and rapidly diluted into C2C12 S30 fraction. After a short incubation at room temperature, the S1 and associated S30 proteins were immunoadsorbed to beads coated with an anti-S1 mAb. The immunopellets were resolved on 10% SDS-PAGE and stained or immunoblotted with Hsp90 and Hsc70 specific antibodies. The Hsp90 antibody detects an 86 kDa band in the complete reaction containing denatured myosin S1. Similarly, the Hsc70 antibody detects a 70 kDa band in this reaction. The Hsp90, and Hsc70 antibodies identify single bands of sizes 86 kDa, and 70 kDa respectively in the S30 used here as positive control.

the completion of motor domain folding (Srikakulam and Winkelmann, 1999). The timing of myosin light chain binding was not examined here, but we will assume that this is an early event in cells as well. The dimerization of the HMM heavy chains via folding of the S2 region of the rod also precedes completion of motor domain folding. This was confirmed here for the cellular pathway of myosin folding. In the reticulocyte lysate assay, an ATP-dependent interaction of HMM with the eukaryotic cytosolic chaperonin (CCT) was demonstrated, but the folding reaction was inefficient. Supplementing the lysate with a cytosolic fraction from muscle cells accelerated folding, suggesting a role for muscle specific factors. We have now shown that Hsc70 and Hsp90, two components found in that cytosolic fraction, interact with newly synthesized myosin in a cytosolic structure that we have termed a myosin maturation complex. Although, we were unable to confirm a role for CCT in the cellular pathway for myosin folding, we believe it may be involved in early folding events associated with step 1 of this pathway.

#### Intermediates in striated muscle myosin folding and assembly

The second step of the pathway involves a newly identified intermediate detected using the forced expression of GFP-myosin. This maturation complex contains partially folded and assembled myosin in association with Hsc70 and Hsp90. The



**Fig. 9.** A model summarizing a proposed myosin folding pathway in striated muscle cells. The molecular chaperones in Step 1 interact with nascent and partially folded myosin molecules. The light chains are not depicted in this drawing but are assumed to associate with the light chain binding region of the myosin head soon after synthesis (Srikakulam and Winkelmann, 1999). This first step involves the initial folding and dimerization of the myosin molecule. Step 2 involves the formation of the myosin maturation complexes containing partially folded myosin and the molecular chaperones Hsc70 and Hsp90. Myosin transit through this intermediate is normally rapid. However, these intermediates accumulate if myofibril assembly is delayed or if Hsp90 activity is inhibited with geldanamycin (GA). Myosin filaments emerge from the maturation complexes containing folded native myosin and are then incorporated into nascent myofibrils in the final step (3) of the pathway.

myosin maturation complexes are most abundant in early stages of muscle differentiation, or when myosin expression is synchronized by virus infection. The disappearance of the myosin maturation complexes coincides with the appearance of striated myofibrils. We hypothesize that myosin usually proceeds rapidly through this intermediate step, forming thick filaments that incorporate into myofibrils. However, if myofibril assembly is delayed, for example, early in the differentiation program when one or several essential components for myofibril assembly are missing or limiting, maturation of myosin is blocked. Partially folded myosin accumulates in loosely organized filament bundles and remains associated with the molecular chaperones, Hsc70 and Hsp90. The chaperones prevent release of active, unregulated 'rogue motors' in the cytoplasm, and the maturation complexes act as a holding state or checkpoint for assembly of the myofibril.

Inhibition of Hsp90 ATPase activity with the ansamycin antibiotic, geldanamycin, also blocks myosin maturation, resulting in the accumulation of all newly synthesized myosin as a partially folded intermediate in complexes containing Hsc70 and Hsp90. This block is reversed after wash-out of the geldanamycin, resulting in disappearance of the maturation complexes and the partitioning of the myosin into striated myofibrils. This synthetic block shows that all of the striated muscle myosin transits through an Hsp90 associated state, indicating that this chaperone-associated intermediate is necessary for muscle myosin maturation and assembly.

Geldanamycin inhibition of Hsp90 activity can trigger proteasome-mediated degradation via an Hsc70/Hsp90-interacting ubiquitin-ligase activity (CHIP) (Hohfeld et al., 2001; Meacham et al., 2001). However, the geldanamycin block introduced in the myosin maturation pathway is reversible with recovery of myofibril assembly after washout of the drug. This outcome is inconsistent with the bulk of the Hsp90 trapped myosin being committed to a degradation pathway. The myosin maturation complexes observed in the absence of inhibitor also do not fit a degradation model since, (1) their accumulation in the cytosol is stage specific, (2) they have relatively long life-times, and (3) they are highly dynamic structures that appear to distribute throughout the cytosol of elongating myocytes. These are not characteristics of a quality control pathway in which proteasome-mediated degradation is generally rapid and intermediates persist only when proteasome activity has been inhibited (Johnston et al., 1998).

Myosin intermediates similar to the ones described here are found in primary cultures of embryonic chicken pectoralis muscle (Lin et al., 1994) and precardiac explants from chick embryos (Rudy et al., 2001). In 6- to 24-hour-old post-mitotic myoblasts and early explanted cardiomyocytes the striated muscle myosin first appears in scattered, irregular clumps or dots that localize independently of thin filaments. It is only later that the two filament systems align and merge to form ordered myofibrils. Thus, the formation of these intermediate complexes is integral to a common myosin maturation and assembly pathway in the C2C12 cell line, primary cultures of differentiating myocytes and explants of cardiomyocytes.

Myosin motor domain folding is the slow step in the pathway and is aided by chaperones. This assertion is based on the analysis of a striated muscle myosin motor domain::GFP chimera, which folds slowly and inefficiently in cell-free assays, does not fold in non-muscle cells, but does fold efficiently in muscle cells (Chow et al., 2002). Therefore, folding of the motor domain is an assisted pathway independent of the assembly function of the rod domain, and it is probably assisted by members of at least two and probably three families of molecular chaperones.

The last step (3 in Fig. 9) of the model shows the myosin emerging fully folded and assembled into thick filaments. This step involves the association of myosin-binding proteins and thick filament-associated proteins that contribute to the regulation of thick filament length and interactions between filaments. These proteins together with titin contribute to the precise organization of the myosin filaments and the incorporation with the thin filaments in the highly ordered structure of the myofibril (Gregorio and Antin, 2000).

### Hsc70 and Hsp90: chaperones mediating myosin maturation

Hsc70 and Hsp90 are major molecular chaperones of the cytosol and nucleus. The role of the Hsp70 family in the folding of newly synthesized proteins, in the protection of proteins during cellular stress and in protein trafficking is well established (Naylor and Hartl, 2001). The functional role of Hsp90 in contrast, is less clearly defined and perhaps more restricted (Young et al., 2001). The most clearly established role of Hsp90 is in the folding and activation of nuclear steroid hormone receptors and signal transduction kinases (Buchner, 1999; Caplan, 1999; Pratt, 1997). Hsp90 participates in each of these activities through a highly coordinated interaction with other chaperones and co-chaperones including: Hsp70/Hsc70, HOP, p23 and Hsp40 (Hernandez et al., 2002; Murphy et al., 2001). Many Hsp90 and Hsc70 interacting co-chaperones share an N-terminal domain containing one to three tandem tetratricopeptide repeats (TPR motifs) that bind a C-terminal recognition peptide of Hsp90 and Hsp70/Hsc70 (Scheufler et al., 2000). The TPR motifs are fused to different functional domains that confer activity or specificity to the chaperone complex to assist in the maturation of target substrates via cycles of ATP-dependent binding and release (Caplan, 1999; Young et al., 2001).

Hsc70 and Hsp90 are abundant proteins present in both muscle and non-muscle cells (Murphy et al., 2001), thus they are probably necessary but not sufficient for striated muscle myosin folding. The Hsc70/Hsp90 folding machinery may require muscle-specific adapters for efficient targeting and folding of muscle myosin. A candidate for just such a role has recently been identified (Ao and Pilgrim, 2000; Barral et al., 1998; Barral et al., 2002; Price et al., 2002). The *C. elegans* UNC-45 protein is a founding member of the UCS family of proteins (Hutagalung et al., 2002). This protein has three basic motifs: an amino-terminal domain with three tandem TPR motifs that is involved in Hsp90 binding; a central domain of unknown function, and a carboxyl-terminal UCS domain that is shared by a growing family of proteins that have a direct interaction with myosin. UNC-45 has been shown to bind directly to both myosin and Hsp90 in a ternary complex (Barral et al., 2002). This interaction may involve the motor domain since the combination of Hsp90 and UNC-45 prevent thermal aggregation of myosin S1.

Vertebrates express two distinct genes that are homologous to UNC-45 (Price et al., 2002). One is expressed generally in all tissues. Expression of the other is limited to skeletal and cardiac muscle and is up regulated during muscle differentiation. The striated muscle UNC-45 homologue has been proposed to be a muscle-specific myosin chaperone (Barral et al., 2002; Price et al., 2002). Reagents for detecting these UNC-45 homologues are not yet available, but it is tempting to predict an association of Hsc70/Hsp90/UNC-45 and striated muscle myosin in the myosin folding intermediates.

### Conclusions

Myofibrillogenesis is a complex pathway requiring the coordinated expression and folding of a large number of muscle-specific proteins, and their assembly into a semi-crystalline structure, the sarcomere (Clark et al., 2002). The

initiation of the muscle-specific gene expression program is transcriptionally coordinated; however, the order of appearance of the major protein components of the sarcomere is stochastic (Lin et al., 1994). This may necessitate cellular mechanisms for holding components in partially folded intermediate states until all the necessary elements are present for organization and assembly of this structure. We conclude that Hsc70 and Hsp90 take part in the initial folding of striated muscle myosin. They participate in a complex pathway that probably includes other known chaperones and perhaps some new muscle-specific adapters or co-chaperones. These chaperones may facilitate the proper folding of myosin and block-off pathway interactions, or they may sequester the partially folded intermediates until the conditions or components necessary for orderly assembly of the larger structure are present. This hypothesis dramatically broadens the role played by molecular chaperones in muscle cells.

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